Investigating the role of microRNAs and Extracellular Vesicles in Cisplatin Resistance in Ovarian Cancer

by

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Abstract

Ovarian cancer claims the lives of more than 150000 women worldwide annually. One of the contributors to this high mortality is resistance to cisplatin chemotherapy. Resistance to cisplatin is multifactorial involving various networks and tiers of regulation and is not completely understood yet. Among the regulatory molecules in the body, microRNAs have a prominent role in physiological and pathological conditions. Extracellular Vesicle (EV) communication between cells could also play a noteworthy role in cisplatin resistance.

The main aim was to identify microRNAs involved in cisplatin resistance in ovarian cancers whose role in this aspect has not been documented yet and to ascertain and validate possible targets. MicroRNAs exhibiting differential levels of expression in sensitive and resistant ovarian cancer cell lines were identified. Gain or loss of function experiments in cell lines validated their involvement in cisplatin resistance; possible targets were confirmed by transient knockdown experiments in ovarian cancer cell lines. miR-21* and miR-31 functionally increased cisplatin resistance in ovarian cancer cells; NAV3 and KCNMA were validated as their respective targets and shown to modulate cisplatin resistance in ovarian cancer cell lines.

A second aim was to explore the role of EVs in cisplatin resistance – to investigate if cisplatin resistance could be transferred between cell lines and to investigate the consequences of preventing EV uptake within a population of cisplatin treated cells. EVs extracted from a cisplatin resistant cell line were transferred onto a cisplatin sensitive cell line; results showed that these EVs could increase cisplatin resistance in the recipient cell line. The response of ovarian cancer cells to cisplatin was analysed following prevention of EV uptake by using heparin, amiloride or dynasore - known inhibitors of EV uptake; results indicate that inhibition of EV uptake increases cisplatin sensitivity significantly.

These results open up future avenues for research regarding the role of microRNAs and EVs in cisplatin resistance with possible therapeutic potential.

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ALL	Acute Lymphoblastic Leukaemia
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia- and RAD3-related
BCA	bicinchoninic acid assay
BER	base excision repair
BKCa	large calcium activated potassium conductance
BSA	Bovine serum albumin
CDE	caveolin dependent endocytosis
CFSE	Carboxyfluorescein succinimidyl ester
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EMT	Epithelial–mesenchymal transition
EV	Extracellular Vesicle
GO	gene ontology
HMG1	nonhistone chromosomal high mobility group 1
HMG2	nonhistone chromosomal high mobility group 2
HR	homologous recombination
IAP	Inhibitors of Apoptosis
ILV	intraluminal vesicles
KCNMA1	Potassium Channel, Calcium Activated Large Conductance Subfamily M
KEGG	Kyoto Encyclopedia of Genes and Genomes
L1CAM	L1 cell adhesion molecule
LNA	locked nucleic acid
lncRNA	long non-coding RNA
miRNA	microRNA
MMR	mismatch repair complex
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVB	multivesicular bodies
NAV3	neurone navigator 3
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PARP-1	poly(ADP Ribose) polymerase 1
PDCD4	Programmed Cell Death 4
PDI	Protein Disulfide Isomerase
PDIA4	Protein Disulfide Isomerase Family A, Member 4
PI3K	Phosphoinositide-3-Kinase
PTEN	Phosphatase And Tensin Homolog
PVDF	Polyvinylidene fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RASA1	RAS P21 Protein Activator (GTPase Activating Protein) 1
RIPA buffer	Radioimmunoprecipitation assay buffer
RISC	RNA induced silencing complex
SEM	standard error of mean
shRNA	short hairpin RNA
siRNA	Small interfering RNA
TCGA	The Cancer Genome Atlas
TLS	trans-lesional synthesis

Chapter 1 INTRODUCTION

1. 1. OVARIAN CANCER

Ovarian cancer is the fifth most common type of cancer in women in the UK (ons.gov.uk, 2012); more than 150,000 women die of ovarian cancer each year worldwide (Fitzmaurice et al., 2015). The overall 5-year survival rate is only about 45% (Siegel et al., 2015). Reasons for this high mortality rate include diagnosis at advanced stages and acquired resistance to chemotherapy.

1.1.1 TYPES OF OVARIAN CANCER

Ovarian cancers are classified based on their histology into several subtypes as reviewed in Jayson et al (Jayson et al., 2014). A newer classification divides them into Type I and Type II tumours (Shih Ie and Kurman, 2004, Kurman and Shih Ie, 2016). These two classifications and molecular changes associated with each type are summarised below.

Type II tumours are high grade tumours and are usually diagnosed in advanced stages progressing rapidly and are very aggressive. The most common of these is the High Grade Serous Carcinoma accounting for almost 70% of ovarian tumours. They show a serous morphology and stain positively for ER, WT1 and CA125. High grade serous cancer is characterised by high rate of proliferation as shown by Ki-67 staining as well as frequent mutations in p53 and BRCA1/ BRCA2 with defective homologous recombination (Kurman and Shih Ie, 2016). Abnormalities in Notch signalling and FOXM1 have also been documented. Four transcriptional subtypes have been described in high grade serous cancers – proliferative, immunologic, mesenchymal and differentiated subtypes based on differential expression (Creighton et al., 2012). High grade serous cancers tend to present in advanced stages when the tumour has spread beyond the confines of the ovary and the pelvis. They are initially highly responsive to chemotherapy but often recur as a resistant tumour; the overall prognosis is poor (Jayson et al., 2014). Other rarer type II tumours include include carcinosarcoma and undifferentiated carcinoma (Kurman and Shih Ie, 2016).

Type I tumours are frequently diagnosed in early stages and are indolent. They develop from atypical borderline precursors and have a better overall prognosis than

type II tumours. They include the following histological types: **Low grade serous:** Serous ovarian cancers are subdivided into high grade and low grade based on the morphology of the nuclei (Hannibal et al., 2012). Low grade serous tumours are indolent and show mutations in PIK3CA, KRAS and BRAF. They present at an earlier stage; however, they are often resistant to conventional chemotherapy for ovarian cancers. MEK inhibitors have been shown to have a promising response in low grade serous cancers (Hunter et al., 2015). **Mucinous:** This subtype of ovarian cancers shows mucinous pathology and are thought to arise from germ cells or transitional cells in the ovary. They show frequent KRAS mutations and HER2 amplification (Perren, 2016). **Endometrioid:** These tumours show squamous metaplasia and are thought to originate from endometriotic nodules. They tend to show MMR deficiency and often show mutations in ARID1a, PI3KCA and PTEN. **Clear cell:** Histopathologically, these tumours show high grade nuclei with a clear cytoplasm; they are thought to originate from endometriotic nodules. They often show mutations in ARID1a and PIK3CA and have a kinase inducing hypoxic drive. They respond poorly to conventional chemotherapy. Other type I tumours include mixed mullerian or seromucinous tumours and Brenner tumours.

There are suggestions that due to the significant differences in the molecular pathology, prognosis and response to chemotherapy, the different subtypes should be treated as different diseases and chemotherapy tailored to each subtype (Kobel et al., 2008). However, the current treatment protocol suggests the same protocol for all the different subtypes (du Bois et al., 2005, Gourley et al., 2014).

1.1.2 DIAGNOSIS AND TREATMENT

Ovarian cancer often presents in advanced stages because the symptoms are non-specific and include abdominal discomfort or pain, lower back pain, flatulence – these symptoms are often attributed to irritable bowel syndrome (Jayson et al., 2014).

By FIGO staging, stage I and II are limited to the pelvis while stage III and IV have spread outside the pelvis (Prat, 2014). In a study investigating stage at diagnosis and ovarian cancer survival in different countries Maringe et al (2012) show that while one year survival for patients diagnosed at stage I is nearly 98%, it drops to 40% in those patients diagnosed in stage IV. There is therefore the necessity to diagnose

ovarian cancer at early stages in order to improve prognosis. Serum CA125 is used as a biomarker for screening; it has been shown by the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) that transvaginal ultrasonography with or without serumCA125 is more sensitive and specific in the detection of ovarian tumours (Menon et al., 2009).

The first line of treatment is surgery combined with chemotherapy with the drug of choice being platinum compounds like cisplatin and carboplatin (du Bois et al., 2005). The initial tumour is very sensitive to chemotherapy (Bogliolo et al., 2015) but in a quarter of the patients there is a platinum resistant recurrence (Chekerov et al., 2013). On recurrence, treatment is further chemotherapy with platinum compounds in combination with other compounds such as taxanes (van der Burg et al., 2014), gemcitabine or pegylated liposomal doxorubicin (Safra et al., 2011); other promising compounds include bevacizumab (Aghajanian et al., 2012), PARP inhibitors (Kaye et al., 2012, Ledermann et al., 2012) and topotecan as reviewed by Bauman et al (Baumann et al., 2012). Drugs to inhibit molecular targets of ovarian cancers are now being developed and studied. These include drugs to inhibit RAS signalling, PI3K/AKT pathway, STAT/JAK/JAK2 pathway inhibitors (Kalachand et al., 2011). Pemetrexed, an antifolate antineoplastic agent which disrupts folate dependent metabolic processes has shown partial response (Miller et al., 2009, Vergote et al., 2009). Intraperitoneal administration of cisplatin has shown a slight improvement in survival (Markman et al., 2001).

1. 2. CISPLATIN

Cis-diaminedichloro platinum(II) or cisplatin is a commonly used anticancer drug. It was first synthesised in 1845 by Peyrone (Cepeda et al., 2007). Its growth reducing properties were discovered only in the 1960s when Rosenberg and co-workers observed decreased growth when they were studying the effect of electrical fields on the growth of *Escherichia coli* caused by platinum compounds released from the platinum electrodes (Rosenberg et al., 1965); this property was later exploited in its use as an anticancer drug. Testicular cancers are highly sensitive to cisplatin; cisplatin is also widely used to treat ovarian, bladder, cervical, head and neck, oesophageal and small cell lung cancer (Gomez-Ruiz et al., 2012). Side effects may be severe and include nephrotoxicity, neurotoxicity and ototoxicity.

1.2.1 MODE OF ACTION

Cisplatin is a neutral inorganic square planar complex (Cepeda et al., 2007). It is taken into cells by both passive (diffusion) and active (copper transporters) uptake mechanisms. Cisplatin is neutral and has to be activated by aquation reactions in which the cis-chloro ligands are replaced with water molecules. This is influenced by the chloride concentration; cisplatin remains neutral when chloride concentration is high, i.e. extracellular fluids (~100 mM) but is activated inside the cell where the chloride concentration falls to a few mM (Siddik, 2003, Cepeda et al., 2007). Activated cisplatin is highly reactive and binds readily with DNA, glutathione, metallothionein and protein. Cisplatin causes cell death through various mechanisms. It is a potent inducer of apoptosis or programmed cell death (described in section 1.2.1.1) (Ormerod et al., 1996, Henkels and Turchi, 1997) but has also been shown to cause necrosis through PARP-1 (poly(ADP Ribose) polymerase 1) (Gonzalez et al., 2001, Cepeda et al., 2007).

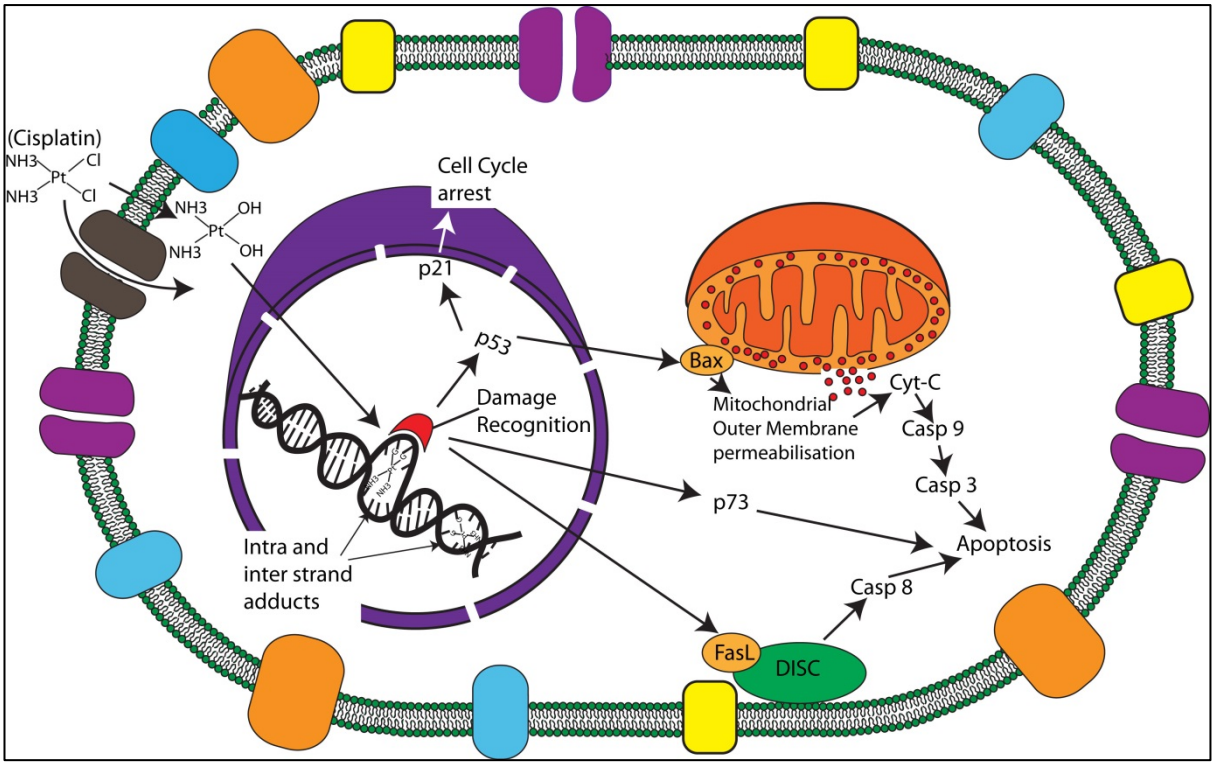

FIGURE 1.1: CISPLATIN MECHANISM OF ACTION
Cisplatin and other platinum compounds, upon uptake by the cell, cause crosslinks in the DNA, bending the helix and damaging the DNA. This damage is recognised by damage recognition proteins which activate p53. This then, depending on other signals, activates Bax which translocates to mitochondrial outer membrane and causes outer membrane permeabilisation releasing cytochrome-C activating the caspase-9 – caspase-3 cascade resulting in apoptosis. p53 may also activate p21 causing cell cycle arrest. Cisplatin can also activate p73 causing apoptosis. The Fas/FasL 'extrinsic' pathway may be triggered by cisplatin causing caspase-8 to come into play and activate apoptosis through caspase-3.

1.2.1.1 Cisplatin induces Apoptosis

- ***Cisplatin binds to DNA and causes DNA damage***

Cisplatin interacts with purine bases to form DNA-DNA inter- and intra-strand and DNA-protein adducts. Of these, the 1,2-d(GpG) intra-strand adduct is most common and considered the most important as it is one of the main initiators of cytotoxicity (Pinto and Lippard, 1985). These intra-strand adducts are shown to cause a physical distortion in the DNA helix (Bellon et al., 1991, Siddik, 2003).

- ***Recognition of the damage by damage recognition proteins***

The damage to DNA is recognised by damage recognition proteins which activate other pathways including proteins involved in apoptosis (Donahue et al., 1990, Chaney and Vaisman, 1999). *hMSH2* or hMutSα (MutS protein homolog 2) component of the DNA mismatch repair complex (MMR) tries to repair the damage;

however if the repair is futile, MMR signals for apoptosis (Fink et al., 1998, Gonzalez et al., 2001, Cepeda et al., 2007). Nonhistone chromosomal high mobility group 1 and 2 proteins (HMG1 and HMG2) may also be activated, which bind to the adduct and shield it from repair.

- ***p53***

p53 is a tumour suppressor protein which is a critical coordinator of the apoptotic pathway; it coordinates the actions of the various repair pathways and can ultimately signal for apoptosis or cell cycle arrest (Meek, 2015). p53 is usually maintained at low levels by MDM2, a RING finger type E3 ligase. After DNA damage and single or double stranded breaks, ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia- and RAD3-related) protein kinases are activated; in addition to initiating DNA-repair mechanisms and activating checkpoints leading to G2 phase arrest, these kinases reduce activity of MDM2 leading to stabilisation of p53 (Damia et al., 2001, Zhao and Piwnica-Worms, 2001, Meek, 2015).

Activated p53 transactivates various proteins including Cdk inhibitor p21waf1/cip1 which is associated with cell cycle arrest and DNA repair. Other proteins activated by p53 include *Gadd45a* gene which causes growth arrest, enhances nuclear activity and protects cells from cisplatin cytotoxicity (Smith et al., 1994, Smith et al., 1997, Delmastro et al., 1997) and the *Bax* proapoptotic gene (Hershberger et al., 2002). p53 is thought to initiate apoptosis based on the cellular context, upstream signals and extent of DNA damage (Meek, 2015). One of the determinants of the fate of the cell is thought to involve members of the Bcl family, which includes proapoptotic genes like Bax, Bad, Bak as well as antiapoptotic factors like Bcl2, Bcl-xl and Bcl-w. Bax-Bcl-2 ratio – induction of Bax as well as cleavage of Bcl-2 by cisplatin increases the ratio (Henkels and Turchi, 1997) – is thought to be pivotal in the initiation of apoptosis (Del Bello et al., 2001, Gonzalez et al., 2001, Cepeda et al., 2007).

- ***Initiation of Apoptosis***

p53 initiates activation of apoptosis by transactivating the Bax gene which is then translocated from the cytosol to the mitochondria, the ‘intrinsic pathway’ (Figure 1.1). The Bax protein then causes mitochondrial membrane permeabilisation and releases cytochrome C, an apoptogenic factor in the intermembrane space of the

mitochondria, which then activates the caspase 9-caspase 3 pathway leading to release of endonucleases and apoptosis (Wang et al., 2000, Makin et al., 2001).

Cisplatin may also induce apoptosis by activating the 'extrinsic' pathway – the fas/fas ligand signalling induces the formation of DISC (death inducing signalling complex) with the fas-associated death domain and caspase-8, which then activates caspase-3 to release endonucleases (Eischen et al., 1997, Micheau et al., 1997, Ferreira et al., 2000).

p73 may also activate the caspase pathway once it has been induced by c-Abl tyrosine kinase; which, in its turn is activated by cisplatin, (Gong et al., 1999). This requires the activity of p38 kinases from the MAPK pathway (Sanchez-Prieto et al., 2002) and cellular proficiency of the mismatch repair pathway (MMR).

Only 5-10% of cisplatin in the cell is bound to the gDNA while the rest is bound to other cellular components with nucleophilic sites such as cytoskeletal microfilaments, thiol containing peptides and proteins (Timerbaev et al., 2006, Cepeda et al., 2007). It has also been suggested that cisplatin may also cause cytotoxicity by binding to proteins in the cytoplasm by their methionine or histidine residues (Cepeda et al., 2007). It is thought that it may bind to ubiquitin and block the ubiquitin-proteasome response thus causing cell death. Cisplatin binds to Hsp90 and specifically blocks its c-terminal ATP binding site which is shown to be a participant in the correct protein folding of proteins involved in signal transduction and cell cycle regulation. It has also been shown recently that by binding to cytoplasmic targets, cisplatin can cause oxidative stress which can trigger mitochondrial outer membrane permeabilisation which then releases caspases, causing apoptosis (Galluzzi et al., 2012).

Resistance to cisplatin occurs when triggering of apoptosis requires a very high concentration of cisplatin; the causes for this are multifactorial and are discussed in Section 1. 4. The genes involved in cisplatin resistance are modulated by different networks of regulating molecules. One new class of regulatory molecule that has emerged in the last two decades is the microRNAs. MicroRNAs are described in the next section.

1. 3. MICRORNAS

Non-coding RNAs have increasingly been recognised in the last two decades as playing an important role in the modulation of many physiological, developmental and pathological conditions. Among these, microRNAs have emerged as important regulators of cellular activity due to their capacity to modify the effect of more than one gene and therefore, whole pathways in physiological and pathological conditions.

MicroRNAs are highly conserved, short single stranded RNAs of 20-22 nucleotides in length generated from endogenous hairpin shaped transcripts by the RNAse III type enzyme DICER (Ambros et al., 2003). The first microRNA was observed in 1993 by Lee et al. in *Caenorhabditis elegans* (Lee et al., 1993b). Since then they have been extensively studied – hundreds of microRNAs have been discovered in various species; a system has been devised for their nomenclature (Ambros et al., 2003) and various physiological and pathological functions of microRNAs have been discovered as reviewed in Erson-Bensan (2014) and Hayes et al. (2014).

1.3.1 FORMATION OF MICRORNAS

The classical mechanism of formation and action of microRNAs is illustrated in Figure 1.2. DNA is transcribed into primary microRNAs, which are hairpin shaped structures that are capped and polytailed, by RNA polymerase II in the nucleus (Lee et al., 2002, Lee et al., 2004). This is then processed by an enzyme called DROSHA along with DGCR8 into a precursor microRNA which is a 60-100nt long stem loop structure with a 5' phosphate and a ~2nt 3' overhang (Lee et al., 2003, Bartel, 2004, Han et al., 2004, Kim, 2005, Esquela-Kerscher and Slack, 2006). Precursor microRNAs are exported out of the nucleus by the transporter RAN GTPase/exportin 5 (Yi et al., 2003, Bohnsack et al., 2004, Lund et al., 2004, Zeng and Cullen, 2004). In the cytoplasm, the precursor miRNA is recognised by its 5' phosphate and the ~2nt 3' overhang and further processed by the enzyme DICER and TAR binding protein 2 into microRNA double stranded duplexes (Bartel, 2004). This duplex is then unwound and one of the strands – now a single stranded microRNA – is loaded into the RISC (RNA induced silencing complex) composed of various components including DICER, TARBP2 and argonaute endonucleases – Ago1 and Ago 2 (Carmell et al., 2002). This forms the miRISC – miRNA including RNA induced silencing complex. Only one strand is loaded into the RISC; the strand not loaded into the RISC complex is known as the passenger strand or the star strand (Winter et al., 2009); this is further discussed in section 1.3.3. The formation of microRNAs may be modified at any of these stages leading to differential expression in different tissues and cells (Obernosterer et al., 2006, Davis et al., 2008, Winter et al., 2009).

1.3.2 MICRORNAS – MECHANISM OF ACTION

The microRNA then directs the complex to target mRNAs by binding to sites with imperfect complementarity; it is thought that the seed region – positions 2–8 – plays a very important role in target recognition (Pillai, 2005, Ebert et al., 2007). Once bound, the translation of the mRNA is repressed or the mRNA is degraded with the argonaute proteins acting as slicers to degrade the mRNA (Pillai, 2005). By this process, microRNAs are able to modulate the expression of target genes and proteins and regulate their transcriptional or post-transcriptional expression. Because of its imperfect complementarity, one microRNA can bind to several targets and one target can be modulated by several microRNAs (Flynt and Lai, 2008, Aigner, 2011).

In addition to the above mechanism, which mainly occurs in the cytoplasm, it has been noticed that some mature miRNAs localise in the nucleus (Meister et al., 2004, Politz et al., 2006, Liao et al., 2010, Jeffries et al., 2011). Argonaute proteins are also seen in the nucleus and found associated with the nuclear miRNAs (Hansen et al., 2011, Zisoulis et al., 2012). They have been shown to regulate other miRNAs and their own transcription (Tang et al., 2012, Liang et al., 2013). They have also been shown to regulate and be regulated by long ncRNAs (Hansen et al., 2011, Li and Yang, 2013) and pseudogenes (Li and Yang, 2013).

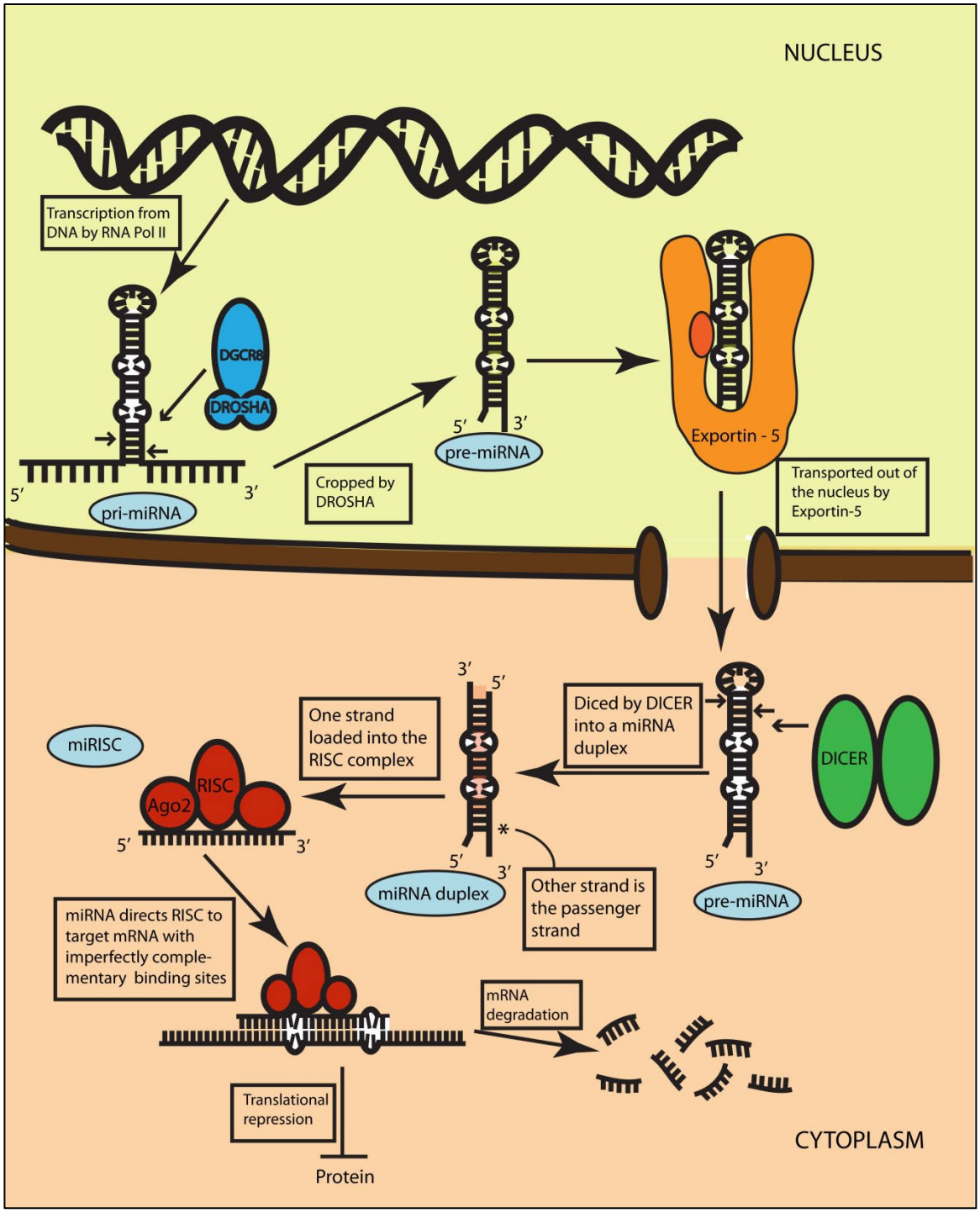

FIGURE 1.2: MICRORNA BIOGENESIS AND MECHANISM OF ACTION

MicroRNAs (miRNAs) are transcribed from DNA into hairpin shaped transcripts called primary microRNA (pri-miRNA), These are processed in the nucleus by the enzyme Drosha into precursor microRNA (pre-miRNA) which is then exported into the cytoplasm by RanGTPase – Exportin-5 complex. In the cytoplasm, it is processed by the enzyme DICER into a miRNA duplex. One strand of the duplex is loaded into the RISC (RNA induced silencing complex) to form the miRISC (miRNA including RISC) and directs it to the target mRNA with imperfectly complementary binding sites and causes mRNA degradation or translational repression.

1.3.3 MICRORNAS PASSENGER STRAND

As described in section 1.3.2, only one strand is loaded into the RISC complex. The other strand (also called the passenger strand or the star strand) of the duplex was thought to be degraded but it has been shown that this strand may also be present and be involved in modulation of proteins (Biasiolo et al., 2011). The choice of the guide strand was thought to be determined by the thermodynamic stability of the base pairs at the 5' end with the less stable pair being loaded into the RISC (Khvorova et al., 2003, Schwarz et al., 2003, Winter et al., 2009). Originally, it was proposed that the stability was determined by a helicase which started unwinding the duplex multiple times but released the end before productive unwinding was accomplished. However no single helicase has yet been identified (Hu et al., 2009). Moreover, it has been shown that in a given tissue, either or both strands of the microRNA may be expressed in varying proportions (, Ro et al., 2007, Packer et al., 2008, Hu et al., 2009, Biasiolo et al., 2011). The dominant strand may be different in different species or in different tissues in the same species. The proportion of the two strands may also change in certain conditions and diseases. As the two microRNA strands are complementary to each other they bind to and modulate different targets or a different sequence on the same target (Ro et al., 2007, Jazdzewski et al., 2009); therefore, they can be synergistic or antagonistic to each other.

1.3.4 PHYSIOLOGICAL FUNCTIONS OF MICRORNAS

MicroRNAs have been shown to modulate numerous physiological cell functions like cell differentiation, cell proliferation, apoptosis, angiogenesis and the cell cycle (Ambros, 2004, Bartel, 2004, Kim, 2005, Aigner, 2011). MicroRNAs are also involved in the cellular response to various stresses; this is reviewed in Jacobs et al; a database is also available to identify previously published studies of microRNAs in stress responses (Jacobs et al., 2013).

1.3.5 MICRORNAS IN CANCER

In addition to their physiological role, microRNAs have been found to be aberrantly expressed in various pathological conditions including cancer (Aigner, 2011). Differential patterns of microRNA expression have been described in tumours as reviewed in (Aigner, 2011, Ress et al., 2015, Anwar and Lehmann, 2015, Huang and

Yu, 2015, Ling et al., 2016, Lim and Yang, 2016). Some microRNAs are well known tumour suppressors – for example, miR-34a let-7, miR146a/b – while others have been shown to be oncogenes, for example miR-21, miR-221/222, miR-582, miR-10b (Esquela-Kerscher and Slack, 2006, Trang et al., 2008, Aigner, 2011, Schraivogel et al., 2011, Frixa et al., 2015). Some have been shown to be both tumour suppressing and promoting depending on the tumour and tissue for example, miR-31, miR-10b, miR-17-92 (Calin and Croce, 2006, Aigner, 2011, Laurila and Kallioniemi, 2013). MicroRNAs have been variously shown to target cell proliferation, metastasis and invasion, apoptosis, angiogenesis, tumour microenvironment thus modifying tumour behaviour (Creighton et al., 2012, Hayes et al., 2014). MicroRNAs, because of their ability to target multiple genes can modify specific networks – miR-21 is associated with proliferation pathways (Pan et al., 2010); miR-31 affects metastasis and invasion (Schmittgen, 2010), miR-34 affects the p53 response (Yamakuchi and Lowenstein, 2009) and miR-17-92 is involved in c-myc pathways (Gurtan and Sharp, 2013). MicroRNAs have also been shown to modulate the response of tumours to various chemotherapeutic drugs (Zheng et al., 2010, van Jaarsveld et al., 2010, Garofalo and Croce, 2013, Magee et al., 2015, Naidu and Garofalo, 2015, Wang et al., 2015a) or radiotherapy (Weidhaas et al., 2007, Marta et al., 2015). The next section discusses the involvement of microRNAs in cisplatin resistance.

1. 4. CISPLATIN RESISTANCE AND MICRORNAS

Cisplatin resistance may be intrinsic or acquired after drug exposure. It has been shown that the resistance to cisplatin is at least two-fold if not higher (Hagopian et al., 1999) as shown in tumour cell lines. Mechanisms of resistance have been studied using cell culture studies; there is generally an agreement with mechanisms encountered clinically (Giaccone, 2000). Often resistance is multifactorial (Teicher et al., 1987, Richon et al., 1987, Kelland et al., 1992, Siddik et al., 1998). Resistance mechanisms can be broadly classified as follows (Galluzzi et al., 2012);

a. pre-target effects relating to
    i. lower intracellular accumulation of cisplatin due to decreased uptake, or increased efflux of the drug or
    ii. lower availability of the drug due to sequestration by GSH (glutathione), metallothionein or other cytoplasmic molecules;
b. on-target effects
    i. involving decrease in DNA damage due to repair by the nucleotide excision repair (NER) system or homologous repair (HR)
    ii. tolerance to DNA damage allowing trans-lesional synthesis or replicative bypass;
c. post-target effects
    i. due to failure to activate apoptosis or by an increase in genes that block apoptosis;
d. off-target effects
    i. involving increase in antioxidant mechanisms or other stress responses.

1.4.1 REDUCED INTRACELLULAR ACCUMULATION – "PRE-TARGET" EFFECTS

Many resistant cell lines have less cisplatin accumulation in the cells (Andrews et al., 1988, Safaei and Howell, 2005). This could be caused by inhibition of drug uptake, inactivation of cisplatin or increase in efflux of the drug (Figure 1.3 and Table 1-1).

Cisplatin uptake

Cisplatin uptake is thought to occur mainly by passive diffusion (Gately and Howell, 1993). Transporters like the copper transporters CTR1 and CTR2 may play a more active role (Safaei and Howell, 2005, Kalayda et al., 2012, Kim et al., 2014) but the

extent of the involvement is still being investigated (Ivy and Kaplan, 2013). Until now, no microRNAs have been shown to be involved in the regulation of these genes.

Cisplatin efflux

One group of transporters involved with efflux from the cell are the ATP-binding cassette (**ABC transporters) transporters**. MicroRNA-130a (ABCB1) (Yang et al., 2012b, Li et al., 2015a), microRNA-130b (ABCB1) (Zong et al., 2014), miR-199a (ABCG2) (Cheng et al., 2012) and miR-128 (ABCC5) (Li et al., 2014a) are validated miRNA-target pairs. **Copper efflux transporters** ATP7A and ATP7B may also be associated with cisplatin response (Inoue et al., 2010, Abada et al., 2012, Li et al., 2012c). The resistance due to the ATP7B is thought to be due to binding and detoxification of cisplatin rather than efflux (Dolgova et al., 2009, Leonhardt et al., 2009, Dmitriev, 2011).

Reduced availability of cisplatin

Cisplatin may be detoxified through binding with other cellular compounds such as glutathione and metallothionein. The thiol groups of glutathione (GSH) bind to cisplatin and inactivate it (Paulusma et al., 1999). Deregulation of glutathione levels or that of the enzymes involved in the metabolism of glutathione and metallothionein can lead to modulation of the cisplatin response (Rocha et al., 2014, Sawers et al., 2014, Jamali et al., 2015, Tuzel et al., 2015, Lee et al., 2015).

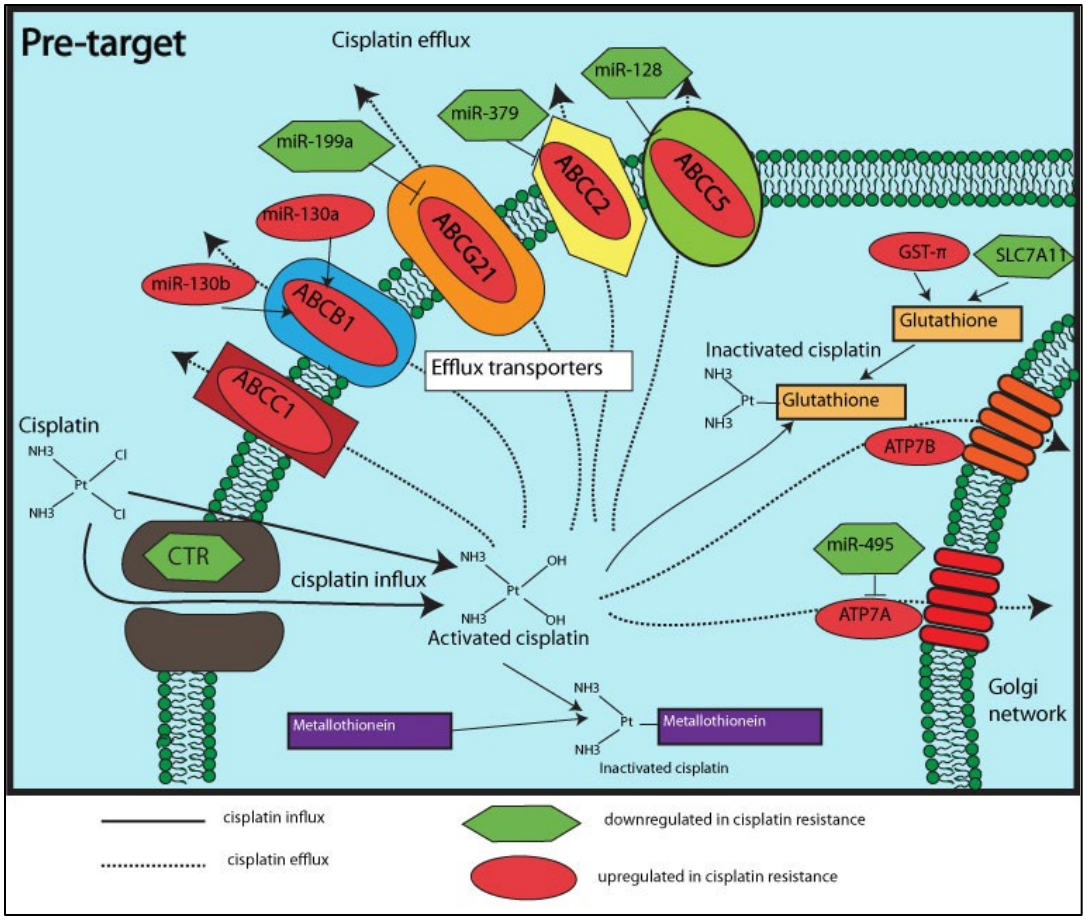

FIGURE 1.3: PRE-TARGET MECHANISMS OF CISPLATIN RESISTANCE

Cisplatin may be taken up by the cell passively or actively through the copper transporter CTR1. Once in the cell, cisplatin is converted to its highly reactive aquated form; this may be exported from the cell by efflux transporters of the ABC family - ABCB1, ABCG2, ABCC1, ABCC2 and ABCC5 or by the copper efflux transporters ATP7a and ATP7b; hence up-regulation of these transporters may be associated with cisplatin resistance. MicroRNAs that modulate these transporters may also be associated with resistance. Cisplatin may also be inactivated by glutathione or metallothionein which can cause resistance.

TABLE 1-1: GENES AND MICRORNAS INVOLVED IN CISPLATIN RESISTANCE THROUGH PRE-TARGET MECHANISMS OF ACTION

Gene	Gene function	Gene deregulation in resistance	MicroRNA	MiRNA Deregulation in resistance	Type of cancer	Reference
CISPLATIN UPTAKE						
CTR1 and 2	copper transporters; also uptake of cisplatin	down				(Kim et al., 2014, Kalayda et al., 2012)
SLC22A2	uptake of cisplatin	down				(Burger et al., 2011)
hMATE	uptake of cisplatin	down				(Burger et al., 2011)
CISPLATIN EFFLUX						
ABCC2	cisplatin efflux	up	miR-379	down	PBMC	(Werk et al., 2014, Ma et al., 2009)
ABCC5	cisplatin efflux	up	miR-128	down	ovarian	(Li et al., 2014a, Weaver et al., 2005)
ABCB1/MDR1	cisplatin efflux	up	miR-130a	up	ovarian	(Yang et al., 2012b, Patch et al., 2015)
ABCB1/MDR1	cisplatin efflux	up	miR-130a	up	ovarian	(Li et al., 2015a)
ABCB1/MDR1	cisplatin efflux	up	miR-130b	up	ovarian	(Zong et al., 2014)
ABCC1/ MRP1	cisplatin efflux	up				(Cai et al., 2011, Zhou and Ling, 2010)
ABCG2	cisplatin efflux	up	miR-199a	down	ovarian	(Cheng et al., 2012, Wu et al., 2015)
ATP7a	copper transporter - cisplatin efflux	up	miR-495	down		(Song et al., 2014, Li et al., 2012c)
ATP7B	copper transporter - cisplatin binding	up				(Safaei et al., 2012, Inoue et al., 2010)
CISPLATIN INACTIVATION						
GST-π	inactivation of cisplatin	up	miR-130b	up	ovarian	(Zong et al., 2014, Jamali et al., 2015, Rocha et al., 2014, Sawers et al., 2014)
SLC7A11	cysteine glutamate exchanger: inactivation of cisplatin	down	miR-27	up	bladder	(Drayton et al., 2014)
KEAP1		down	miR-141	up	ovarian	(van Jaarsveld et al., 2013)
metallothionein	inactivation of cisplatin	up	miR-23a	up	gastric	(An et al., 2013, Tuzel et al., 2015)

1.4.2 EXTENT OF DNA DAMAGE AND TOLERANCE– "ON-TARGET" MECHANISMS

The extent of DNA damage is determined by the amount of drug bound to DNA (Fraval and Roberts, 1979). However the effects of this damage can be decreased by increased DNA repair or by tolerance to the DNA damage. The overall DNA repair capacity in peripheral lymphocytes was shown to correlate inversely with overall survival in non-small-cell lung cancer treated with platinum chemotherapy (Wang et al., 2011a). The mechanisms of "on-target resistance" are described in section 1.4.2.1 and summarised in Figure 1.4 and Table 1.2.

1.4.2.1. Increased DNA repair

Intrastrand crosslinks make up 80% of the crosslinks caused by cisplatin while interstrand crosslinks (ICLs) make up less than 10% of cisplatin DNA adducts (Fichtinger-Schepman et al., 1987). Intrastrand crosslinks are removed by nucleotide excision repair (NER) or base excision repair (BER) while interstrand crosslinks are repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) (Ceccaldi et al., 2015). These DNA repair pathways influence DNA repair and consequently, cisplatin sensitivity. The genes and microRNAs involved in modulating these pathways are listed in Figure 1.4 and Table 1.2.

Nucleotide excision repair (NER) pathway recognises and repairs DNA damage caused by cisplatin; enhanced activity of genes in this pathway can increase cisplatin resistance. **Base Excision repair** (BER) could play a role in the extent of DNA cisplatin crosslinks. By this mechanism, platinum is removed as soon as it binds to the DNA before crosslinks are formed (Caiola et al., 2015). MicroRNAs involved in modifying these pathways are shown in Figure 1.4 and Table 1.2.

H**omologous Repair** (HR) results in highly accurate repair using the sister chromatid as a template strand. A study by Wang et al (2011b) suggests that blocking homologous repair results in far greater cisplatin sensitivity than blocking NER. **Non Homologous End Joining** (NHEJ) is a process by which the two ends are simply joined together repairing double stranded breaks in an error prone fashion. MiR-101 has been shown to downregulate NHEJ in lung cancer cells (Yan et al., 2010). **DNA mismatch repair** (MMR) system is involved in the recognition of DNA damage caused by cisplatin

(Stewart, 2007). However, upregulation of genes involved in this pathway are associated with increase in chemosensitivity; it is possible that the MMR system attempts to repair the DNA damage but failing to do so, activates apoptosis (Vaisman et al., 1998, Fink et al., 1998, Adachi et al., 2010, Zeller et al., 2012).

The role of BRCA1/ BRCA2 in the mechanisms of repair of DNA damage in relation to cisplatin resistance and ovarian cancer is worthy of special mention. BRCA1 is essential for HR but also is suggested to play a role in NHEJ while BRCA2 primarily facilitates and is essential for HR (Roy et al., 2012). The effect of a germline mutation of BRCA1/ BRCA2 on ovarian cancer is two-fold – on the one hand, it increases the risk of ovarian cancer (King et al., 2003); however, the same defect in homologous repair makes these tumours sensitive to cisplatin. It has been shown that in tumours with BRCA 1/2 mutation which develop resistance to cisplatin, there is a second deletion mutation reverting to wild-type BRCA reading frame (Edwards et al., 2008, Sakai et al., 2008, Swisher et al., 2008, Sakai et al., 2009). BRCA 1 or 2 mutation is associated with significantly improved progression free survival (Vencken et al., 2011, Vencken et al., 2013, Rudaitis et al., 2014, Harter et al., 2016). While BRCA1/2 defects may arise through germline or somatic mutations as well as hypermethylation, Gu et al have shown a miRNA signature of miR-146a, miR-148a and miR-545 that target BRCA1 or 2 is associated with overall survival and progression free survival in wild type BRCA1/2 ovarian tumours (Gu et al., 2015, Moschetta et al., 2016). Similarly Sun et al have shown that miR-9 targets BRCA1 and is associated with progression free survival as well as platinum sensitivity in ovarian cancers (Sun et al., 2013). These studies suggest that miRNA modulation of BRCA may also play a role in chemosensitivity.

1.4.2.2. Tolerance to DNA damage

Translesion synthesis (TLS) allows the cell to bypass the lesion by "replicative bypass or trans-lesion synthesis"(Clauson et al., 2013). miR-93 - *POLH* (Srivastava et al., 2015) and miR-96 - *REV-1* (Wang et al., 2012) are miRNA-target pairs shown to increase chemoresistance. POLH and REV-1 are genes involved in translesional synthesis.

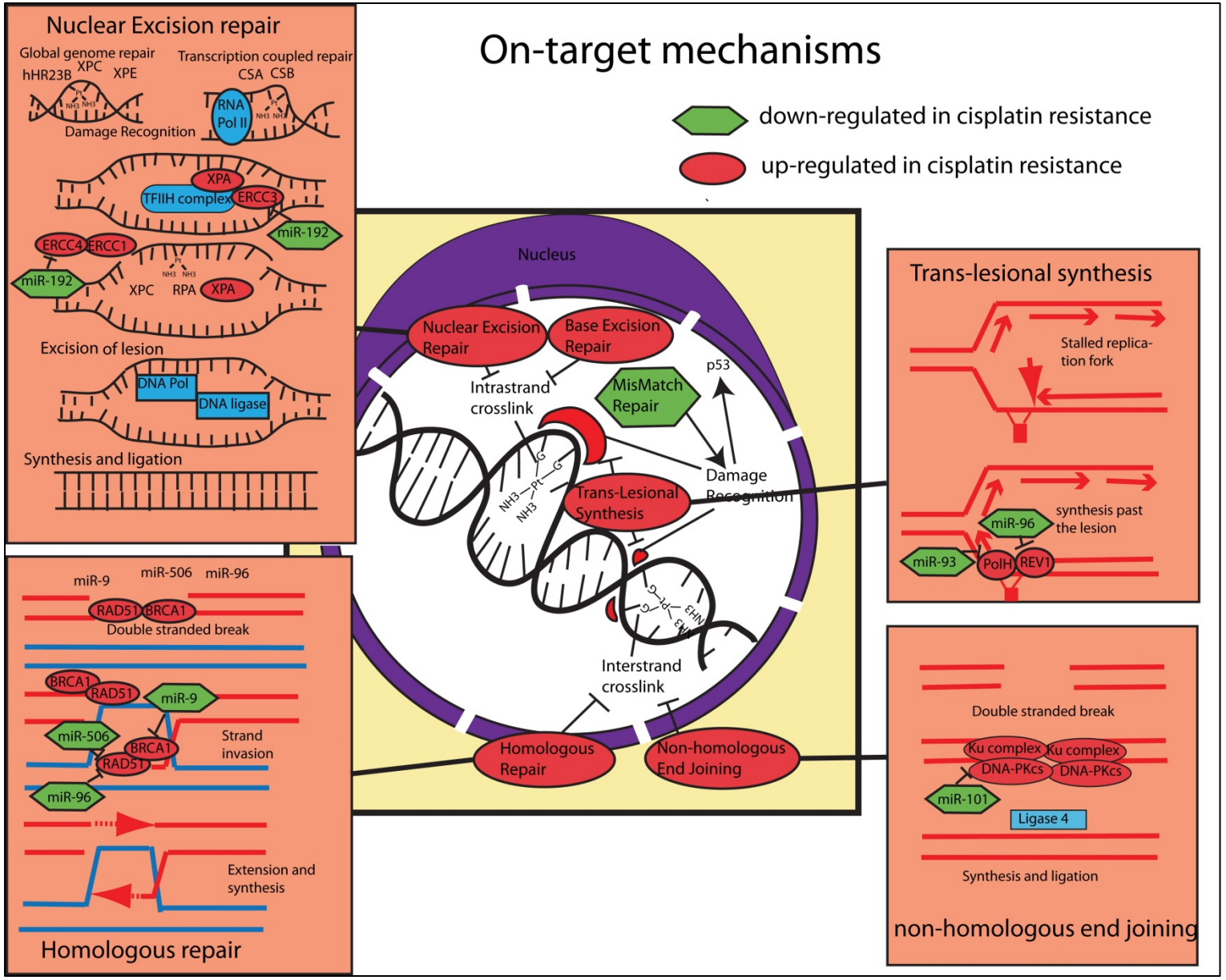

FIGURE 1.4: ON-TARGET MECHANISMS OF CISPLATIN RESISTANCE

The extent of DNA damage caused by cisplatin can be reduced by the action of repair pathways – nuclear excision repair pathway, homologous repair pathway, non-homologous end joining. Trans-lesional synthesis allows tolerance of the damage. The genes involved and the microRNAs are shown.

TABLE 1-2: GENES AND MICRORNAS INVOLVED IN CISPLATIN RESISTANCE THROUGH ON-TARGET MECHANISMS OF ACTION

Gene	Gene function	Gene deregulation in resistance	MicroRNA	MiRNA Deregula-tion in resistance	Type of cancer	Reference
ERCC1	NER	up				(Lee et al., 1993a, Arora et al., 2010)
XPA	NER	up				(Ferry et al., 2000, Lee et al., 1993a)
ERCC3	NER	up	miR-192	down	hepatoma	(Xie et al., 2011, Arora et al., 2010)
ERCC4	NER	up	miR-192	down	hepatoma	(Xie et al., 2011)Xie et al., 2011
ENG	upregulates SIRT1 (XPA deacetylation) NER	up	miR-370	down	ovarian	(Chen et al., 2014b, Ziebarth et al., 2013)
BRCA1	HR	up	miR-9	down	ovarian	(Sun et al., 2013, Rudaitis et al., 2014, Vencken et al., 2013)
BRCA2	HR	up				(Vencken et al., 2013, Rudaitis et al., 2014)
RAD51	HR	up	miR-506	down	ovarian	(Liu et al., 2015)
RAD51	HR	up	miR-96	down	ovarian	(Wang et al., 2012)
Ku complex	NHEJ	up				(Li et al., 2015b, Tian et al., 2007)
DNA-PKcs	NHEJ	up	miR-101	down	lung cancer	(Tian et al., 2007, Yan et al., 2010, Li et al., 2015b)
POLH (Pol η)	TLS	up	miR-93	down	ovarian	(Srivastava et al., 2015, Wang et al., 2015b)
REV1	TLS	up	miR-96	down	ovarian	(Wang et al., 2012)
MLH1	MMR; signal for apoptosis	down	miR-155	up	colorectal cancer	(Valeri et al., 2010, Zeller et al., 2012)
MSH2	MMR; signal for apoptosis	down				(Zeller et al., 2012)

1.4.3 REDUCED APOPTOTIC RESPONSE – "POST-TARGET" MECHANISMS

Cisplatin induces apoptosis through the caspase cascade via two different pathways- fas/fas ligand signalling (extrinsic pathway) or cytochrome–c release (intrinsic pathway). In cells with defective apoptosis systems, there is an increase in the levels of DNA damage required to initiate apoptosis (Gonzalez et al., 2001). The main components of the pathways are described below and summarised in Table 1-3 and Figure 1.5.

1.4.3.1. p53

p53 is a major coordinator in the initiation of the intrinsic apoptotic pathway and is thought to usually support apoptosis (Fan et al., 1994, Segal-Bendirdjian et al., 1998); however some studies are contrary and show that disruption of p53 function sensitises to cisplatin (Fan et al., 1995, Hawkins et al., 1996). miR-34, known to be a tumour suppressor, may be induced by p53 activity but can also, in turn, increase the activity of p53, forming a positive feedback loop (Yamakuchi and Lowenstein, 2009).

1.4.3.2. Cell cycle arrest

ATM and ATR, in addition to activating p53 also cause a cell cycle arrest through mediating checkpoints (Meek, 2015). This disruption in cell cycle points is essential for DNA damage repair and may lead to resistance (Shah and Schwartz, 2001).

1.4.3.3. Bcl2 family

Modulation of the Bcl2 family of apoptotic regulators may also contribute to resistance. Down-regulation of the pro-apoptotic members (Bax, Bad, Bak) or up-regulation of anti-apoptotic members (Bcl2, Bcl-xl, Bcl-w and Mcl-1) can be associated with resistance (Hata et al., 2015). MicroRNAs modulating this pathway are summarised in Table 1-3.

1.4.3.4. PI3K-Akt pathway

PI3K-Akt pathway is a major modulator of the apoptotic cascade as reviewed in (Steelman et al., 2011). Akt, induced by PI3K promotes *MDM2*; this decreases p53 activation, inactivates pro-apoptotic Bad protein and inhibits procaspase-9 thus reducing intrinsic apoptosis (Mayo and Donner, 2001). Akt also affects the extrinsic apoptotic pathway by causing Foxo-3 protein to re-localise to the cytoplasm, which causes down regulation of fas ligand and therefore, decreased apoptosis (Steelman et

al., 2011). Other proteins are known to negatively regulate Akt such as PTEN, ALK7 (Zhang et al., 2006) and PDCD4 (Wei et al., 2012). mTOR (mammalian target of rapamycin) is a downstream target of the PI3K/ Akt pathway and is shown to modulate resistance to cisplatin (Dobbin and Landen, 2013). Various microRNAs summarised in Table 1-3 are shown to target Akt and associated proteins.

1.4.3.5. Inhibitors of apoptosis

Apoptosis can also be decreased by Inhibitors of Apoptosis proteins (IAPs) such as XIAP (Asselin et al., 2001) and survivin (Karczmarek-Borowska et al., 2005, Ikeguchi et al., 2002) which may be regulated by microRNAs.

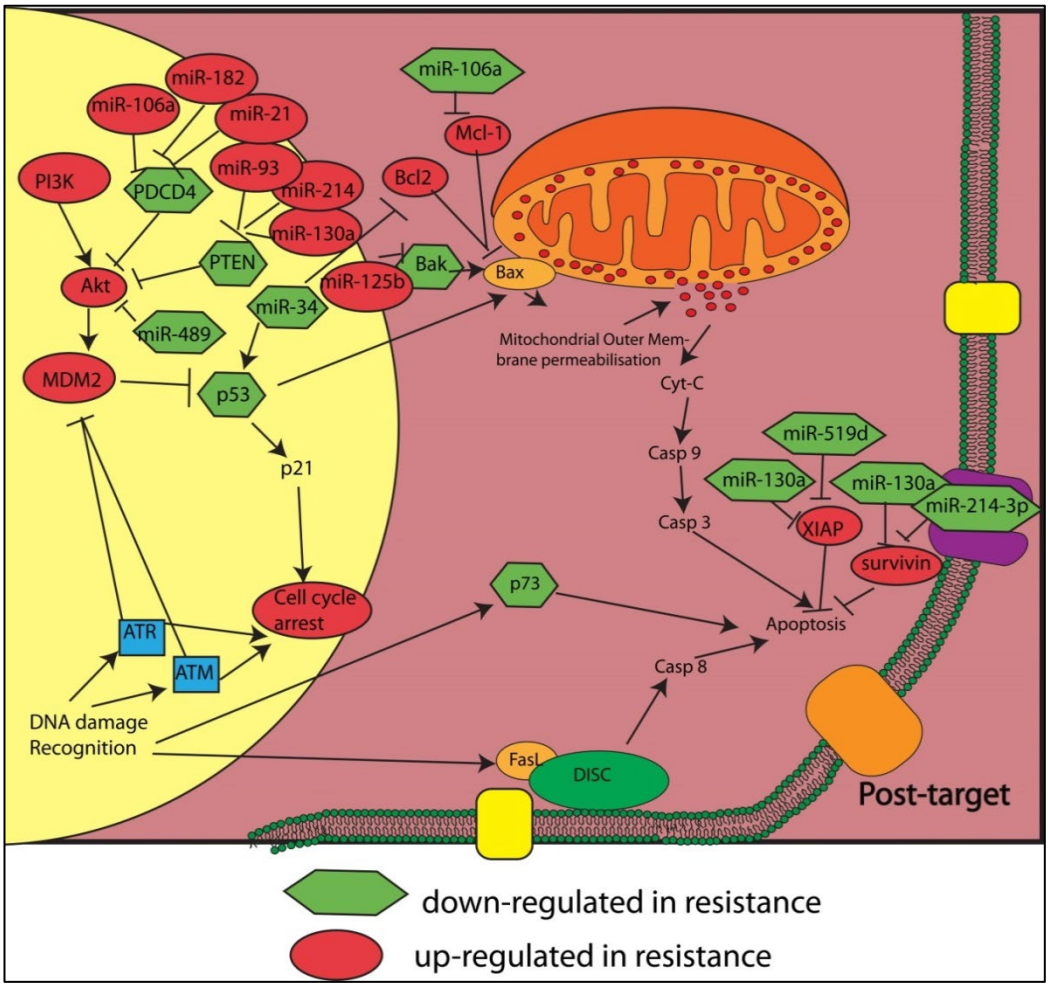

FIGURE 1.5: POST-TARGET MECHANISMS OF RESISTANCE

Once DNA damage is recognised, p53 is activated which in turn activates the apoptotic cascade. PI3K-Akt pathway can decrease p53 and cause resistance; Akt in turn may be downregulated by PTEN and PDCD4. The antiapoptotic Bcl2 and Mcl1 can decrease apoptosis while Bak-I can increase apoptosis. Inhibitors of apoptosis such as XIAP and survivin may also inhibit apoptosis. The miRNAs that affect these pathways are shown in the figure. PTEN – phosphatase and tensin homolog, PDCD4 - Programmed Cell Death 4, XIAP – x-linked inhibitor of apoptosis, Cyt-c - Cytochrome C

TABLE 1-3: GENES AND MICRORNAS INVOLVED IN CISPLATIN RESISTANCE THROUGH POST-TARGET MECHANISMS OF ACTION

Gene	Gene function	Gene deregulation in resistance	MicroRNA	MiRNA Deregula-tion in resistance	Type of cancer	Reference
p53	coordinator of apoptosis	usually down	miR-34	down		(Yamakuchi and Lowenstein, 2009)
CSF1	enhances effects of p53	up	miR-130b	down	ovarian	(Yang et al., 2012a)
TP53INP1	limits activation of p53, p73 and NF-κB	down	miR-569	up	ovarian	(Chaluvally-Raghavan et al., 2014)
CDC6	checkpoint mediated growth arrest	up	miR-29a	down	ovarian	(Creighton et al., 2012, Kan et al., 2008)
TIMELESS		up	miR-29a	down	ovarian	(Creighton et al., 2012, Yoshida et al., 2013)
Bcl2	Antiapoptotic	up	miR-34	down	gastric and pancreatic	(Ji et al., 2009, Ji et al., 2008)
Mcl-1	Antiapoptotic	up	miR-106a	down	ovarian	(Rao et al., 2013)
Bak1	Proapoptotic	down	miR-125b	up	ovarian	(Kong et al., 2011)
CCND1	maintenance of anti-apoptotic Bcl2 and Bcl-xl	up	let-7e	down	ovarian	(Cai et al., 2013, Biliran et al., 2005)
Akt3	decreases p53 activation	up	miR-489	down	ovarian	(Wu et al., 2014)
PTEN	downregulates Akt	down	miR-130a	up	ovarian	(Li et al., 2015a)
PTEN	downregulates Akt	down	miR-214	up	ovarian	(Yang et al., 2008)
PTEN	downregulates Akt	down	miR-93	up	ovarian	(Fu et al., 2012)
ALK7	downregulates Akt	down	miR-376c	up	ovarian	(Ye et al., 2011)
PDCD4	downregulates Akt	down	miR-106a	up	ovarian	(Li et al., 2014c)
PDCD4	downregulates Akt	down	miR-182	up	ovarian	(Wang et al., 2013a)
PDCD4	downregulates Akt	down	miR-21	up	ovarian	(Chan et al., 2014)
PDCD4	downregulates Akt	down	miR-21	up	ovarian	(Echevarria-Vargas et al., 2014)

Table 3.1 contd..

Gene	Gene function	Gene dereg-ulation in resistance	MicroRNA	MiRNA Deregula-tion in resistance	Type of cancer	Reference
PDCD4	downregulates Akt	down	miR-21	up	ovarian	(Liu et al., 2013a)
mTOR	target of AkT	?	mir-199a		ovarian	(Wang et al., 2013b)
mTOR	target of AkT	?	miR-497	?	ovarian	(Xu et al., 2015a)
XIAP	Inhibitor of apoptosis	up	miR-130a	down	ovarian	(Zhang et al., 2013)
XIAP	Inhibitor of apoptosis	up	miR-519d	down	ovarian	(Pang et al., 2014)
Survivin	inhibitor of apoptosis	up	miR-335	down	gastric	(Yang et al., 2015)
Survivin	inhibitor of apoptosis	up	miR-214-3p	down	oesophageal cancer	(Phatak et al., 2015)
COL1A1	ERK signalling resist apoptosis	up	miR-29a/b/c	down	ovarian	(Yu et al., 2013)

1.4.4 OTHER FACTORS LINKED TO PLATINUM RESISTANCE – "OFF-TARGET" MECHANISMS

De-regulation of some cellular pathways which are not directly involved in processing of cisplatin or responses to cisplatin damage are shown to increase resistance to chemotherapy. These "off-target" mechanisms are of significant interest. The off-target mechanisms are described below and summarised in Table 1-4.

1.4.4.1. Autophagy

Autophagy is one of the cellular responses to stress in which organelles are consumed within lysosomes recycling damaged components (Cecconi and Levine, 2008). In cancer chemotherapy, however, autophagy is one of the mechanisms of programmed cell death and can modulate cisplatin cytotoxicity by increasing sensitivity to the drug (Garcia-Cano et al., 2015, Leisching et al., 2015). In contrast, some studies have shown that enhanced autophagy can cause increased resistance to cisplatin (Ren et al., 2010). MicroRNAs modulating this response are shown in Table 1-4.

1.4.4.2. Epithelial-mesenchymal transition

Epithelial–mesenchymal transition (EMT) is an important process in the invasion and metastasis of cancer cells and is characterised by a loss of epithelial properties of cancer cells such as cell-cell adhesion and a gain of mesenchymal markers and properties such as greater motility as reviewed by De Craene and Berx (2013). EMT, additionally has been shown to have an important role in chemoresistance (Fischer et al., 2015). A recent study has identified a group of eight microRNAs, including miR-200a and miR-506, deregulation of which may be involved in the maintenance of a mesenchymal subtype (Yang et al., 2013a, Sun et al., 2015,). Other microRNAs involved are summarised in Table 1-4.

1.4.4.3. Cancer Stem cells

Cancer stem cells are resistant to chemotherapy and can influence response to cisplatin in spite of forming only a small proportion of cells in the tumour (Bapat et al., 2005, Yan et al., 2014). Table 1-4 shows microRNAs involved in this pathway.

1.4.4.4. Tumour microenvironment

Most of the mechanisms discussed thus far modify the cell itself; other mechanisms may cause chemoresistance by modifying the tumour microenvironment. One case in point is miR-484 which induces cisplatin sensitivity (Vecchione et al., 2013) *in vivo* but not *in vitro*. It was discovered that this microRNA, secreted by the tumour cells, is taken up by the endothelial cells and causes a decrease in angiogenesis by suppressing VEGFB and VEGFR; this leads to hypoxia and necrosis of the tumour cells.

1.4.4.5. Other mechanisms

Other microRNAs have been shown to modify the response of ovarian cancer tumours to cisplatin through interaction with components of the chromatin modifying complexes or transcription factors. These are summarised in Table 1-4.

TABLE 1-4: GENES AND MICRORNAS INVOLVED IN CISPLATIN RESISTANCE THROUGH OFF-TARGET MECHANISMS OF ACTION

Gene	Gene function	Gene deregulation in resistance	MicroRNA	MiRNA Deregulation in resistance	Type of cancer	Reference
mTOR	downregulates autophagy	up	miR-199a	down	ovarian	(Wang et al., 2013b)
mTOR	downregulates autophagy	up	miR-497	down	ovarian	(Xu et al., 2015a)
ATG14	upregulates autophagy	up	miR-152	down	ovarian	(He et al., 2015)
Twist1	regulation of EMT	up	miR-186	down	ovarian	(Zhu et al., 2015)
ZEB1	regulation of EMT	up	miR-200	down	ovarian	(Jabbari et al., 2014)
CRIM1	affects control of EMT	down	miR-193b*	up	ovarian	(Ziliak et al., 2012)
Notch1	Modulation of ovarian cancer stem cells	up	miR-449a	down	ovarian	(Zhou et al., 2014)
JAG	Modulation of ovarian cancer stem cells	up	miR-199b-5p	down	ovarian	(Liu et al., 2014b)
CD44	marker of cancer stem cells	up	miR-199a	down	ovarian	(Cheng et al., 2012)
VEGFB	affects angiogenesis	up	miR-484	down	ovarian	(Vecchione et al., 2013)
VEGFR2	affects angiogenesis	up	miR-484	down	ovarian	(Vecchione et al., 2013)
HDAC4	chromatin modifying complexes	down	miR-302b	up	ovarian	(De Cecco et al., 2013)
EZH2	chromatin modifying complexes	up	let-7e	down	ovarian	(Cai et al., 2013)
EZH2	chromatin modifying complexes	up	miR-101	down	ovarian	(Liu et al., 2014a)
DNMT1	chromatin modifying complexes	up	miR-152	down	ovarian	(Xiang et al., 2014)
DNMT1	chromatin modifying complexes	up	miR-185	down	ovarian	(Xiang et al., 2014)
DNMT3A	chromatin modifying complexes	up	miR-29a	down	ovarian	(Creighton et al., 2012)
DNMT3B	chromatin modifying complexes	up	miR-29a	down	ovarian	(Creighton et al., 2012)
CBX1	chromatin modifying complexes	up	miR-29a	down	ovarian	(Creighton et al., 2012)
MYBL2	transcription	up	miR-29a	down	ovarian	(Creighton et al., 2012)
Bmi-1	may be involved in EMT or maintenance of cancer stem cells	up	miR-128	down	ovarian	(Li et al., 2014a)

1.4.5 *MicroRNAs and cisplatin resistance in ovarian tumours*

In ovarian tumours, while various microRNAs have been shown to be associated with cisplatin resistance, there is no single specific miRNA signature to predict cisplatin resistance (Creighton et al., 2012). This reflects the complex nature of cisplatin resistance and therefore the many miRNAs involved in modulation of this resistance. Some of the microRNAs highlighted in chemo resistance have previously been shown to be deregulated in ovarian cancers such as the miR-200 family (Muralidhar and Barbolina, 2015), let-7 family (Wang et al., 2012), miR-214 (Penna et al., 2015) and miR-21 (Gao et al., 2016). The studies listed in this section for the various mechanisms of cisplatin resistance have used different models to establish the role of the microRNAs such as cell line studies, xenografts, quantification in ovarian tissues or in analysis of publically available datasets such as TCGA. While cell line studies are useful to identify potential microRNAs, clinical relevance is usually indicated by *in vivo* studies. miR-484 has been shown to affect cisplatin resistance *in vivo* by affecting angiogenesis in xenograft models (Vecchione et al., 2013). miR-9 also appears to play an important role in chemoresistance by downregulating BRCA1 in xenografts (Sun et al., 2013). Some microRNAs such as miR-199a have been shown by two different labs to show a similar deregulation increasing the degree of confidence in the biological relevance of these miRNAs (Cheng et al., 2012, Wang et al., 2013). Though there is no complete consensus on which microRNAs can be used as biomarkers to predict chemotherapy response, as more studies highlight the various mechanisms of pathways by which microRNAs affect cisplatin response, there is the possibility that a panel of microRNAs may distinguish the drug response characteristics of ovarian tumours, and thus enable effective choice of drugs.

The above described summary provides a glimpse into the complexity of cisplatin resistance modulation and the possible variations in the types of tumours and resistance mechanisms; the tables indicate the possibility that many more microRNAs could potentially be involved in this regulation of cisplatin response. In order to successfully tackle the problem of cisplatin resistance, more research is needed to understand and exploit this tier of modulation.

Interestingly, microRNAs can be transferred between cells by means of membrane bound vesicles called extracellular vesicles (EVs) as described in section 1.5.3. This provides an exciting avenue of research regarding the interaction between cells in the acquisition of cisplatin resistance. The next section describes EVs and their functions with special reference to their involvement in drug resistance.

1. 5. EXTRACELLULAR VESICLES

Extracellular vesicles are membrane contained vesicles released by cells into the extracellular environment; they are shown to contain proteins, lipids, RNA and sugars and can thus transfer information between cells (Fevrier and Raposo, 2004, Yanez-Mo et al., 2015).

EVs were first described in the process of transferrin receptor shedding from reticulocytes (Harding et al., 1984) as a means of eliminating obsolete proteins (Johnstone et al., 1991). Later it was discovered that a similar process of EV release in antigen presenting cells could functionally increase T- cell proliferation in *in vitro* studies (Raposo et al., 1996) as well as induce anti-tumour responses *in vivo* (Zitvogel et al., 1998). Since then it has been shown that EVs are released by most tested cell lines (Chaput and Thery, 2011, Samir EL-Andaloussi et al., 2013).

1.5.1. EXTRACELLULAR VESICLES – TYPES AND BIOGENESIS

EVs are identified based on morphological and biochemical criteria. Morphologically, they are membrane bound vesicles with a diameter of 50-90 nm (Fevrier and Raposo, 2004). They are thought to be of three types (Yanez-Mo et al., 2015):

a. Microvesicles/ microparticles/ ectosomes: these are caused by an outward budding and fission of the plasma membrane
b. Exosomes: these are formed within the endosomal network and released by the fusion of multivesicular bodies with the plasma membrane (Johnstone et al., 1987)
c. Apoptotic bodies: these are blebs from cells undergoing apoptosis.

Though these three types of vesicles have different characteristics, most EV isolation procedures yield a mixture of all three types; therefore there is a lack of consensus on specific markers for each type (Yanez-Mo et al., 2015).

Microparticle biogenesis involves budding out from the plasma membrane and release as vesicles; they tend to be 0.1 μM – 1 μM in size (Gong et al., 2013). Their contents include plasma membrane, surface proteins and cytoplasmic material from the parent cell (Bebawy et al., 2009, Jaiswal et al., 2012). **Exosome** biogenesis involves a series of steps: initiation, endocytosis, multi-vesicular body (MVB) formation and exosome secretion (Johnstone et al., 1987, Kharaziha et al., 2012,). These are shown in Figure 1.6. The MVB formation is dependent on Tsg101 while hepatocyte growth factor substrate (Hrs) is essential for accumulation of ILVs (intraluminal vesicles) in the MVBs (Razi and Futter, 2006). The MVBs then may be degraded by fusion with a lysosome, fuse with the golgi network for recycling of cargo or fuse with the plasma membrane resulting in release of exosomes (Kharaziha et al., 2012). **Apoptotic Bodies** are formed by separation of the blebs formed by cells undergoing programmed cell death or apoptosis.

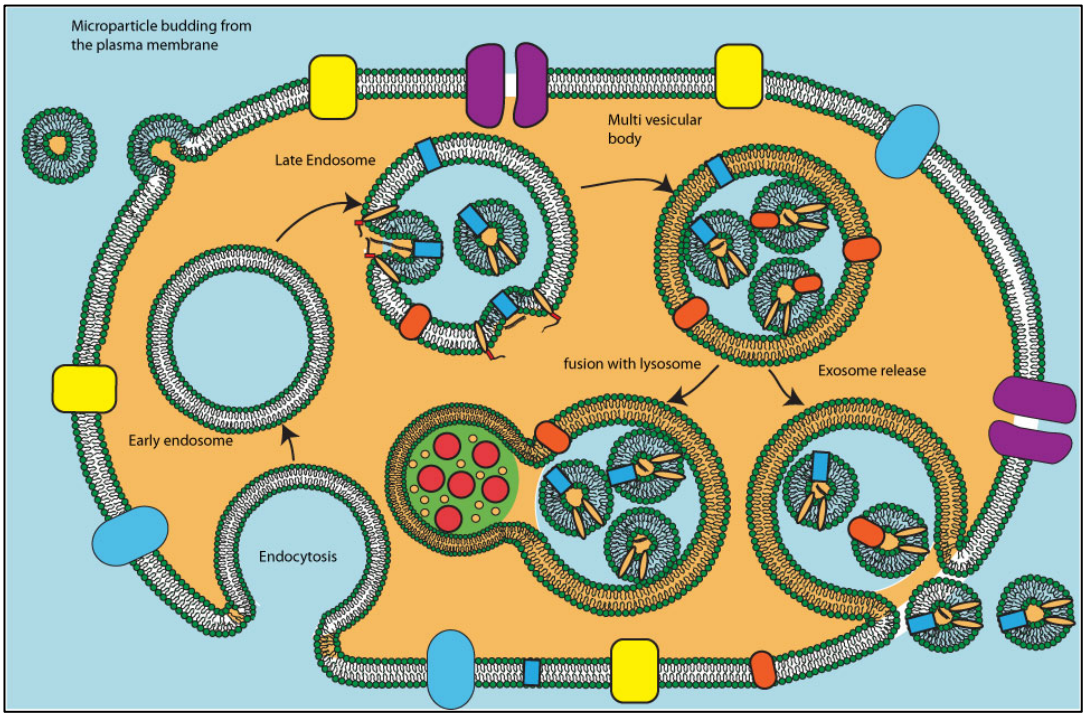

FIGURE 1.6: MICROPARTICLE AND EXOSOME BIOGENESIS
Microparticles are generated by budding of the plasma membrane. Exosome biogenesis involves invagination of the plasma membrane to form endosomes, invagination within the endosomes to form a multi vesicular body. The multivesicular body can fuse with the plasma membrane to release exosomes or fuse with a lysosome for degradation.

1.5.2. EXTRACELLULAR VESICLE ISOLATION:

EVs have been identified in plasma, serum, amniotic fluid, saliva and urine as well as in supernatant from *in vitro* cell cultures as reviewed in Yanez-Mo et al (Yanez-Mo et al., 2015). The most common method used for isolation of EVs is differential ultracentrifugation in which the supernatants of viable cell cultures are centrifuged at low speed to remove cells and cell debris; EVs are then pelleted at 100 000 g (Raposo et al., 1996, Fevrier and Raposo, 2004). Additionally, any protein can then be removed by flotation on a sucrose gradient – EVs float at a density of 1.13 g/ml to 1.19 g/ml (Fevrier and Raposo, 2004, Kharaziha et al., 2012). Other methods described include size exclusion chromatography, filtration and polymer based precipitation (Taylor and Shah, 2015, Szatanek et al., 2015).

1.5.3. CONTENTS OF EXTRACELLULAR VESICLES:

EVs contain lipids, protein, nucleic acids and sugars as reviewed in Yanez-Mo et al (Yanez-Mo et al., 2015).

1.5.3.1. **Proteins:** Various studies have characterised the protein content of EVs by proteomic analysis (Wubbolts et al., 2003, Mears et al., 2004, Staubach et al., 2009, Graner et al., 2009). EVs contain common proteins reflecting their biogenesis as well as specific proteins characteristic of cell / tissue type. These are summarised in Table 1-5. These proteins have been used as markers for extracellular vesicles. In addition EVs derived from different cell types contain other specific proteins. Sorting of proteins into MVBs and EVs based on the local membrane curvature determines the shape size and composition of EVs (Ramamurthi, 2010, Yanez-Mo et al., 2015). Tetraspannins are thought to influence the assimilation of receptors into EVs (Perez-Hernandez et al., 2013). ESCRT proteins are also thought to sort proteins into EVs based on ubiquitination of the protein (Katzmann et al., 2001, Reggiori and Pelham, 2001).

TABLE 1-5: PROTEIN CARGO IN EVS

Protein class	proteins	function	reference
Tetraspannins	CD9, CD63, CD81, CD82	indicate the origin of EVs from MVBs	(van Niel et al., 2001)
Intracellular membrane transport and signalling proteins	heat shock proteins (hsc73), annexins, Gi2α		(Thery et al., 1999, Thery et al., 2001)
Lipid raft associated proteins	CD55 and CD59	protect EVs from complement mediated lysis	(Clayton et al., 2003, Rabesandratana et al., 1998)
	CD109		(Sakakura et al., 2015)
	HSP70		(Staubach et al., 2009)
Antigen presentation molecules	MHC class II and class I molecules		(van Niel et al., 2001)
ESCRT proteins	Alix, TSG101	biogenesis, cargo sorting and release of EVs, indicate origin of EVs from MVBs	(Thery et al., 2001)

1.5.3.2. **Nucleic acids:** Most studies indicate the presence of RNA of different types in the extracellular vesicles - including intact mRNA, mRNA fragments, long non coding RNA and microRNAs.

- **mRNA:** EVs have been shown to transfer functional mRNA between cells (Ratajczak et al., 2006, Valadi et al., 2007, Ridder et al., 2015). In a study using breast cancer cells, EVs were shown to transfer mRNA with metastatic and oncogenic potential (Rodriguez et al., 2015). EVs were also shown to contain inhibitors of apoptosis (Valenzuela et al., 2015). It has been suggested that there is an enrichment of 3'UTR mRNA fragments in EVs (Batagov and Kurochkin, 2013). A specific consensus sequence consisting of a 25 nucleotide sequence with a short CTGCC core domain with a miR-1289 binding site is thought to target mRNAs into EVs (Bolukbasi et al., 2012).
- **microRNA:** One study showed that there are, on average, 0.00825 microRNAs in an EV; i.e. about 1 microRNA in a hundred EVs (Chevillet et al., 2014). Studies suggest that miRNAs sorting into EVs might involve EXOmotif GGAG and its binding to sumoylated hnRNPA2B1 (Villarroya-Beltri et al., 2013). Studies have shown that the microRNAs transferred via EVs are functional once internalised into the recipient cell and can repress

target mRNAs (Montecalvo et al., 2012, Stoorvogel, 2012, Chen et al., 2014a). Taylor and Gercel-Taylor showed that EVs derived from the blood of ovarian cancer patients had increased miRNA levels which mirrored the miRNA composition of the original tumour (Taylor and Gercel-Taylor, 2008). miR-451 and miR-21 were shown to be transferred from glioblastoma by EVs which were avidly taken up by microglia (van der Vos et al., 2016). In a recent study, the cargo of microparticles was shown to include, in addition to miRNAs, transcripts encoding for enzymes in the miRNA biogenesis machinery – DROSHA, DICER and Ago2 (Jaiswal et al., 2012). Melo et al showed that, EVs derived from cancer cells also contained precursor miRNAs and DICER, Ago2 and TRBP and that the pre-miRNAs were processed into mature miRNAs in the EVs (Melo et al., 2014). It has been suggested that there is an enrichment of 3'UTR mRNA fragments in EVs; the potential implication is that this may cause microRNA modulation in the recipient cell as these fragments can compete with the recipient cellular RNA for miRNA binding (Batagov and Kurochkin, 2013).

- **lncRNA:** Long non-coding RNAs with low expression levels in cells are found to be enriched in EVs (Gezer et al., 2014).

1.5.3.3. **Lipids:** EVs are shown to contain sphingomyelin, ceramide, cholesterol and glycolipid GM3 (Wubbolts et al., 2003) which are associated with lipid rafts.

1.5.3.4. **Glycosylation:** The glycosylation patterns of EVs were shown to differ from parent cell membrane (Krishnamoorthy et al., 2009, Batista et al., 2011); moreover, the pattern of glycosylation was shown to be altered in pathological conditions (Escrevente et al., 2011). It has been suggested that EV uptake by target cells may be influenced by glycosylation patterns (Batista et al., 2011, Escrevente et al., 2011, Yanez-Mo et al., 2015).

1.5.4. EXTRACELLULAR VESICLE UPTAKE

EVs affect other cells by surface interaction or by internalisation or uptake. There is a growing body of research suggesting that the contents of extracellular vesicles are transferred into the cell – functional mRNA is transferred to recipient cells (Valadi et al., 2007), siRNA delivered through EVs knockdown gene targets and luciferin substrate delivered via EVs induced bioluminescence in luciferase expressing mouse

dendritic cells (Montecalvo et al., 2012). The uptake of EVs is studied by using lipid membrane dyes such as PKH26 or chemical compounds such as CFSE to stain the EVs and quantify their presence in the recipient cells.

EV uptake is thought to involve protein interactions with target cell membrane (Christianson et al., 2013, Svensson et al., 2013). Proteins that have been proposed to be involved in uptake include tetraspanins (Rana et al., 2012), integrins such as CD11a, CD51 and CD61 (Morelli et al., 2004) and proteoglycans such as heparan sulfate (Christianson et al., 2013). Various mechanisms have been proposed for the uptake of EVs by recipient cells as reviewed in (Mulcahy et al., 2014) and summarised below. Indeed, uptake is thought to occur through more than one mechanism (Escrevente et al., 2011); therefore internalisation cannot be entirely prevented by one inhibitor.

1.5.4.1. **Phagocytosis:** This involves the uptake of opsonised particulate matter by phagocytic cells such as macrophages (Swanson, 2008); this has been thought to be a mechanism of EV uptake (Feng et al., 2010); this is further supported by the fact that dynamin inhibition decreases the uptake of EVs (Feng et al., 2010, Fitzner et al., 2011).

1.5.4.2. **Clathrin mediated endocytosis** is one of the mechanisms thought to play a role in extracellular vesicle uptake. This involves invagination of the outer membrane by the sequential assembly of clathrin along with heterotetrameric clathrin adaptor AP2 and Eps15 (Cocucci et al., 2012) followed by cargo capture facilitated by interaction with receptors (Traub and Bonifacino, 2013); dynamin facilitates the pinching off of the vesicle into the cytoplasm (Morlot and Roux, 2013); auxilin and hsc70 (a member of the hsp70 family) assist the uncoating of the clathrin coated vesicles within the cell (Kirchhausen et al., 2014). The involvement of clathrin mediated endocytosis in uptake of extracellular vesicles is indicated by various studies: chlorpromazine, which inhibits clathrin mediated endocytosis, decreases uptake of extracellular vesicles in ovarian cancer recipient cells (Escrevente et al., 2011); inhibition of dynamin2 in phagocytic cells inhibited EV internalisation (Feng et al., 2010, Fitzner et al., 2011), expression of a dominant negative mutant of Eps15 led to a reduction in EV uptake (Feng et al., 2010).

1.5.4.3. **Caveolin dependent endocytosis** (CDE) is shown to be a mechanism of uptake of particles. It involves cave-like invagination of the plasma membrane which can be internalised into the cell; the caveolae are shown to be enriched in cholesterol and sphingolipids and are sensitive to cholesterol depletion. The main proteins involved in caveolae formation are caveolin-1 and cavin-1; dynamin facilitates the assembly and internalisation of the caveolae (Lajoie and Nabi, 2007, Briand et al., 2011). Inhibition of dynamin2 decreases uptake of EVs (Feng et al., 2010, Fitzner et al., 2011). Specific knockdown of caveolin-1 decreased the uptake of EVs in a nasopharyngeal cell line (Nanbo et al., 2013); but increased EV uptake in mouse embryonic fibroblast cells (Svensson et al., 2013), indicating CDE as a possible cell specific mechanism of EV uptake.

1.5.4.4. **Macropinocytosis** is a type of endocytosis characterised by the formation of a cup shaped macropinosome by invaginated membrane ruffles which fuse with each other or the plasma membrane to enclose extracellular fluid along with its contents (Swanson, 2008); EVs have been shown to be taken up in the extracellular fluid (Fitzner et al., 2011). Macropinocytosis may occur spontaneously or be stimulated by Epidermal Growth Factor (EGF), K-ras and small GTPase Rac1 (Swanson, 2008, Nakase et al., 2015); it requires the activity of Na+/H+ ion exchange (West et al., 1989). The Na+/H+ exchange can be blocked by amiloride thus blocking uptake of EVs (West et al., 1989, Fitzner et al., 2011).

1.5.4.5. **Heparan sulfate proteoglycans (HSPG)** are cell surface proteoglycans that can bind to particulates such as viruses and facilitate their uptake into cells (Shukla et al., 1999, Schafer et al., 2015). This mechanism has been shown to be important for EV uptake as treatment of recipient cells with heparin, which competitively binds to HSPG, inhibits EV uptake (Christianson et al., 2013, Franzen et al., 2014).

1.5.4.6. **Lipid raft mediated uptake:** Lipid rafts are areas in the plasma membrane in which altered phospholipid content results in tightly packed and ordered regions enriched in cholesterol (Simons and Ehehalt, 2002). They are known to contribute to viral particle uptake and have been shown, more recently, to take part in EV uptake (Svensson et al., 2013, Escrevente et al., 2011).

1.5.5. FUNCTIONS

Functionally, EVs can alter pathways in the recipient cells by transfer of nucleic acids or proteins (Valadi et al., 2007, Montecalvo et al., 2012). Additionally, release of EVs can modulate pathways within the donor cell. For example, CD82 and CD9 are shown to decrease Wnt-signalling by exporting β-catenin in EVs out of the cell, decreasing levels within cells (Chairoungdua et al., 2010).

Physiologically, EVs can affect multiple physiological processes by virtue of their function in transferring mRNA, microRNA and proteins. They are thought to be involved in antigen presentation, cell survival, coagulation and repair of tissues as reviewed in (Yanez-Mo et al., 2015). EVs are shown to play an important role in the immune response; this is suggested by their role in antigen presentation (Zitvogel et al., 1998, van Niel et al., 2001, Andre et al., 2002) and induction of T cell (Thery et al., 2002) and humoral immune responses (Van Niel et al., 2003). Recently they have also been shown to be involved in the release of Leukotriene B4, a secondary attractant from neutrophils (Majumdar et al., 2016).

1.5.6. EVS IN CANCERS

Studies suggest that patients with cancer have higher number of circulating EVs in the blood as compared to healthy patients and as well as a higher microRNA content (Taylor and Gercel-Taylor, 2008, Rabinowits et al., 2009). Studies have shown that lung cancer derived EVs exhibit a different proteomic profile with enrichment of some proteins including EGFR, GRB2 and SRC as compared to normal cell derived EVs (Clark et al., 2015). The protein profile and indeed the morphological appearance of EVs derived from multidrug resistant tumours was shown to be different from EVs derived from their sensitive counterparts in a study using lung and leukemia cell lines (Lopes-Rodrigues et al., 2015). EVs may affect tumorigenesis by modifying tumour inducing ability (Melo et al., 2014), proliferation (Clark et al., 2015), viability (Kogure et al., 2011) and metastatic capability (Gorczynski et al., 2016) of tumour cells. The tumour microenvironment may be modified by EV-mediated communication between the tumour and cancer associated fibroblasts (Maida et al., 2015, Sanchez et al., 2015) and mesenchymal stem cells (Wang et al., 2016). EVs are able to modulate angiogenesis, an important factor in cancer progression (Hegmans et al., 2004, Al-Nedawi et al., 2008, Hood et

al., 2009, Taverna et al., 2012). EVs have been shown to modulate the anti-tumoral response by affecting the immune response, T-cell activation and natural killer cell induction (Wolfers et al., 2001, Admyre et al., 2006, Graner et al., 2009, Hellwinkel et al., 2015).

1.5.6.1. EVs and response to chemotherapeutic drugs

EVs have also been shown to be involved in cancer chemotherapy resistance through various mechanisms, reviewed recently by Sousa et al (Sousa et al., 2015). These are discussed below.

- **Sequestration of drug and oncogenic material:** EVs themselves could serve as vehicles to sequester quantities of drug thus making the donor cells more resistant to the drug (Sousa et al., 2015). Microparticles from breast cell lines and ALL (Acute Lymphoblastic Leukaemia) cell lines were shown to sequester daunorubicin; moreover, it was shown that these particles contained P-gp drug transporter in an inside out orientation thus enabling the particles to actively absorb the drug in the presence of ATP (Gong et al., 2013); adriamycin was also shown to accumulate in EVs in drug resistant breast cancer cells (Ma et al., 2014). Ifergan et al showed an ABCG2 dependent increase in the concentration of riboflavin and mitoxantrone in EVs derived from mitoxantrone resistant MCF7 cells and C-1305 resistant non-small cell lung cancer cells (Ifergan et al., 2005, Ifergan et al., 2009). Chapuy et al showed a mechanism of sequestration of cytotoxic drugs in multivesicular bodies associated with lysosomes (Chapuy et al., 2008). Along the same lines, Akao et al showed that colon cancer cells became more resistant to 5-fluorouracil by exporting tumour suppressor microRNAs miR-34a and miR-145 within EVs (Akao et al., 2014). In another study, in prostate cancer cells treated with fludarabine; decreased export of oncogenic miR-485 in EVs was found; this increased the levels of miR-485 within the donor cell and through repression of NF-YB (nuclear transcription factor Y beta) increased levels of *MDR1* and

CCNB2 genes; miR-485 was shown to modulate resistance to fludarabine (Lucotti et al., 2013).

- **Drug efflux pumps:** The presence of drug efflux pumps in EVs have been of interest as these can then be taken up by recipient cells and develop drug resistance (Sousa et al., 2015). ***ABCB1 (MDR1/P-glycoprotein):*** P-glycoprotein mediated transfer of drug resistance between cells has been documented by co-cultures of neuroblastoma cells with their multidrug resistant counterparts; in fact P-glycoprotein was also shown to be transferred to the stromal fibroblasts (Levchenko et al., 2005); a similar study in breast cancer cells also yielded similar results (Pasquier et al., 2011). This transfer was shown to be transfer of protein rather than transfer of the gene due to the short time taken for the expression of P-glycoprotein (Bebawy et al., 2009). Moreover EVs were also shown to mediate transfer of drug resistance from resistant cells to sensitive cells probably through transfer of P-glycoprotein (Lv et al., 2014). Corcoran et al showed a transfer of docetaxel resistance in prostate cancer cells through EVs; the EVs were shown to be enriched in P-glycoprotein (Corcoran et al., 2012); this was confirmed by Kharaziha et al (Kharaziha et al., 2015). ***ABCG2:*** Studies have shown the sequestration of drugs such as mitoxantrone, topotecan, methotrexate and riboflavin in large ABCG2 rich extracellular vesicles (Ifergan et al., 2005, Ifergan et al., 2009, Goler-Baron and Assaraf, 2011, Goler-Baron et al., 2012). ***ABCC1:*** Microparticles were shown to mediate transfer of MRP1 between drug sensitive and drug resistant leukaemia cell lines (Lu et al., 2013).
- **Other proteins:** Annexin A3 has been shown to be associated with platinum resistance in ovarian cancer cells and to be enriched in EVs from cisplatin resistant ovarian cancer cells (Yin et al., 2012). Another protein, TrpC5, transferred by EVs, has been shown to increase drug resistance in breast cancer cells and been shown to cause sequestration of adriamycin and induce P-glycoprotien expression; this is also taken up by endothelial cells (Ma et al., 2014).

- **microRNAs:** EVs have been suggested as an important vehicle for transfer of microRNAs between cells as the microRNAs are protected from degradation by RNAses (Koga et al., 2011). It has been suggested that additional protection is provided by associated Ago2 complexes (Li et al., 2012b). Various studies show that microRNAs in EVs provide potential biomarkers for drug resistance (Taylor and Gercel-Taylor, 2008, Skog et al., 2008, Rabinowits et al., 2009, Gercel-Taylor et al., 2012, Aushev et al., 2013). In a study in prostate cancer cells, four microRNAs (miR-598, miR-34a, miR-146a, miR-148a) were shown to be enriched in derived EVs mirroring the parent cell; of these miR-34a was shown to knockdown Bcl-2 thus modulating response to docetaxel (Corcoran et al., 2014). A specific microRNA panel (miR-1228*, miR-1246, miR-1308, miR-149*, miR-455-3p, miR-638 and miR-923) was shown to be enriched in EVs derived from multidrug resistant ALL (acute lymphoblastic leukaemia) and breast cancer cell lines (Jaiswal et al., 2012). In breast cancer cell lines, adriamycin and docetaxel resistant cell line derived EVs could increase chemotherapy resistance in the sensitive cell line and were found to be enriched in specific miRNAs (Chen et al., 2014a); tamoxifen resistance was increased by miR-221/222 transfer through EVs (Wei et al., 2014).
- **Other mechanisms:** Takahashi et al showed that EV mediated transfer of linc-ROR and linc-VLDLR could increase chemotherapy resistance (Takahashi et al., 2014b, Takahashi et al., 2014c). A novel mechanism of drug resistance was described in B-cell lymphomas where CD20 on EVs bound to rituximab, an anti CD20 chimeric antibody used for therapy, as well as to complement; thus shielding cells from the drug; approximately 50% of the drug was found to bind to EVs (Aung et al., 2011).

1.5.6.2. EVs in therapy

The presence of EVs in blood, plasma and other secreted fluids suggests a possible role for EVs as biomarkers. EVs from patients with cancer were shown to mirror the microRNA profile of the tumour itself indicating the possibility of their use as

biomarkers (Taylor and Gercel-Taylor, 2008, Rabinowits et al., 2009,). A recent paper has shown that microRNA profiling of EV content could predict the presence of metastatic sporadic melanoma (Pfeffer et al., 2015). EV microRNA was shown to reflect the removal of tumours in patients after resection in lung cancer (Aushev et al., 2013). In another study involving pancreatic cancers, exoDNA and exoRNA sequencing of EVs derived from a pleural effusion or from blood could identify mutations and copy number profiles (San Lucas et al., 2015). It has been suggested by one study that long non coding RNA, lnc-p21, in EVs may potentially differentiate prostate cancer from benign disease (Isin et al., 2015).

It has recently been suggested that specific molecular signature of EVs derived from prostate cancer may predict response to docetaxel (Kharaziha et al., 2015). Similarly, evidence suggests that the protein profile of EVs derived from multidrug resistant cancers differs from that of their sensitive counterparts (Lopes-Rodrigues et al., 2015).

The participation of EVs in eliciting immune responses has sparked an interest in the use of EVs for anti-tumoral vaccines (Chaput et al., 2003, Chaput et al., 2004b, Chaput et al., 2004a).

1.5.7. Extracellular Vesicles in Ovarian Cancers

In ovarian cancers, EVs in the serum of patients with ovarian cancer were shown to be enriched in miR-21, miR-141, miR-200a/b/c, miR-203, miR-205 and miR-214 (Taylor and Gercel-Taylor, 2008). Meng et al (2016) showed that exosomal miR-200a/b/c and miR-373 was higher in ovarian cancer patients. It has also been shown that the microRNA profile of EVs from peritoneal or pleural effusions of patients with ovarian cancer could be associated with progression free survival (Vaksman et al., 2014). Cappelleso et al (Cappellesso et al., 2014) demonstrated that miR-21 and PDCD4 levels in EVs from peritoneal effusions mirrored the levels in cystadenoma and serous carcinoma tumours. Annexin A3 has been shown to be associated with platinum resistance in ovarian cancer cells and to be enriched in EVs from cisplatin resistant ovarian cancer cells (Yin et al., 2012). Yi et al (2015) have shown that EVs from high grade ovarian cancer have the potential to regulate angiogenesis. These studies indicate the functional relevance of EVs in ovarian cancer – from the transport of microRNAs and proteins affecting various signalling pathways to

phenotypic effects such as angiogenesis, EVs can modulate cells in neighbouring and distant sites; EVs are also potential biomarkers for the various phenotypic characteristics of cancers.

It can be seen that cisplatin resistance in ovarian cancer cells is a multifactorial complex process, the nuances of which are still being investigated; microRNAs and cell-cell communication by extracellular vesicles represent newer variables in the modulation of the response to cisplatin chemotherapy. These two aspects require further research if the various facets of cisplatin resistance are to be understood and overcome.

1. 6. AIMS OF THE PROJECT

Based on the gap in the knowledge identified above, this project aims

- To identify microRNAs with a novel role in cisplatin resistance in ovarian cancer cells
- To elucidate possible target genes and mechanisms of action of these microRNAs
- To analyse aspects of the transfer of drug resistance between cells by extracellular vesicles

Chapter 2 MATERIALS AND METHODS

General materials and methods are described here. Methods specific to each chapter are described in the materials and methods section at the beginning of each chapter.

2.1. CELL CULTURE

The main cell lines used in this project are the cisplatin sensitive ovarian cancer cell line A2780 (Parker et al., 1991) and its cisplatin resistant derivatives MCP1 and CP70 (Parker et al., 1991). Other cell lines used to validate experiments include ovarian cancer cell lines OVCAR-5, OVCAR-8 (Stinson et al., 1992), IGROV-1 (Benard et al., 1985) and SKOV-3 (Fogh, 1975). Human Ovarian Cancer cell lines A2780, MCP-1, CP-70 and OVCAR-8 were cultured in RPMI (Invitrogen Gibco) supplemented with 10% v/v heat inactivated foetal bovine serum (Invitrogen Gibco). Human Ovarian Cancer Cell lines OVCAR-5 and IGROV-1 were cultured in DMEM/F-12 (Invitrogen Gibco) supplemented with 10% v/v heat inactivated foetal bovine serum (Invitrogen Gibco). Cells were sub-cultured every 5-7 days using 0.05% v/v trypsin/EDTA (Fisher, 15400054) and fresh media was added every 2-3 days.

2.2. MTT ASSAY

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was made up as 2 mg/ml of MTT (Sigma) in phosphate buffered saline (pH: 7.2 ± 0.05, Gibco). 100 µl of this solution was added to the 100 µl of growth medium already present in each test well of the 96-well plate. The plate was then incubated for 2 hours at 37°C. During this time the MTT is taken up by the viable cells and transformed into formazan salts which are purple coloured. All the liquid was then pipetted out of the wells leaving the coloured salts at the bottom of the wells. These were then dissolved in 100 µl MTT solvent (4 mM HCl and 0.1% v/v IGEPAL® CA-630 (Sigma) in Isopropanol) was added to each well to dissolve the salts to form a coloured solution. The strength of the solution was quantified by reading the absorbance of each well on a plate reader at 570 nm wavelength with a reference filter at 630nm.

2.3. SULFURHODAMINE B ASSAY

The protein dye sulfurhodamine B can be used to quantify proliferation by measuring protein content in fixed cells; in a mildly acidic environment it binds to basic amino acids in a cell and in a mildly basic environment it can be extracted from the cells

(Voigt, 2005). All growth media was removed from the wells. Cells were washed once with 100 µl phosphate buffered saline (Fisher Bioreagents) and then fixed with 100 µl/well of 25% v/v acetic acid in methanol for 2 hours at 4°C. The fixative was then removed and cells were washed with distilled water, stained with 100 µl 0.4% w/v sulfurhodamine B sodium salt (Sigma) in 1% v/v acetic acid for 30 minutes. The stain was then removed, Cells were washed with 1% v/v acetic acid and air-dried. The stain was then dissolved in 100 µl/ well 10mM TRIS base (Fisher - BPE152-1, 0.121 g in 100 mls) solution and read on a plate reader at 570 nm.

2.4. CISPLATIN TREATMENTS

Optimal cisplatin concentrations were determined by treating cells seeded in 96-well plates with varying concentrations of cisplatin for 3 hours 48 hours after seeding. MTT assay was done 48 hours after cisplatin treatment. The dose response curves for A2780, MCP1 and CP70 are shown in Figure 3.1 and the curves for OVCAR-5, OVCAR-8 and IGROV-1 are shown in appendix A – Figure A-2. IC50 was estimated using GraphPad Prism. For OVCAR-5 though the IC50 was estimated at about 100µM, when this concentration was used in the cells, there was >60% cell death; hence the concentration was lowered to 50µM to give 50% cell death.

Cells were seeded in 96 well plates (day 0) at optimal concentrations (Table 2-1). On day 1, any experimental intervention such as miRNA, siRNA or shRNA treatment was completed as described in Section 2.5. On each plate, in addition to the controls for the treatment, one group of cells was left untreated as an internal control. On day 2, half of the wells in all groups including the internal controls were treated with cisplatin (Fisher; stock solution - 16.7 mM made up in PBS) for 3 hours diluted in media to optimal concentrations (Table 2-1) or at varying concentrations for dose response curves. After 3 hours, the media containing cisplatin was removed, cells were washed with PBS and fresh media was added. On day 4, viability assays such as MTT or sulfurhodamine B were performed. The internal controls were analysed; the percentage of absorbance of cisplatin treated cells to untreated cells in this group is expected to be within the range of 40% to 60%. If the readings were beyond this range, the plate was excluded from the analysis for the experiment.

TABLE 2-1: OPTIMAL CELL CONCENTRATIONS AND CISPLATIN CONCENTRATIONS USED FOR EACH CELL LINE

Cell Line	Cell concentrations (per well) in 96-well plate	IC50	Cisplatin concentration (μM)
A2780	10 000	19.5 to 26.25	20
MCP1	10 000	76.26 to 94.71	80
CP70	6 000	123 to 141.5	150
OVCAR-5	15 000	76.2 to 130.1	50
OVCAR-8	10 000	88.8 to 126	80
IGROV-1	15 000	35.07 to 49.15	50

2.5. DRUG TREATMENTS

16F16 (Sigma, SML0021) was used to inhibit PDIs at a final concentration of 2.5 μM in media. Paxilline (Sigma, P2928) was used to block BKCa channels at a final concentration of 10 μM in media. Heparin (Sigma, H3393), amiloride (Sigma, A3085) and dynasore (Sigma, D7693) – EV uptake inhibitors were diluted in media to final concentrations of 10 μg/ml, 50 μM and 50 μM respectively. Guggulsterone (Sigma, G5168) and Bexarotene (Sigma, SML0282) were used at final concentrations of 50μM and 5μM respectively.

2.6. MIRNA, SIRNA AND SHRNA TRANSFECTION

Cells were seeded in 96-well plates at optimal concentrations. After 24 hours, microRNA mimics, **miR-21*mimic** (Dharmacon, Thermo, UK, C-301023-01-0005), **miR-31 mimic** (Dharmacon, Thermo, UK, C-300507-05) or **miRNA mimic negative control 2** (Dharmacon, Thermo, UK, CN-002000-01-20) were transfected at 5 nM using Dharmafect 3 transfection reagent (0.1 μl/ well). For microRNA inhibitor experiments, miRIDIAN hairpin inhibitors; **miR-21* inhibitor** (Dharmacon, Thermo, UK, IH-301023-02-0005), **miR-31 inhibitor** (Dharmacon, Thermo, UK, IH-300507-06-0005) or **inhibitor negative control** (Dharmacon, Thermo, UK, IN-001005-01-05) were transfected at 50nM using Dharmafect 3 transfection reagent (GE Dharmacon, DZT-2003-02) (0.1 μl/ well). **NAV3**

knockdown siRNA (SASI_Hs01_00215078) – (CUCUAAUUUAAAUAGGAGA[dT][dT]) and **siRNA negative control** (MISSION® siRNA Universal Negative Control #2 SIC002) (both from Sigma), **PDIA4 knockdown siRNAs** (Thermo Fisher Scientific s225164 and s225165 - 4392420) and **siRNA negative control** (Fisher 4390843) were transfected at 50 nM concentrations using Dharmafect 3 transfection reagent (0.1 µl/ well). Three shRNAs designed to target different areas of KCNMA1 mRNA were used to knockdown KCNMA1; the sequences are as follows **KCNMA1 shRNA1**-CCGGGCGTAGTATTCAAACCAGTATCTCGAGATACTGGTTTGAATACTACGCTTTTT - TRCN0000000209, **KCNMA1 shRNA2** – CCGGCCCAATAGAATCCTGCCAGAACTCGAGTTCTGGCAGGATTCTATTGGGTTTTT - TRCN0000000210 and **KCNMA1 shRNA3**– CCGGTGGCAGAAATACTACTTGGAACTCGAGTTCCAAGTAGTATTTCTGCCATTTTT - TRCN0000000211. As a control, a scrambled negative shRNA (Sigma, UK, SHC002V) was used. They were transfected at concentrations of 50ng plasmid per well of 96-well plate using Fugene (0.25 µl/ well) (Roche, UK). Cells were harvested for RNA or protein extraction 24 hours after treatment with miRNA, siRNA or shRNA. For experiments involving drug resistance, cells were treated with cisplatin or doxorubicin 24 hours after transfection with miRNA mimics, inhibitors, siRNA or shRNAs.

2.7. WESTERN BLOTTING

Total protein was extracted from cells using RIPA buffer (50 mM Tris HCl pH 8 (Fisher, T/P631/48), 150 mM NaCl, 1% v/v IGEPAL-CA630 (Sigma), 0.25% sodium deoxycholate, 0.01% SDS, 10 mM EDTA (Sigma - E4884) and 2 mM EGTA (VWR, 0732-50GP) and quantified by the BCA assay using standardised solutions of BSA (Thermo Fisher Scientific). Approximately 20 µg of protein was prepared in SDS-PAGE loading dye with DTT (Fisher FQ-R0861) and heated to 100ºC for 10 minutes. Samples were loaded onto a 12% denaturing polyacrylamide gel (BioRad, 12% Mini-PROTEAN® TGX Stain-Free™ Gel #456-8046), electrophoresed and transferred to a PVDF membrane (Bio-Rad, 170-4156). The blot was blocked with 5% BSA in TBS-0.05% tween (TBST) for 2 hours at room temperature (RT). Primary antibodies were diluted to 1:10,000 (anti-GAPDH antibody, Abcam, AB8245), 1:500 (anti-KCNMA1 antibody, Abcam, AB104711) or

1:1000 (Anti-NAV3 antibody, Sigma N4288-200UL) and incubated with the blot overnight at 4°C. Membranes were washed and incubated for 1 hour at RT in 1:5,000 (NAV3) or 1:10,000 (KCNMA1) secondary-HRP labelled antibody (170-6515, Bio-Rad) and washed. The blots were then immersed in ECL solution (Clarity Western ECL solution, BioRad, 170-5060) and digitally imaged Bio-Rad Chemi-Doc MP system and analysed using ImageLab software.

2.8. QRT-PCR

24 hours following transfections the cells were washed with PBS and extracted straight from the well using TRI-reagent (Sigma) (1ml for 10 minutes). The RNA was extracted and analysed for quality and quantity using a Nanodrop and Bioanalyzer 2100. Taqman miRNA assays (Thermo Fisher) were used as per manufacturer's instructions to quantify miR-31, miR-21* and a reference gene, RNU58a. Quantification was performed using the 2ΔΔCt method. For measuring NAV3, KCNMA1 and PDIA4 levels the RNA was DNAse I (Sigma) treated, cDNA was then synthesised (HiCap RNA-to-cDNA kit, Life technologies, UK). 30ng of cDNA was used per 20µl SYBR green PCR reaction along with 1µl each of forward and reverse primers (10µM) (Sigma) and 10µl of SYBR green mastermix (SensiMix SYBR, Bioline, UK); the reactions were subjected to 40 cycles of PCR with 10 seconds of denaturation at 95°C, 10 seconds of annealing at 60°C and 20 seconds of elongation at 72°C in Bio-Rad CFX96 RT-PCR system. No-template and no-RT reactions gave no or negligible signal (Ct values of 36 or over). NAV3, PDIA4 and KCNMA1 were quantified using the 2^-ΔΔCt method with GAPDH as reference gene. Statistical significance was assessed using the Student's t-test. RNA was extracted by me, the qPCRs for Mir-21*, Mir-31, NAV3 and KCNMA1 were performed by Dr. Pink; results are published in Pink et al. (2015) and Samuel et al. (2015) and are referred to and discussed in chapters 2 and 3. For PDIA4 qPCR, I performed the qPCR. PDIA4 primers used were – forward 5' - TCCATGGCAACTTCTTCCC, reverse 5' - CATTGCGGACGAAGAGGAC .

2.9. MICRORNA MICROARRAY

A2780, MCP1 and CP70 cells were washed with PBS and total RNA was extracted using Trireagent (Sigma). Three biological replicates for each cell line were obtained and 2 µg RNA aliquots were sent to Exiqon for miRCURY LNA microarray

analysis. RNA quality was assessed on the Agilent Bioanalyzer 2100 (only samples with RIN value >8 were used). and the samples were individually labelled with Hy3™, and a pool of all nine samples was Hy5™ fluorescent labelled (power labelling kit, Exiqon, Denmark). Samples were then hybridised to the miRCURY LNA array (5th generation). Image analysis was carried out using the ImaGene 8.0 software (BioDiscovery Inc, USA). Quantified signals were background corrected and normalised using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm to minimise the intensity-dependent differences between the dyes. To identify differentially expressed miRNAs a 2-tailed t-test was applied and Bonferroni correction was applied.

2.10. NIMBLEGEN GENE MICROARRAY

A2780 cells were seeded in 6-well plates and allowed to settle for 24 hours. They were then transfected with microRNA-21* mimics or control microRNA at 5nM using Dharmafect 3 transfection reagent (0.1 µl/well). 24 hours later, cells were washed with PBS and total RNA was extracted using TRIreagent (Sigma) and DNase treated using a Qiagen RNeasy miniprep kit. The RNA quality was verified with Agilent Bioanalyzer 2100; only samples with RIN value >8 were used. 100 ng of RNA was taken to generate cDNA using the Transplex complete whole transcriptome amplification kit (Sigma). Nimblegen Gene microarray was performed with these samples. In summary, the cDNA sample was RNase (Sigma) treated and cDNA was precipitated following the Nimblegen Expression Array protocol. Samples were labelled with Nimblegen dual-colour DNA labelling kit, hybridised to a Nimblegen 12plex x 135k gene Human transcriptome microarray and washed according to the supplier's protocol. The array slide was scanned at 3 µm resolution on an InnoScan 700 microarray scanner and converted into TIFF images using MAPIX version 5.1 software. The TIFF images were then aligned to their Nimblegen design files and converted into probe intensity values using the Nimblegen DEVA software. This data was then Loess normalised using R statistical program and then quantile normalised for array variation using DNAstar (ArrayStar Inc.). Significant difference of gene expression between three biological replicates was assessed by fold change and t-test using the DNAstar software. RNA was extracted for this procedure by me, the microarray was performed by Dr. Pink.

2.11. RNA SEQUENCING

RNA was extracted from two biological replicates of A2780 and CP70 cells, and one MCP1 sample using TRIreagent (Sigma), and 10 μg of each was sent to Source BioScience to be sequenced on an Illumina Genome Analyser IIx. The single-end 38 base-pair reads were aligned using Illumina's pipeline software. Transcript abundance was estimated using Cufflinks and results presented as reads per kilobase of transcript per million mapped reads. Relative levels of transcripts between samples were compared using DESeq. Genes that were significantly and concordantly altered in MCP1 and CP70 cells were analysed using the DAVID functional annotation tool (Version 6.7) to assess enriched KEGG pathways and gene ontology molecular function results.

2.12. STATISTICAL ANALYSIS

Student's t-test was used to analyse statistical significance for point to point comparisons in the bar charts. GraphPad Prism was used to calculate IC50s for curves and to analyse significance in differences between IC50s of curves; this uses the extra sum-of-squares F test to determine if the IC50s are significantly different between curves. Pearsons correlation was used to identify correlations between IC50 for cisplatin and gene expression levels in NCI 60 cell line panel dataset (GEO GDS1761 / 2618). Gene expression levels between resistant and sensitive tumours in the TCGA ovarian cancer dataset were compared using a student's t-test; these analyses were done using Microsoft Excel software. For all experiments at least three biological replicates were performed to enable statistical comparisons. For experiments involving A2780 cells, experiments were repeated to ensure experimental repeatability; thereby giving a larger number of replicates. P-values in figures are depicted as follows: <0.05 - *, <0.01 - **, <0.001 - ***, <0.0001 - ****.

Chapter 3 MICRORNA-21* INCREASES CISPLATIN RESISTANCE IN OVARIAN CANCER CELLS

3.1 INTRODUCTION

As described in section 1. 1, ovarian cancer claims more than 150 000 lives worldwide every year (Fitzmaurice et al., 2015). One contributor to the high mortality rate is resistance to platinum drugs, the main drugs used for chemotherapy. The resistance to cisplatin is multifactorial and regulated by many networks of genes; one emerging ubiquitous group of modulators is the microRNAs which have been shown to be involved in many physiological and pathological cellular processes including cisplatin resistance. Though several microRNAs have been shown to regulate cisplatin resistance, there are indications that many more are involved. Hence one of the aims of the project (Section 1. 6) was to identify microRNAs that have a role in the cisplatin resistance of ovarian cancer cells for which such a role has not been described thus far. Paired ovarian cancer cell lines A2780 and cisplatin resistant derivative CP70 were used to identify microRNAs that could possibly be involved in the cisplatin resistance of this paired cell line.

This chapter describes the identification of microRNAs potentially involved in cisplatin resistance and the validation of miR-21* as one of the microRNAs involved in cisplatin resistance and the validation of NAV3 as its target gene. MicroRNA-21* was of special interest as it is a passenger strand microRNA. Only one strand of the microRNA duplex (see section 1: Introduction), thought to be determined by the thermodynamic stability of the base pairs at the 5' end, is loaded into the RISC (RNA induced silencing complex) (Kim, 2005, Han et al., 2006, Winter et al., 2009). The other strand is known as the passenger strand or the star strand and was originally thought to be degraded (Matranga et al., 2005, Winter et al., 2009). However, recent studies have shown that the proportion of the 5p and the 3p strands varies between tissues and species (Ro et al., 2007, Biasiolo et al., 2011); it may be altered by changes in temperature (Potla et al., 2015). Passenger strands have been shown to have a functional role in thyroid cancer and can target genes that are different from the guide strand (Jazdzewski et al., 2009). Both the guide strand and the passenger strand of miR-17 and miR-582 were found to modulate tumour growth

in prostate cancer and bladder cancer, respectively (Uchino et al., 2013, Yang et al., 2013c), while the passenger strand of miR-221 has been shown to promote colorectal cancer tumour growth (Yuan et al., 2013). The two strands of miR-28 were shown to act on different targets and have opposing effects on migration and invasion in colorectal cancers (Almeida et al., 2012). Similarly, miR-125-5p and miR-125-3p were shown to have opposing effects on cell migration and invasion in NSCLC (non-small cell lung carcinoma) (Jiang et al., 2010).

MicroRNA-21* was specifically shown to be induced by berberine treatment in the hepatocellular carcinoma cell line HepG2 and to inhibit the cell growth in hepatomas (Lo et al., 2013). There have been indications of its involvement in breast cancer; levels were increased in a group of breast cancer tissues; overexpression of miR-21* resulted in increased proliferation in breast cancer cell lines and enhanced activity of the Akt pathway (Aure et al., 2013). MiR-21* was also overexpressed in chemoresistant triple negative breast cancer (Ouyang et al., 2014). A study of laryngeal cancers has shown a 1.8 fold upregulation of miR-21* (Lu et al., 2014) while there was also significant upregulation in a group of 30 cervical cancers (Han et al., 2015). In cardiac tissue, miR-21* was found to be enriched in EVs derived from cardiac fibroblasts and was shown to mediate cardiac hypertrophy (Bang et al., 2014). On the other hand, another study showed that miR-21* overexpression suppressed transverse aortic constriction induced cardiac hypertrophy (Yan et al., 2015). MiR-21* was also found to be increased in kidneys of patients with acute kidney injury after kidney transplants (Wilflingseder et al., 2014). Moreover, a recent publication has shown an increase in miR-21* correlates with tumour progression in squamous cell carcinoma of the skin (Ge et al., 2015). These studies suggest that miR-21* may have a significant role in cancer.The aim of this aspect of the project was also to identify possible mechanisms and gene targets by which miR-21* could cause resistance to cisplatin. In published literature, few targets have been validated for miR-21*. MiR-21* was shown to decrease proliferation in hepatocellular carcinoma by targeting human methionine adenosyltransferases 2A and 2B (Lo et al., 2013). MiR-21* was found to increase expression of L1CAM (L1 cell adhesion molecule), which is associated with poor prognosis in cancers, through knockdown of CALM1, a negative regulator in renal cancer cell lines; miR-21* was

also shown to increase migration of the cells (Doberstein et al., 2014). MiR-21* was shown to enhance the activity of the AKT pathway in breast cancer (Aure et al., 2013) and in cardiac tissue by the down regulation of *HDAC8* (histone deacetylase-8) (Yan et al., 2015). In cardiac tissue, miR-21* was shown to knockdown *SORB2* (sorbin and SH3 domain containing protein 2) and *PDLIM5* (PDZ and LIM domain 5), which are deregulated in cardiac pathologies (Bang et al., 2014). However none of these are associated with cisplatin or drug resistance. This chapter also describes the identification of *NAV3* as a target gene of miR-21* involved in cisplatin resistance.

3.2 MATERIALS AND METHODS

Materials and methods specific to this chapter are described here. Other general methods are described in Chapter 2.

3.2.1 LUCIFERASE ASSAY

A luciferase assay was done in order to validate NAV3 as a direct target of miR-21*. A2780 cells were stably transfected with Lentiviral vector pLSGNAV3 3′UTR-RenSP (Mission® 3′UTR Lenti GoClone NAV3 from Sigma) and selected with puromycin (0.5 µg/ml). Stable cells were seeded at 20 000 cells/well in 96-well plates and transfected with miR-21* mimics or negative control mimic (Dharmacon, Thermo, UK) at 5 nM with 0.1 µl/ well of Dharmafect 3 transfection reagent. After 24 hrs, cells were lysed (using the lysis solution from Dual-Glo luciferase assay system from Promega), 75 µl of Renilla substrate (Dual-Glo Luciferase assay system from Promega) was added and luminescence quantified on a luminometer.

3.2.2 PDI INHIBITOR TREATMENT

In order to assess the effect of knockdown of PDIA4 on cisplatin resistance, cells were treated with PDI inhibitor 16F16 (Sigma, SML0021) diluted in media at 2.5 µM for 1 hour prior to cisplatin treatment.

3.2.3 APOLIVE-GLO MULTIPLEX ASSAY

In order to quantify viability and apoptosis, the ApoLive-Glo Multiplex assay (Promega) was performed as per manufacturer's protocol. Briefly, 20 µl of viability

reagent (fluorogenic live cell protease marker) was added to each well. After 30 minutes of incubation, fluorescence was quantified at UVEx/530Em using a Bio-Rad imager and intensity quantified with ImageLab 5.1. Then 100µl of Caspase-Glo® 3/7 reagent (luminogenic caspase 3/7 substrate) was added to each well. Luminescence was quantified after 30 minutes of incubation.

3.3 RESULTS

3.3.1. CISPLATIN RESPONSE CURVES OF CELL LINES

The cell lines used in this project are the A2780, an ovarian serous adenocarcinoma cell line and MCP-1 and CP-70s, cisplatin resistant derivatives of the A2780. The cell growth characteristics of these cell lines were monitored by MTT assay and optimal cell numbers to be seeded for week long experiments in 96-well plates were determined to be 10000 cells/well for A2780s and MCP1 and 6000 cells/well for CP70s. Cisplatin dose response curves (Figure 3.1) were plotted for the three cell lines by treating cells in 96-well plates with varying concentrations (0 - 400µM) of cisplatin; cell viability was assessed by the MTT assay. A2780 is seen to be the most cisplatin sensitive cell line with 50% cell death occurring around 20 µM cisplatin treatment; CP70s are the most cisplatin resistant with 130 µM cisplatin required for similar percentage of cell death. The cisplatin resistance exhibited by MCP1 appears to be higher than that of the A2780s but lower than that of the CP70s with 50% cell death occurring around 80µM cisplatin. Other cell lines used in the project are ovarian cancer cell lines, OVCAR-5, OVCAR-8 and IGROV-1. Cisplatin concentration curves were also plotted for these cell lines (shown in appendix A, Figure A-2) and optimal concentration for cisplatin treatment was determined to be 50 µM, 80 µM and 50 µM respectively as described in section 2.4.

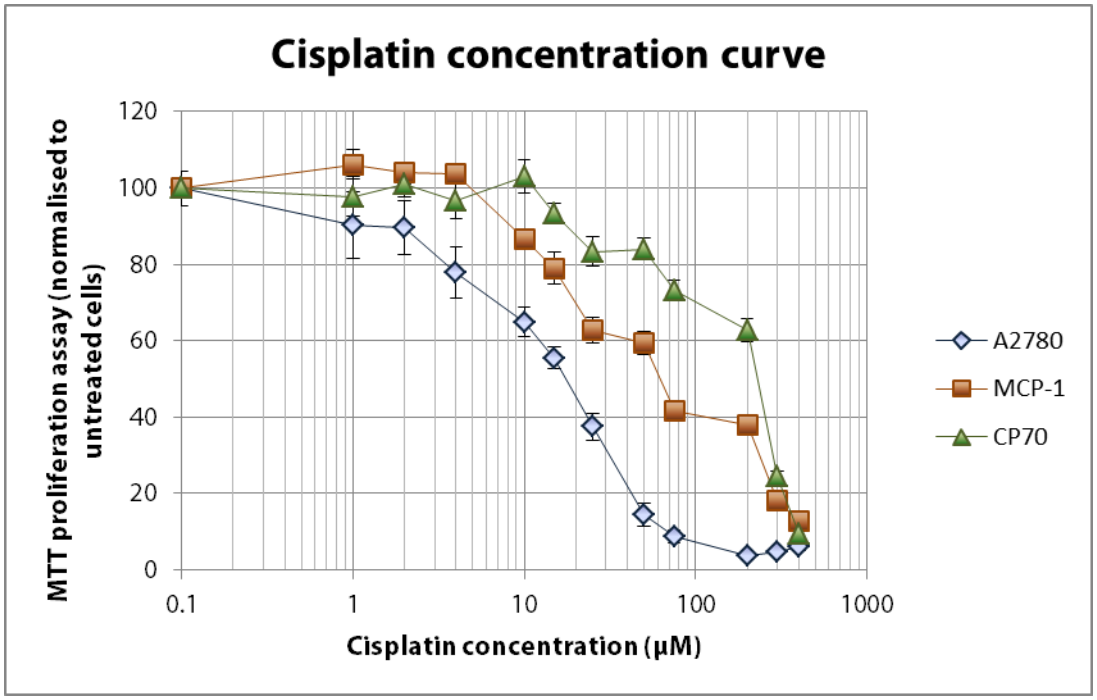

FIGURE 3.1: CISPLATIN DOSE RESPONSE CURVES FOR A2780, MCP1 AND CP70
A2780, MCP1 and CP70 cells seeded in 96-well plates were treated with increasing concentrations of cisplatin for 3 hours. Cell viability was assessed after 48 hours by the MTT assay. Absorbance was normalised to untreated cells in each group; error bars show SEM from at least 6 biological replicates for each point of the graph. IC50 calculated by GraphPad PRISM is as follows: A2780 – 22.64±1.721 µM, MCP1 – 84.83±4.751 µM and CP70 – 131.9±4.673 µM indicating that CP70s are the most resistant to cisplatin while A2780s are the most cisplatin sensitive cell line of the three cell lines.

3.3.2. MIRNA MICROARRAY INDICATES MICRORNAS POSSIBLY INVOLVED IN RESISTANCE

In order to identify microRNAs that are up- or down-regulated in the resistant cell lines, total RNA extracted (TRI-reagent) from A2780, MCP1 and CP70 was sent to Exiqon for miRCURY LNA miRNA microarray analysis. Only microRNAs with at least a 2-fold change between sensitive and resistant cell lines and a significant p-value < 3.91*E-05 were classified as showing differential expression. Results are shown in Table 3-1, and Figures 3.2 – 3.4. In total, 46 microRNAs showed differential expression between one of the resistant cell lines and the sensitive cell line. 25 microRNAs were upregulated in the CP70 cell line (Table 3-1) when compared with the A2780 cell line while 18 microRNAs were downregulated (Table 3-1). Expression levels of 13 microRNAs were higher (Table 3-1) and 9 microRNAs were lower (Table 3-1) in the MCP1 cells as compared to the A2780 cells. 8 microRNAs were concordantly upregulated and 7 microRNAs were downregulated

in both resistant cell lines while 4 microRNAs showed discordant regulation between the resistant cell lines. MicroRNA-31 shows the highest fold change with expression 50-fold higher in the CP70 cells than the A2780 cells. The heat map from the microarray showing all the microRNAs with significant changes in expression in all the replicates is shown in Figure 3.2. Blue indicates levels lower than the average levels in all samples while red indicates levels higher than the average. Figure 3.3 shows a volcano plot of the fold changes in CP70 vs A2780 against the p-values from two tailed T-test while Figure 3.4 shows the volcano plot of fold changes in MCP1 over A2780 against p-values (Two tailed T-test). MiR-21* and miR-31 datapoints are highlighted. In Figure 3.3, miR-31 clearly shows the highest fold change and a significant p-value; miR-21* is among the microRNAs with significantly different expression between the two cell lines.

TABLE 3-1: MICRORNAS SIGNIFICANTLY DEREGULATED IN THE RESISTANT CELL LINES
miRNA microarray was performed on total RNA extracted from the A2780, MCP1 and CP70; results were normalised, Lowess corrected and Bonferroni correction applied. Table shows all microRNAs that were significantly deregulated in the resistant cell lines – CP70 and MCP-1 – as compared to the resistant cell line. Three replicates were used for each sample. Only microRNAs with at least 2-fold change and p-value < 3.91*E-05 were chosen.

microRNA	fold change CP70 vs A2780	p-value CP70 vs A2780	fold change MCP1 v A2780	p-value MCP1 v A2780
hsa-miR-31	50.36275	1.28E-07		
hsa-miR-10a	21.81537	2E-06		
hsa-miR-31*	15.33747	6.85E-07		
hsa-miR-222	9.921415	1.26E-06	3.477058	2.16E-05
hsa-let-7i	6.938651	3.63E-06		
hsa-miR-221	6.513637	5.04E-06	2.924624	3.55E-05
hsa-let-7g	5.740532	2.31E-06		
hsa-let-7c	5.211375	2.53E-05	2.470035	0.000138
hsa-miR-29b	4.023443	2.77E-05	2.496424	0.000272
hsa-miR-9	4.020124	2E-05		
hsa-miR-21	3.908365	6.74E-06	2.006828	0.000191
hsa-miR-9*	3.823692	4.24E-06		
hsa-miRPlus-E1186	3.409833	1.46E-05	2.367232	7.49E-05
hsa-miR-182	3.166715	7.6E-05		
hsa-miR-21*	2.994392	4.65E-06	2.012074	0.000508
hsa-miR-96	2.941224	1.41E-05		
hsa-let-7b	2.883473	9.58E-06		
hsa-miR-27b*	2.463407	8.96E-06	2.505888	0.00016
hsa-miR-132	2.40558	1.3E-05		

Table 3-1 contd..

hsa-miR-29c	2.387018	2.71E-05		
hsa-miR-27b	2.25834	1.77E-05		
hsa-let-7a	2.22355	0.000621	0.49531	0.001118
hsa-miR-218	2.222012	0.000134	0.314613	0.000156
hsa-miR-29a	2.135448	3.54E-07		
hsa-miR-212	2.028627	8.4E-05		
hsa-miR-18a*			2.002815	5.6E-06
hsa-miR-19a			2.46716	0.000177
hsa-miR-18a			2.399199	7.85E-05
hsa-miR-18b	0.498287	2.16E-05		
hsa-miRPlus-E1238	0.48176	2.28E-05		
hsa-miR-602	0.459987	1.03E-05		
hsa-miR-17	0.42707	3.23E-05		
hsa-miR-210	0.417759	1.21E-05	0.253264	5.53E-06
hsa-miR-92a	0.409684	9E-05		
hsa-miR-106a	0.394816	2.98E-05		
hsa-miRPlus-F1181	0.372698	3.1E-05		
hsa-miR-19b	0.335621	5.65E-06		
hsa-miR-100	0.327802	3.07E-05	0.30231	3.49E-05
hsa-miR-135a	0.266752	0.000257	2.953007	0.000578
hsa-miR-106a*	0.219328	5.24E-06	0.230747	1.05E-05
hsa-miR-34a	0.143713	1.12E-06	0.133853	1.56E-06
hsa-miR-143	0.111691	1.12E-06		
hsa-miR-20b	0.089706	3.71E-08	0.119124	2.95E-06
hsa-miR-363	0.055433	6.75E-07	0.036125	2.27E-06
hsa-miR-199b-5p	0.053188	6.55E-06	0.336708	0.00032
hsa-miR-335	0.049947	6.13E-06	2.290361	0.000423

significantly upregulated compared to the A2780 cell line

significantly downregulated compared to the A2780 cell line

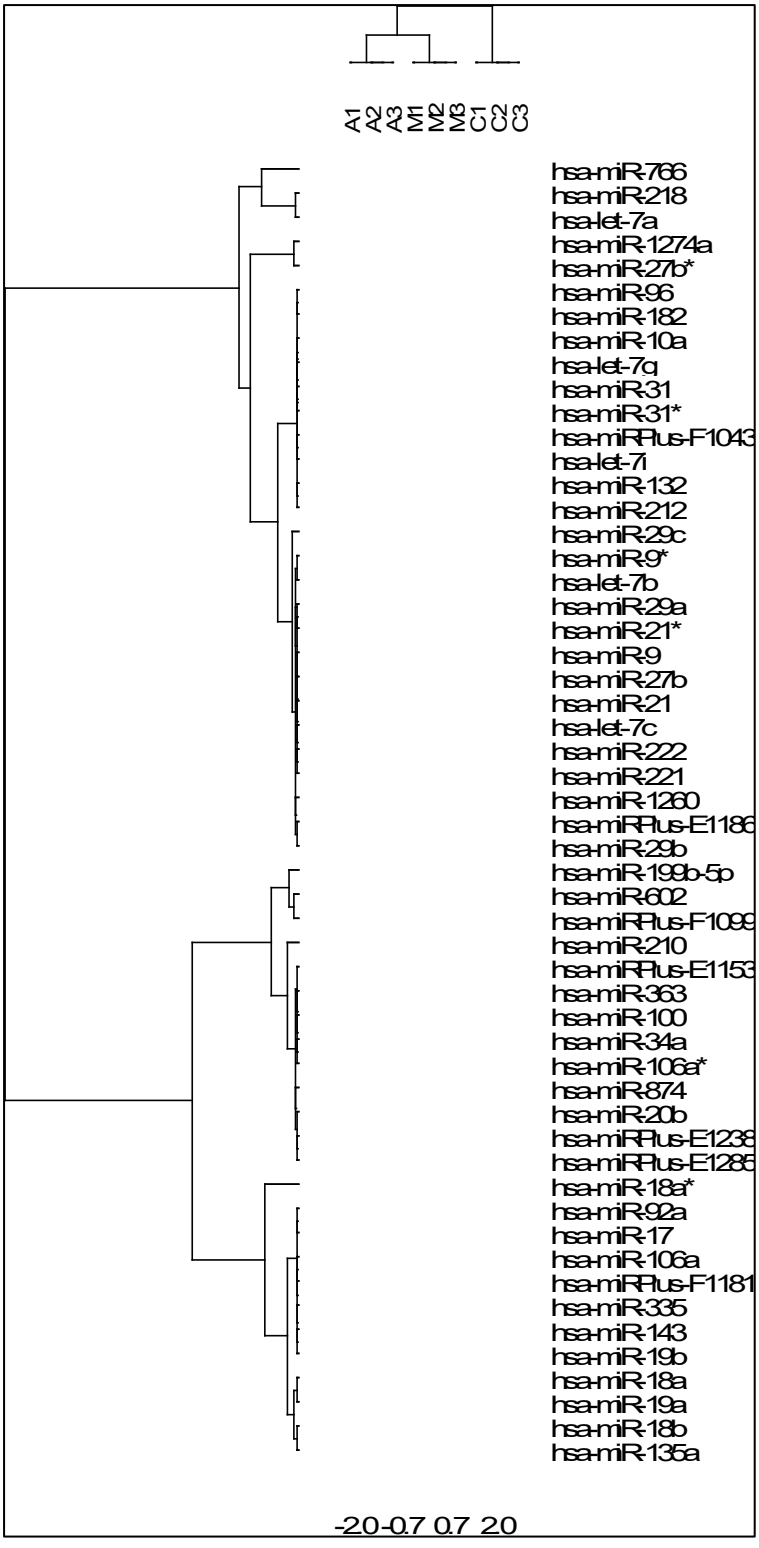

FIGURE 3.2: MICROARRAY HEAT MAP

MicroRNA microarray was performed on total RNA extracted from the A2780, MCP1 and CP70; results were normalised, Lowess corrected and Bonferroni correction applied. Only microRNAs with a fold change of at 2-fold and significant p-value < 3.91*E-05 were further assessed. Blue indicates levels lower than the average of all the samples while red indicates levels higher than the average.

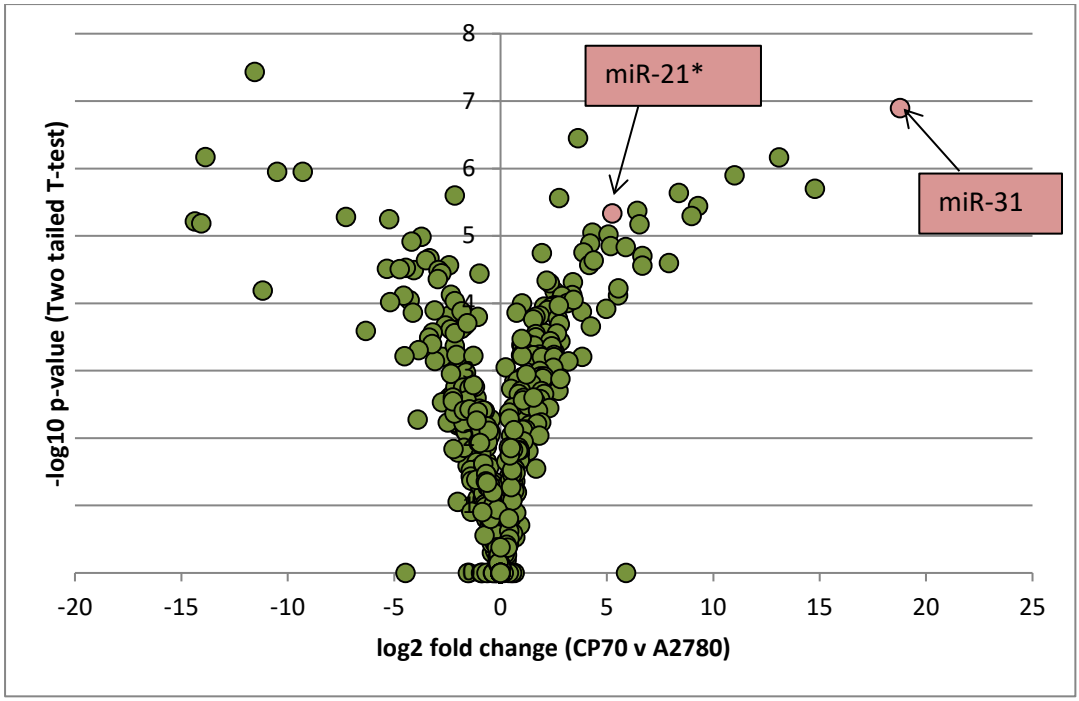

FIGURE 3.3: VOLCANO PLOT SHOWING DIFFERENTIALLY EXPRESSED MICRORNAS IN CP70 VS A2780

The microRNA microarray results of differentially regulated microRNAs in CP70 vs A2780 are depicted here as a volcano plot. The log2 of the fold change values are plotted against –log10 p-values (Two tailed t-test) on the Y-axis. MiR-31 and miR-21* datapoints are highlighted.

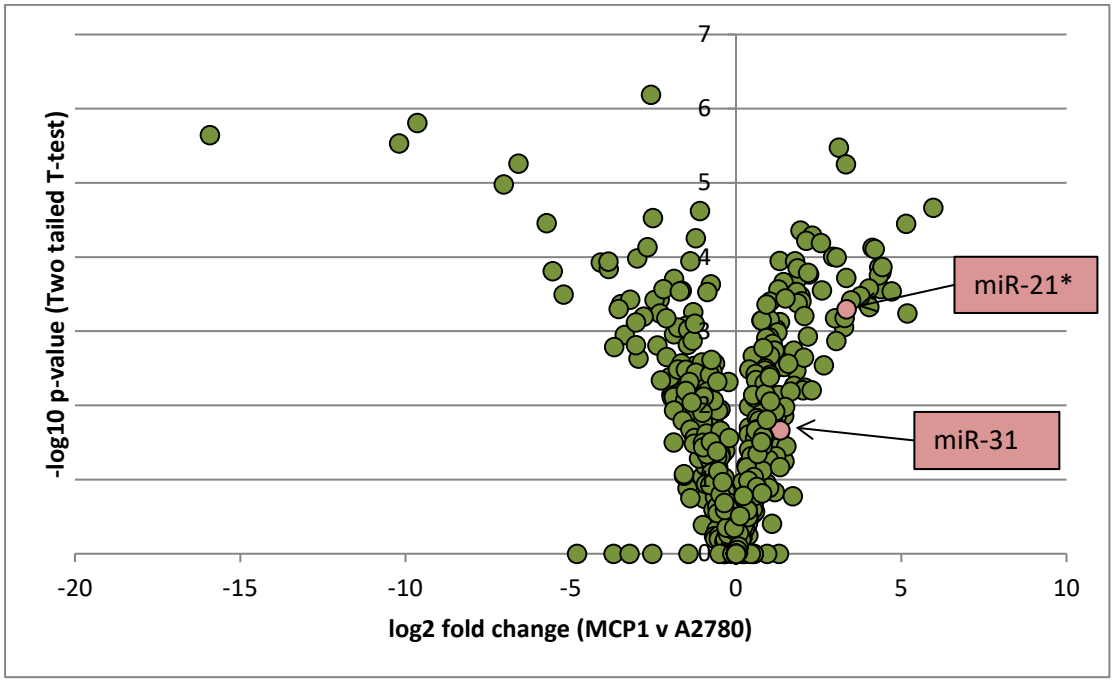

FIGURE 3.4: VOLCANO PLOT SHOWING DIFFERENTIALLY EXPRESSED MICRORNAS IN MCP1 VS A2780

The microRNA microarray results of differentially regulated microRNAs in MCP1 vs A2780 are depicted here as a volcano plot. The log2 of the fold change values are plotted against -log10 p-values (Two tailed t-test) on the Y-axis. MiR-31 and miR-21* datapoints are highlighted.

These results indicate the these microRNAs are differentially regulated in the resistant phenotype and merit further investigation regarding their role in the regulation of a resistant phenotype. Of these microRNAs, miR-21* was of special interest as it is a passenger strand and miR-31 showed the highest fold change between the sensitive and resistant cell lines. This chapter discusses the investigation of miR-21* and cisplatin resistance; microRNA-31 and its association with cisplatin resistance is presented in Chapter 4.

3.3.3. *MicroRNA-21* and cisplatin resistance*

3.3.3.1. *MicroRNA-21* levels are higher in cisplatin resistant cell lines*

In order to confirm the results of the microarray, miR-21* levels were estimated by Taqman miRNA qPCR assay in total RNA extracted from the A2780 and CP70 cells; the procedure was performed by Dr. Ryan Pink. The results are presented in Pink et al (2015). Expression of miR-21* was nearly 20-fold higher in the cisplatin resistant CP70 cell line as compared to A2780 cell line.

3.3.3.2. *MicroRNA-21* and cisplatin resistance in ovarian cancer cell lines*

Gain or loss of function experiments were carried to establish if microRNA-21* modulated cisplatin resistance.

Raising levels of miR-21* increases resistance

To investigate if increasing the levels of miR-21* within cells changes resistance, A2780 cells in half of the wells on a 96-well plate of were transfected with miR-21* mimics alongside control cells transfected with a neutral microRNA (Section 2.5) and then treated with cisplatin (Section 2.4) at 20 μM. The response was quantified as cell viability by the MTT assay (Section 2.2). The results indicate that A2780 cells treated with miR-21* mimic have a 20% higher percentage of survival than those treated with controls (Students t-test, p-value < 0.0001) (Figure 3.5).

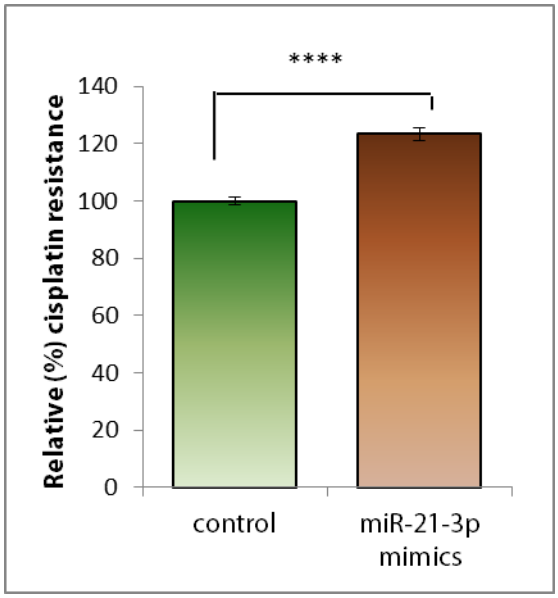

FIGURE 3.5: MICRORNA-21* INCREASES RESISTANCE TO CISPLATIN IN A2780 CELLS
A2780 cells in 96 well plates were treated with 5nM miR-21* mimics or negative control miRNA and then subjected to 20 μM cisplatin treatment for 3 hours. The percentage of cell viability (measured by the MTT assay 48 hours after cisplatin treatment) after cisplatin treatment as compared to that of untreated cells in each group was then normalised to control and compared by the student's t-test; error bars show SEM of 64 biological replicates. The results show an increase in resistance after treatment with microRNA-21* mimics by about 20% (p-value<0.0001).

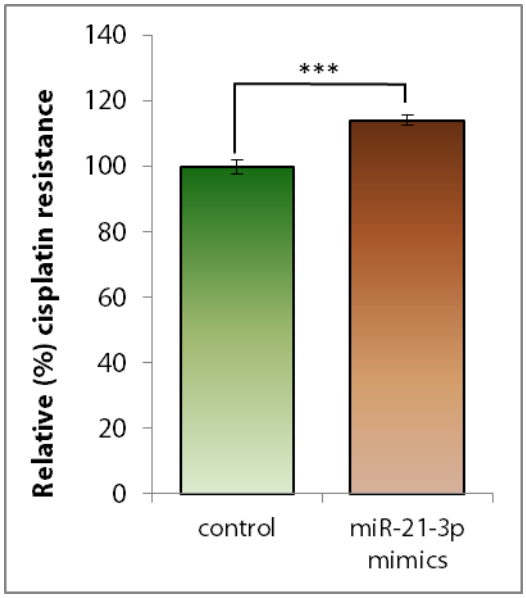

FIGURE 3.6: MICRORNA-21* INCREASES CISPLATIN RESISTANCE IN A2780 (SULFURHODAMINE B ASSAY)
A2780 cells in 96 well plates were treated with 5nM microRNA-21* mimics or negative control miRNA and then subjected to 20 μM cisplatin treatment for 3 hours. The percentage of cell viability (as measured by the Sulfurhodamine B assay 48 hours after cisplatin treatment) after cisplatin treatment as compared to that of untreated cells in each group was then normalised to control and compared by the student's t-test; error bars show SEM of 12 biological replicates. The results show an increase in resistance after treatment with microRNA-21* mimics by about 15% (p-value<0.001).

Similar results were obtained when the cisplatin response of miR-21* mimic treated and control treated A2780 was quantified by the sulfurhodamine assay (Section 2.3) (Figure 3.6). MiR-21* mimic treated group showed a 15% increase in cell viability as compared to the control treated cells (students t-test, p-value < 0.001). This shows that miR-21* mimics increase cisplatin resistance.

Cisplatin concentration curves were experimentally plotted using MTT assay after miR-21* mimic treated A2780 cells and negative control treated A2780 cells were treated with varying concentrations of cisplatin (Figure 3.7). The IC50s were then calculated using GraphPad PRISM; miR-21* mimic treatment was shown to raise the IC50 from 9.056μM to 13.15μM; this shift in the curve was shown to be significant using the extra sum-of-squares F test in GraphPad PRISM (p-value = 0.0051).

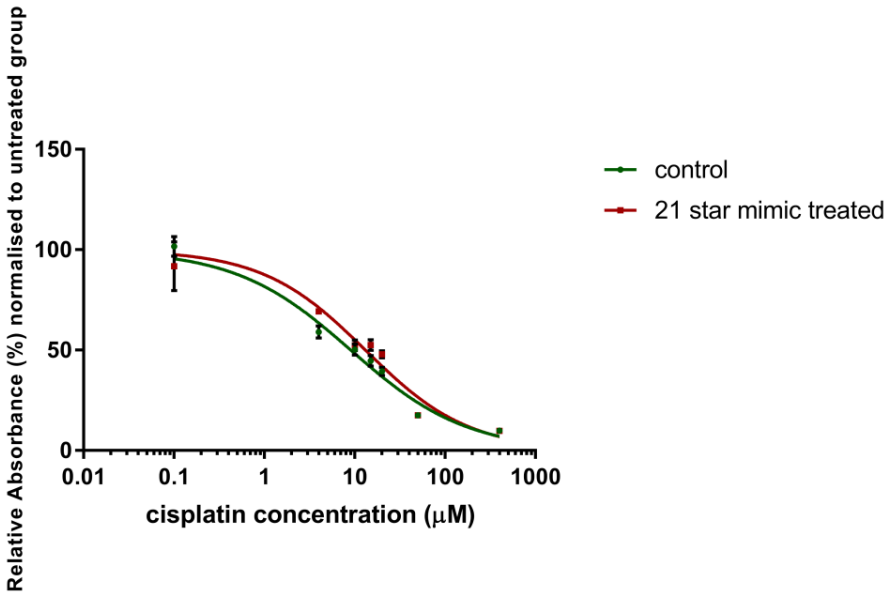

FIGURE 3.7: MIR-21* MIMIC TREATED CELLS SHOW INCREASED CISPLATIN RESISTANCE
A2780 cells seeded in 96-well plates were treated with 5nM miR-21* mimics or 5nM negative control miRNA 24 hours after seeding. 24 hours later, they were subjected to different concentrations of cisplatin for 3 hours. Cell viability (as measured by the MTT assay) after a further 48 hours has been normalised to untreated cells in each group. Error bars show SEM of at least 5 biological replicates for each point. IC50 of the control cells was calculated (GraphPad PRISM) as 9.056; treatment with miR-21* mimic raised it to 13.15; this difference was shown to be significant (p-value 0.0051) by the extra sum-of-squares F test in GraphPad PRISM.

In order to validate the results, the experiments were repeated in three other ovarian cancer cell lines – MCP1, OVCAR-5 and IGROV-1. Treatment with miR-21* mimics increased cisplatin resistance in these cell lines (Figure 3.8). MCP1, the cisplatin resistant derivative of the A2780 cell line showed a significant increase in cell viability of about 20% after cisplatin treatment in the miR-21* mimic treated wells as compared to the control (p-value < 0.001). OVCAR-5 and IGROV-1 showed an increase in cisplatin resistance by about 20% while it was more pronounced in OVCAR-8 with cell viability increasing by about 30% in the miR-21* mimic treated group. This confirms that miR-21* mimics transfection increases cisplatin resistance in a range of ovarian cancer cell lines.

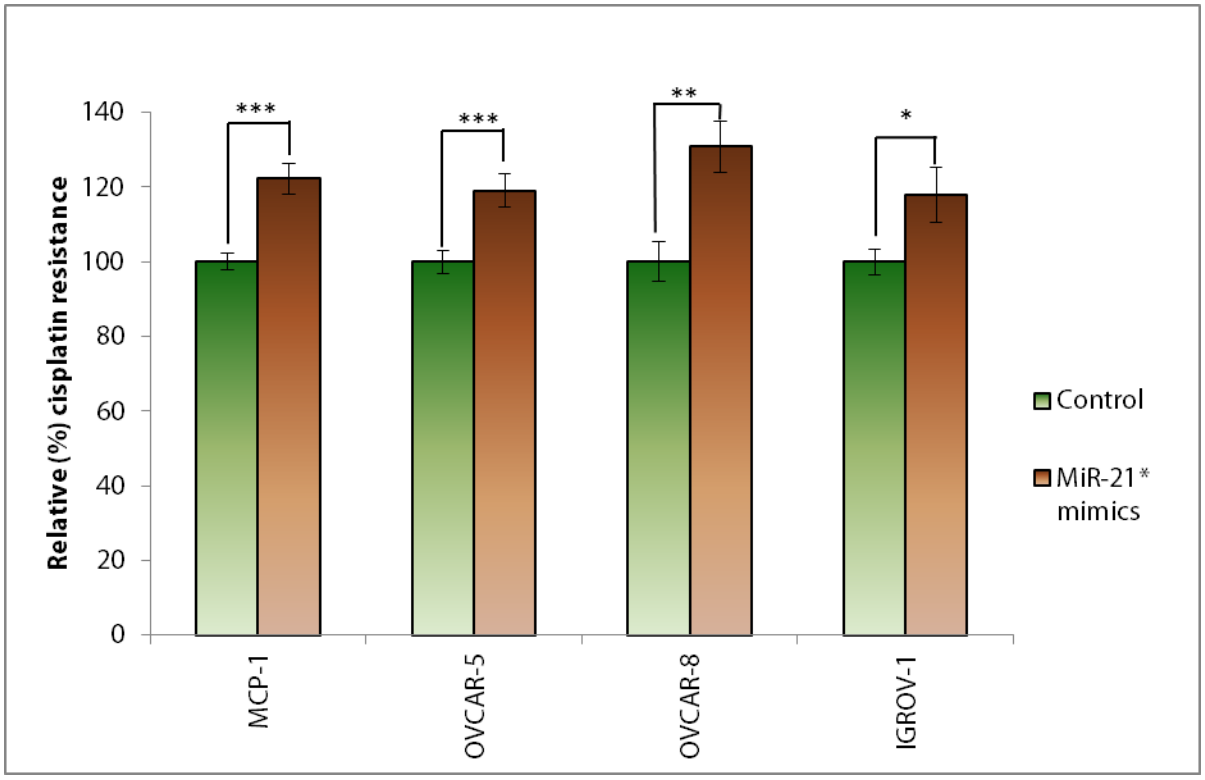

FIGURE 3.8: MIR-21* MIMIC TRANSFECTION INCREASES RESISTANCE IN A PANEL OF OVARIAN CANCER CELL LINES

Cells seeded in 96 well plates were treated with 5nM miR-21* mimics or control; after 24 hours they weresubjected to cisplatin treatment for 3 hours. MTT assay was performed 48 hours later; the percentage of cell viability (measured by the MTT assay) after cisplatin treatment as compared to that of untreated cells in each group was then normalised to control and compared by the student's t-test; error bars show SEM from at least 9 biological samples. The results show an increase in cisplatin resistance after treatment with miR-21* mimics in all the cell lines.

Inhibiting miR-21* decreases resistance

In order to investigate the effect of inhibiting miR-21*, specific hairpin inhibitor of miR-21* were transfected into cells alongside a negative control siRNA and response quantified by MTT assay 48 hours after cisplatin treatment. In A2780 cells, cell viability decreased after cisplatin treatment by about 10% in the miR-21* inhibitor treated group as compared to the control (Figure 3.9, p-value = 4.37E-06). In the CP-70s, the resistant cell line, the decrease was marginally more marked with a 15% decrease in survival in the miR-21* inhibitor treated group (Figure 3.10; p-value = 0.003477). This indicates that inhibiting miR-21* decreases cisplatin resistance in A2780s and cisplatin resistant CP70s.

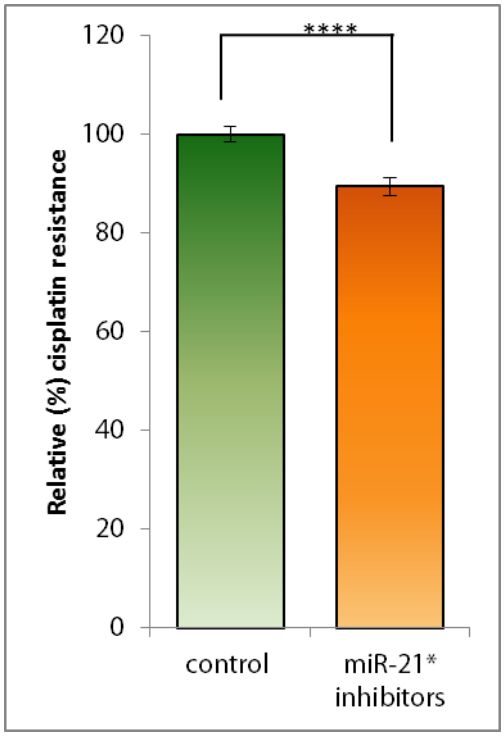

FIGURE 3.9: MIR-21* INHIBITORS DECREASE RESISTANCE IN A2780 CELLS

A2780 cells were treated with 5nM miR-21* inhibitors alongside negative controls; 24 hours later treated with 20 μM cisplatin for 3 hours; cell viability was assessed by MTT assay 48 hours later. Results were assessed as percentage of cell survival after cisplatin treatment as compared to untreated cells and then normalised to control and compared using the students t-test; error bars show SEM of 20 biological replicates. Results show a decrease in survival after miR-21* inhibitor treatment.(p-value < 0.0001)

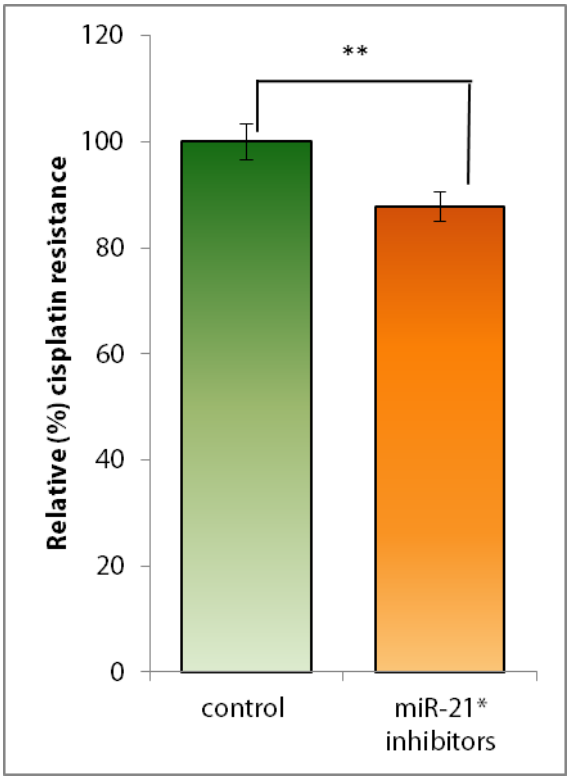

FIGURE 3.10: MIR-21* INHIBITORS DECREASE RESISTANCE IN CP-70 CELLS

CP70 cells were treated with 5 nM miR-21* inhibitors alongside negative controls; 24 hours later treated with 150 μM cisplatin for 3 hours; cell viability was assessed by MTT assay 48 hours later. Results were assessed as percentage of cell survival after cisplatin treatment as compared to untreated cells and then normalised to control and compared using the students t-test; error bars show SEM of 20 biological replicates. Results show a substantial decrease in survival in the miR-21* inhibitor treated group as compared to the control.(p-value = 0.003477)

The effects of miR-21* inhibitor treatment were also tested in the other ovarian cancer cell lines (Figure 3.11). In the OVCAR-5 cells there was a small (5%) decrease in survival in the wells treated with miR-21* inhibitors prior to cisplatin treatment (p-value < 0.05) while in the IGROV-1 cell line there was a substantial (30%) decrease in cell viability in the miR-21* inhibitor treated group as compared to the control (p-value < 0.001). The very marginal decrease in the OVCAR-8 cell cisplatin resistance upon inhibitor treatment was not significant. These results show

that, pre-treatment with miR-21* inhibitor decreases cell survival on cisplatin treatment.

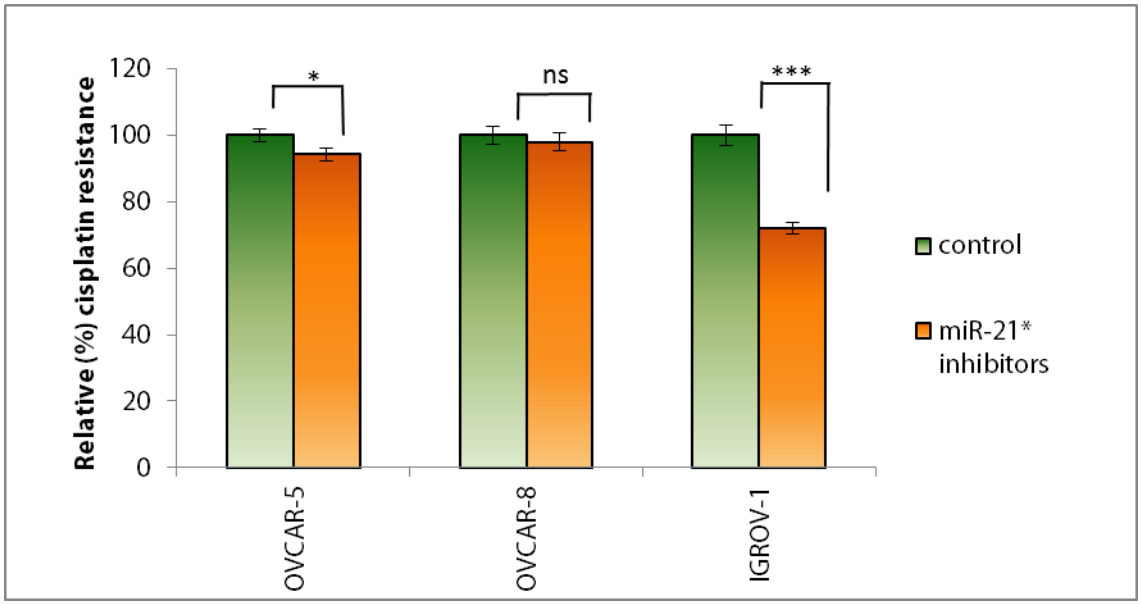

FIGURE 3.11: MIR-21* INHIBITORS DECREASE RESISTANCE IN OVARIAN CANCER CELL LINES

OVCAR-5, OVCAR-8 and IGROV-1 cells were treated with 50 nM miR-21* inhibitors alongside negative controls; 24 hours later treated with appropriate cisplatin concentration for 3 hours; cell viability was assessed by MTT assay 48 hours later. Results were assessed as percentage of cell survival after cisplatin treatment as compared to untreated cells and then normalised to control and compared using the students t-test; error bars show SEM of at least 6 replicates. Results show a decrease in survival after miR-21* inhibitor treatment in OVCAR-5 and IGROV-1 cell lines (Student's t-test p-values <0.05 and <0.001 respectively); however there is no significant change in resistance in the OVCAR-8 cell line.

*3.3.4. IDENTIFICATION OF GENE TARGET OF MIR-21**

In order to identify possible gene targets through which miR-21* modulates cisplatin resistance three approaches were used:

1. Microarray was used to quantify gene expression levels in microRNA-21* mimic treated A2780 and control treated A2780 cells to identify genes with differential expression. The results of the microarray are described in Section 3.3.4.1.
2. RNA sequencing of total RNA from A2780, MCP1 and CP70s to identify genes with lower expression in CP70s than in A2780s. The results of the RNA sequencing are described in Section 4.2.3.1.

3. Predicted targets of miR-21* were identified through online prediction program miR-walk which also shows predictions by miRANDA, PICTAR, PITA and Targetscan (Dweep et al., 2011). Results are discussed in section 3.3.4.3.

3.3.4.1. GENE EXPRESSION MICROARRAY

Gene expression microarray (method described in section 2.10; this microarray was performed by Dr Pink) was used to identify genes with a lower expression level in miR-21* mimic treated A2780s. 5nM microRNA-21* mimics were transfected in A2780 cells alongside control; total RNA extracted from both sets of cells was converted to cDNA, labelled using the dual-labelling Nimblegen Expression Array protocol, hybridised to a Nimblegen human transcriptome microarray, processed and scanned, converted to probe intensity, Loess- and quartile-normalised. 774 genes showed significant down-regulation with p-value < 0.05. Table 3-2 shows 30 down-regulated genes with the highest fold change in the microRNA-21* mimic treated cells. The results are also shown as a volcano plot (Figure 3.12) with log2 fold change (miR-21* treated A2780 vs control A2780) plotted against –log10 p-value (two tailed T-test). NAV3 datapoint is highlighted.

TABLE 3-2: TOP 30 DOWNREGULATED GENES IN MIR-21* MIMIC TREATED A2780 CELLS

Number	gene	Fold change	P value
1	DSPP	0.30447	0.00057
2	OR4Q3	0.361882	0.00628
3	OR5J2	0.368845	0.0307
4	LOC647315	0.404726	0.00673
5	CHRM2	0.410338	0.0113
6	KRTAP26-1	0.412532	0.000397
7	OR2F1	0.415295	0.00977
8	OR51F2	0.417786	0.0127
9	DSG4	0.422472	0.00613
10	OR2T8	0.432284	0.0431
11	OR2T33	0.432441	0.0368
12	KRTAP21-1	0.433447	0.0236
13	KRTAP19-1	0.439084	0.0284

Table 3-2 contd..

Number	gene	Fold change	P value
14	DSG4	0.443013	0.00968
15	KRTAP13-1	0.445072	0.0191
16	KRTAP19-1	0.452724	0.0298
17	LOC646036	0.462944	0.0146
18	OR52N5	0.475751	0.0198
19	OR52E2	0.478061	0.00204
20	KRTAP21-2	0.480694	0.0318
21	NAV3	0.491796	0.00106
22	OR10K1	0.493486	0.0235
23	OR10G4	0.495873	0.0413
24	RASSF6	0.502593	0.00777
25	OR2T4	0.503748	0.0381
26	OR1C1	0.506809	0.000438
27	LOC642974	0.507505	0.0136
28	LOC646738	0.511915	0.0132
29	LOC642691	0.517265	0.025
30	OR4K1	0.51914	0.0364

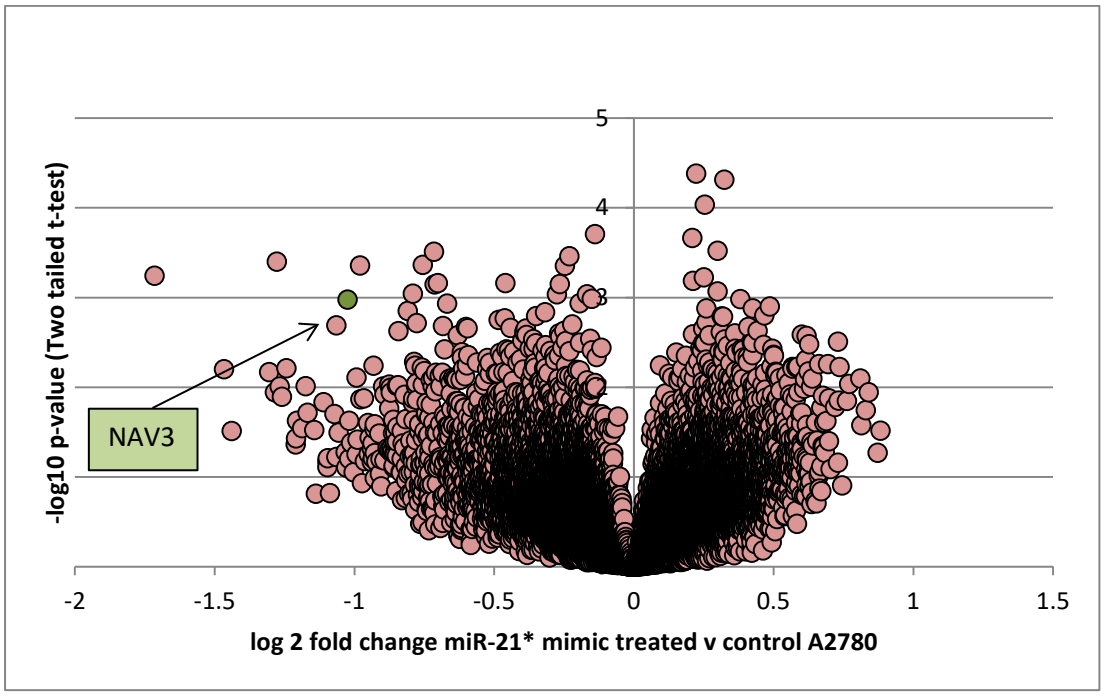

FIGURE 3.12: VOLCANO PLOT MIR-21* VS CONTROL A2780

Volcano plot showing microarray results - log 2 fold change of miR-21* mimic treated A2780 vs control A2780 against –log10 p-value (Two tailed T-test). NAV3 datapoint is highlighted.

3.3.4.2. *RNA SEQUENCING OF A2780, MCP1 AND CP70 CELL LINES:*

RNA sequencing (data presented in Section 2.11) was used to identify genes with decreased level of expression in CP70 as compared to A2780. These results are discussed in section 4.2.3.1. 21 genes that were significantly and concordantly down regulated in the CP70 and MCP1 cell lines and miR-21* mimic treated A2780s are shown in Table 3-3.

3.3.4.3. *MIRWALK PREDICTION OF TARGETS FOR MIR-21**

The online target prediction site miRWalk which also uses predictions from miRANDA, PICTAR, PITA and Targetscan was used to identify the presence of predicted target sites for miR-21* on the 21 genes of interest. The number of programs that predicted direct target sites for miR-21* on each gene are shown in Table 3-3. Only ten of these genes were predicted to be possible direct targets for microRNA-21*.

TABLE 3-3: TABLE SHOWING GENES WITH SIGNIFICANT DOWNREGULATION IN BOTH MIR-21* MIMIC TREATED A2780S AND IN CP70S

gene	Fold change MiR-21* v control A2780	P value	fold change CP70 v A2780	No of programs predicting target sites for miR-21*
NAV3	0.491796	0.00106	0.043308	2
MAMDC1	0.578199	0.0133	0.025884	2
DUSP15	0.6347	0.0117	0.294859	0
MMP12	0.688634	0.00947	0.155521	1
MCF2L	0.747838	0.0493	0.322626	0
GUCY1B3	0.769102	0.0327	0.009633	0
FGFR1	0.774261	0.0411	0.319566	0
LRRC54	0.815706	0.0215	0.42271	2
PRTG	0.82748	0.0188	0.293472	2
PRAME	0.83008	0.00444	0.205112	0
CRISPLD1	0.837649	0.0337	0.054499	0
HRC	0.852772	0.038	0.024003	0
RAD51L1	0.876691	0.0337	0.093278	1
CASP1	0.886228	0.0394	0.025706	0
CFH	0.889898	0.000916	0.26174	0
RTTN	0.89005	0.00836	0.062524	0
TRPM3	0.89691	0.0123	0.013509	2
DREV1	0.897284	0.0389	0.234848	1
SLC7A2	0.898151	0.0315	0.266712	1
GPC6	0.900474	0.0311	0.035161	0
PDIA4	0.902532	0.0474	0.4086	2

3.3.4.4. CORRELATION WITH CISPLATIN RESISTANCE IN NCI60 PANEL OF CELL LINES

In order to further whittle down possible gene targets of miR-21*, gene expression levels from RNA sequencing data of ovarian cancer tumours was obtained from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). The tumours were divided into resistant and sensitive; the change in expression levels of the genes of interest between the resistant and sensitive tumours were analysed; the fold change and T-test values are given in Table 3-4. Of the genes of interest, only PDIA4 and RTTN showed a slight but significant change in expression levels with down regulation in resistant tumours.

Analysing the correlation of the genes with the cisplatin IC50 of established NCI60 panel cell lines would provide further indication that the gene could be involved in

cisplatin resistance. Target genes of miR-21* that are involved in cisplatin resistance would be negatively correlated with the IC50 in the cell lines. Therefore GEO dataset (GEO GDS1761 / 2618) with gene expression data of all the NCI60 panel of cell lines acquired by microarray was obtained from the GEO website along with the cisplatin IC50 of these cell lines. The two datasets were analysed to identify correlations between expression of gene of interest and IC50. The ovarian cancer tumours from the TCGA dataset were divided into resistant and sensitive; the change in expression levels of the genes of interest between the resistant and sensitive tumours were analysed using Student's t-test. PDIA4 showed a significant decrease in resistant tumours in the TCGA dataset (Figure 3.13). In the dataset with the NCI60 panel cell lines, while analysis of all the cell lines did not show any significant correlation (Pearson's correlation and p-values are shown in Table 3-4), analysis of the ovarian cancer cell lines on the panel appeared to show a significant negative correlation between cisplatin resistance and PDIA4. Figure 3.14 shows the correlation between PDIA4 and cisplatin IC50 of all the cell lines from the NCI60 panel – no significant correlation or trend is seen; however, on analysis of the ovarian cancer cell line subset (Figure 3.15), a very strong negative correlation appears to exist (Pearsons -0.917; p-value < 0.05). As PDIA4 appears to correlate with both cisplatin resistance in the NCI60 panel and chemotherapy resistance in the TCGA dataset, it was further investigated.

TABLE 3-4: GENES OF INTEREST CORRELATION WITH TCGA OVARIAN CANCER GENE EXPRESSION DATA AND NCI60 PANEL GENE EXPRESSION –CISPLATIN IC50 DATA

gene	Fold change MiR-21* vs control A2780	fold change CP70 v A2780	No of programs predicting target sites for miR-21*	TCGA data correlation	t-test value	Pearsons correlation NCI60 panel	p-value	Pearsons correlation NCI60 panel – ovarian	p-value
NAV3	0.492	0.043	2	0.821	0.148	-0.038	0.775	-0.038	0.942
MAMDC1	0.578	0.026	2	0.890	0.686	NA		NA	
MMP12	0.689	0.156	1	0.578	0.146	-0.199	0.127	-0.649	0.163
LRRC54	0.816	0.423	2	1.013	0.936	NA		NA	
PRTG	0.827	0.293	2	1.105	0.607	NA		NA	
RAD51L1	0.877	0.093	1	1.047	0.502	NA		NA	
TRPM3	0.897	0.014	2	0.794	0.262	NA		NA	
DREV1	0.897	0.235	1	0.990	0.851	-0.159	0.224	0.037	0.944
SLC7A2	0.898	0.267	1	1.175	0.560	NA		NA	
PDIA4	0.903	0.409	2	0.789	0.002	0.045	0.733	-0.917	0.01

NA - Not available

- Significant change/correlation

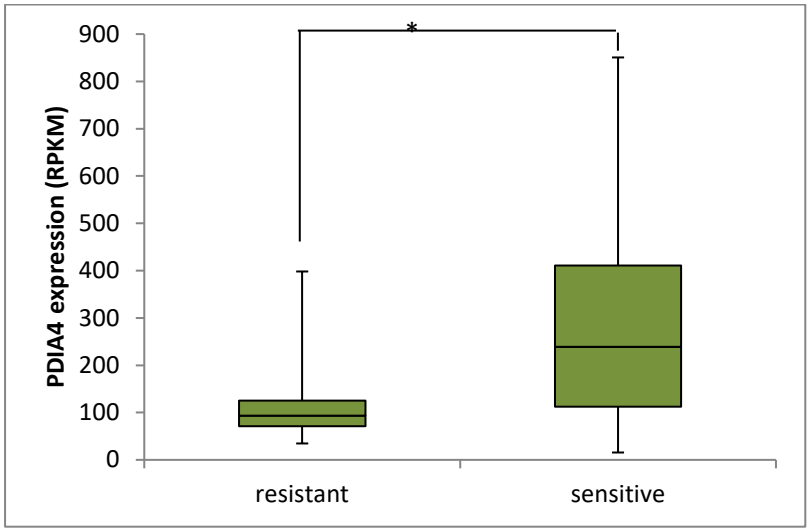

FIGURE 3.13: PDIA4 EXPRESSION IN SENSITIVE AND RESISTANT OVARIAN CANCERS IN TCGA DATA

RNA sequencing data was downloaded for the ovarian cancer dataset from The Cancer Genome Atlas along with clinical data indicating resistance or sensitivity to chemotherapy. The expression levels (RPKM) of PDIA4 were compared between the sensitive set and the resistant set of ovarian tumours; fold changes are presented in Table 3-4. The expression of PDIA4 was significantly different between the sensitive and the resistant set (student's t-test p-value = 0.002).

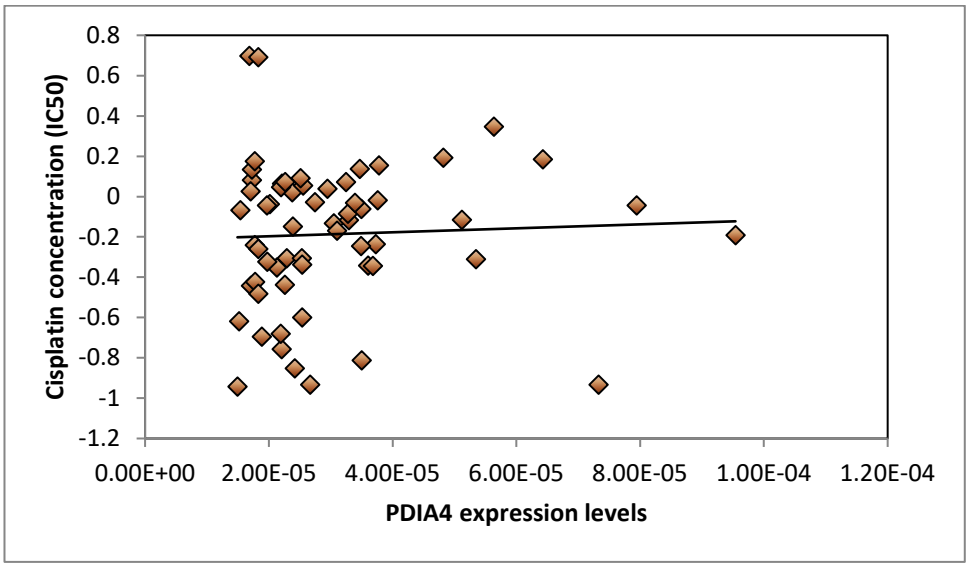

FIGURE 3.14: PDIA4 EXPRESSION LEVELS AND CISPLATIN IC50 IN NCI60 PANEL
Data set GEO GDS1761 / 2618 was obtained from the GEP website. PDIA4 expression levels from this dataset for the cell lines in the NCI60 panel were plotted with cisplatin IC50 for the cell lines. No significant correlation is seen.

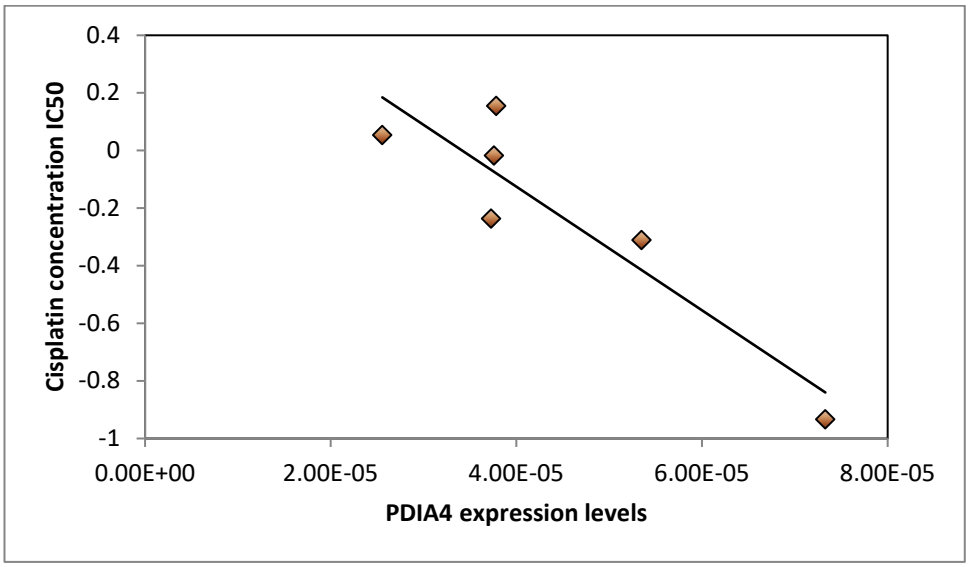

FIGURE 3.15: PDIA4 EXPRESSION LEVELS AND CISPLATIN IC50 IN OVARIAN CANCER SUBSET OF NCI60 PANEL
Expression level of PDIA4 in the ovarian cancer subset of NCI60 panel of cell lines and corresponding cisplatin IC50 were plotted. Data from GEO website: GEO GDS1761 / 2618. A strong negative correlation can be clearly seen (Pearsons -0.917, p-value < 0.05).

3.3.5. PDIA4 AND CISPLATIN RESISTANCE

Loss of function experiments were done to ascertain if PDIA4 had a role in cisplatin resistance. PDI inhibitor 16F16 was used to decrease PDI function. In published

literature, PDI inhibitor 16F16 has been used at various concentrations from 2µM (Liu et al., 2015) to 50µM (Zhang et al., 2014). Hoffstrom et al. (2010) and Kaplan et al., (2015) showed that the EC50 was 1.5µM while Liu et al (2015) showed effects on cancer cells from about 2µM. Hence, the A2780 cells were treated with 2 µM 16F16. A2780 cells were treated with 2 µM 16F16, a general PDI inhibitor 1 hour prior to treatment with cisplatin at 20 µM for 3 hours. Viability was assayed after 48 hours by MTT assay (Section 2.2). Results (Figure 3.16) showed a significant increase in resistance by about 20% (students t-test p-value <0.0001). These results indicate that PDI inhibitors increase cisplatin resistance in A2780 cells.

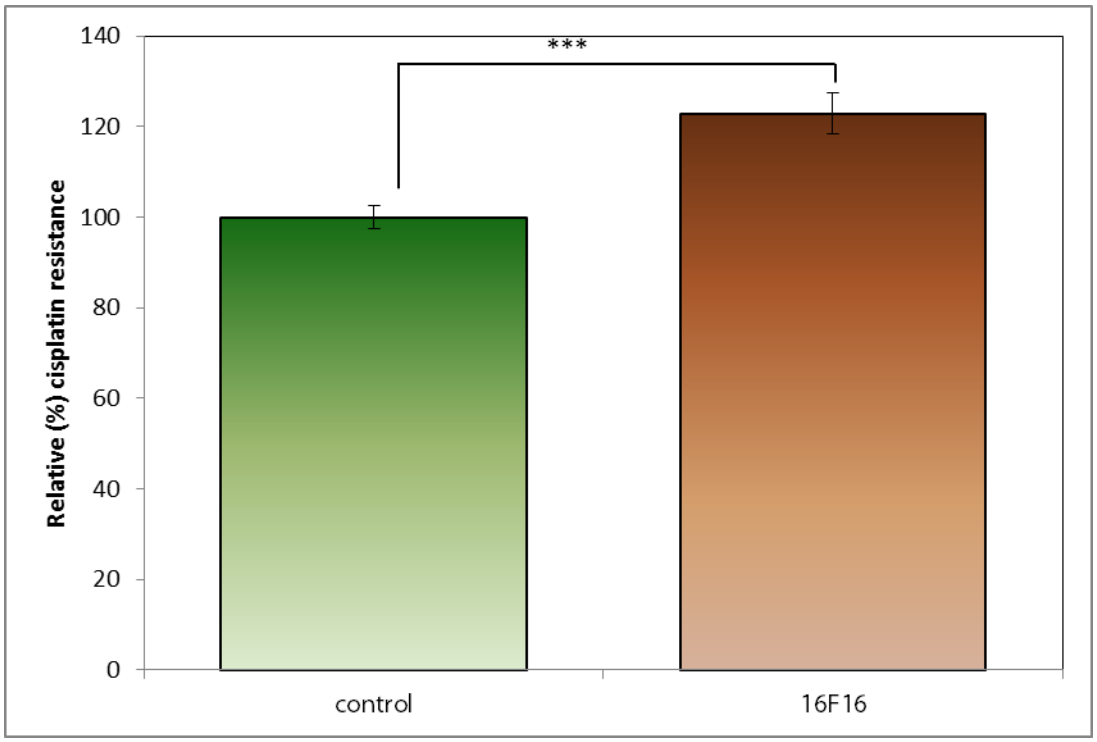

FIGURE 3.16: PDI INHIBITOR 16F16 INCREASES RESISTANCE TO CISPLATIN IN A2780 CELLS

A2780 cells seeded in 96-well plates were treated with 2 µM 16F16 for 1 hour prior to treatment with cisplatin for 3 hours. Cell viability was assessed by the MTT assay 48 hours later; resistance values were quantified as a percentage of cisplatin untreated cells in each group and normalised to control; error bars show SEM of 15 biological replicates. Results showed a significant increase in resistance in 16F16 treated group (students p-value < 0.0001).

Two specific siRNAs designed to knockdown PDIA4 at two different regions were obtained from Fisher Scientific (see section 2.5). Knockdown of PDIA4 was confirmed by RT-qPCR with SYBR Green (method and primers described in section 2.7). Data was analysed by the $2^{\Delta\Delta Ct}$ method with GAPDH as the reference gene. Results

are shown in Figure 3.17; PDIA4 siRNA1 showed >60% knockdown (p-value < 0.001) and PDIA4 siRNA2 showed 25% knockdown (student's t-test p-value < 0.01).

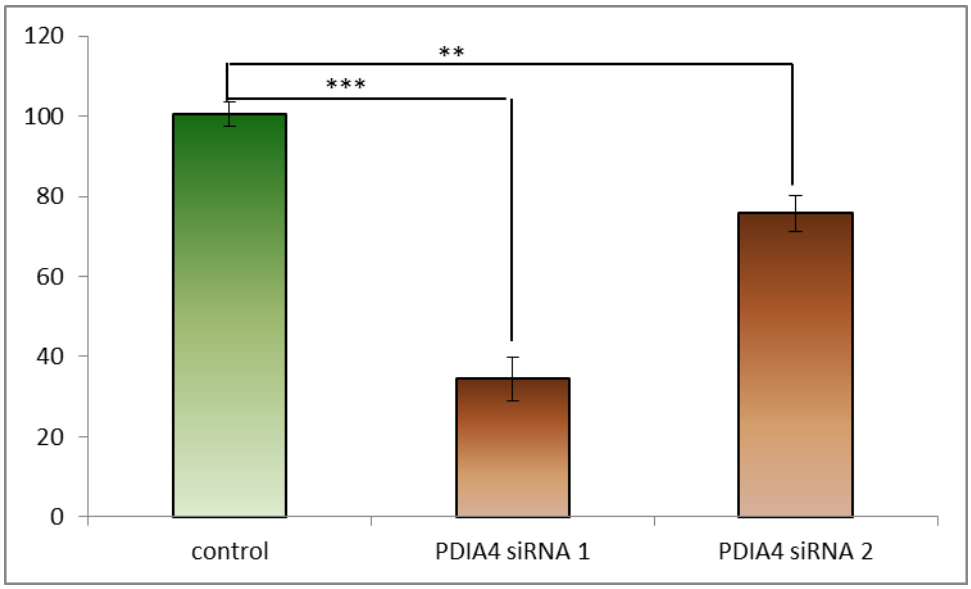

FIGURE 3.17: PDIA4 SIRNA KNOCKDOWN VALIDATED

30ng of cDNA was used per 20µl SYBR green PCR reaction along with 1µl each of forward and reverse primers (10µM) (Sigma) and 10µl of SYBR green mastermix (SensiMix SYBR, Bioline, UK); the reactions were subjected to 40 cycles of PCR with 10 seconds of denaturation at 95°C, 10 seconds of annealing at 60°C and 20 seconds of elongation at 72°C in Bio-Rad CFX96 RT-PCR system.Data was analysed by the 2^ΔΔCt method normalised to GAPDH; the result is shown normalised to the control transfected cells.

A2780 cells were then treated with 50nM PDIA4 knockdown siRNA1 or siRNA2 alongside control siRNA prior to treatment with cisplatin. Cell viability was assessed by MTT assay (section 2.2). Unexpectedly, specific knockdown of PDIA4 had no significant effect on cisplatin resistance in A2780 cells (Figure 3.18). Therefore genes of interest were re-evaluated using the approaches described before.

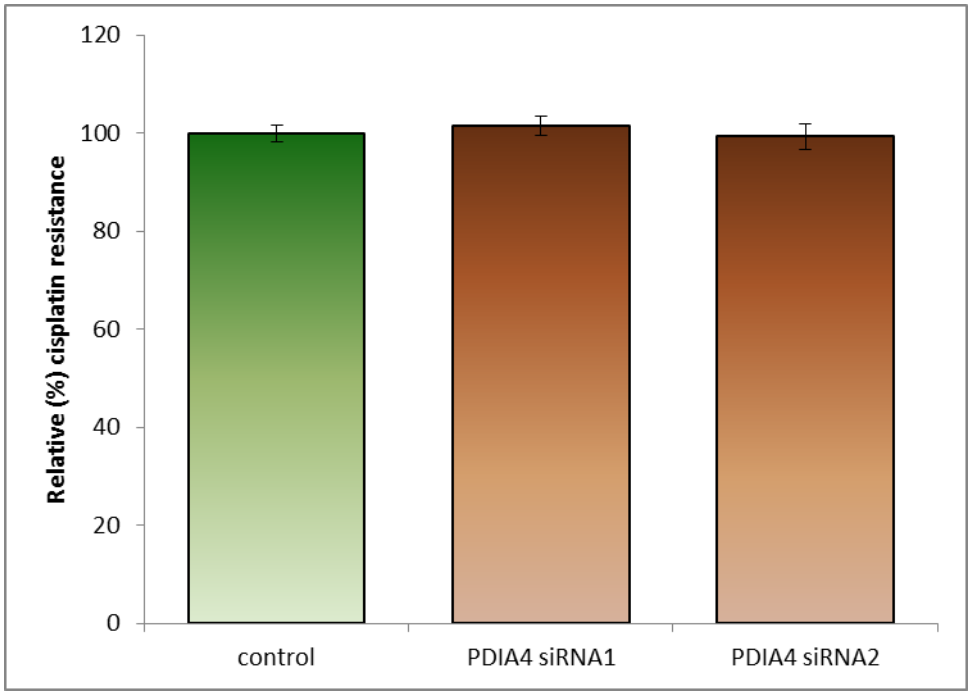

FIGURE 3.18: *PDIA4* KNOCKDOWN DOES NOT AFFECT CISPLATIN RESISTANCE IN A2780 CELLS

A2780 cells seeded in 96-well plates were treated with 50nM PDIA4 knockdown siRNA1 or siRNA2 or control siRNA for 24 hour prior to treatment with cisplatin for 3 hours. Cell viability was assessed by the MTT assay 48 hours later; resistance values were quantified as a percentage of cisplatin untreated cells in each group and normalised to control; error bars show SEM of 25 biological replicates. Results showed no significant change in resistance PDIA4 knockdown groups..

3.3.6. NAV3 AND CISPLATIN RESISTANCE

Neurone navigator 3 (NAV3) with a significant fold change of 0.492 in the miR-21* treated cells (Section 3.3.4.1), a significant fold change of 0.0433 in the CP70 cells (Section 3.3.4.2) and predicted by both miRWalk and MiRanda to have a target site for miR-21* (Section 3.3.4.3) was further investigated. The TCGA dataset, however did not show a significant difference between the resistant and sensitive tumours for expression of NAV3 (Figure 3.19).

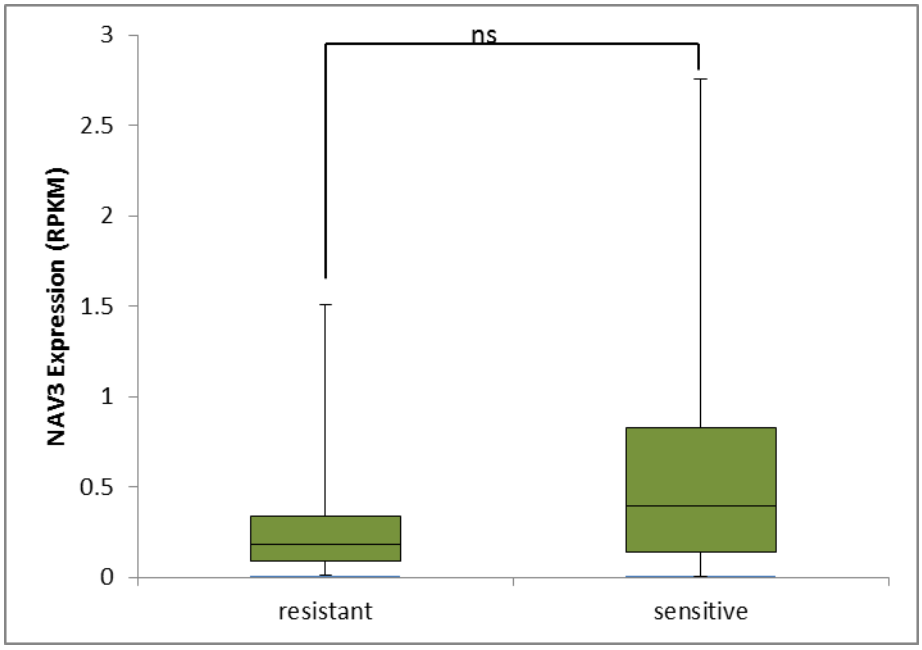

FIGURE 3.19: *NAV3* DOES NOT SHOW SIGNIFICANT DIFFERENCE BETWEEN SENSITIVE AND RESISTANT TUMOURS IN TCGA DATASET

RNA sequencing data was downloaded for the ovarian cancer dataset from The Cancer Genome Atlas along with clinical data indicating resistance or sensitivity to chemotherapy. The expression levels (RPKM) of NAV3 were compared between the sensitive set and the resistant set of ovarian tumours; fold changes are presented in Table 3-4. The difference between the sensitive and resistant sets were not significant.

3.3.6.1. NAV3 AND RESISTANCE IN OVARIAN CANCER CELL LINES

NAV3 was transiently knocked down using 50 nM specific NAV3 knockdown siRNA (Section 2.5) in A2780 cells in a 96-well plate alongside cells treated with negative control siRNA (MISSION® siRNA Universal Negative Control #2 SIC002 from Sigma). Knockdown was validated in A2780 by western blot (Pink et al., 2015). Half of the wells in each group were then treated with 20 µM cisplatin and proliferation assessed by MTT assay (Section 2.2). Results show about 10% higher survival after cisplatin treatment in NAV3 silenced cells as compared to the control cells (Student's t-test p-value <0.001) (Figure 3.20).

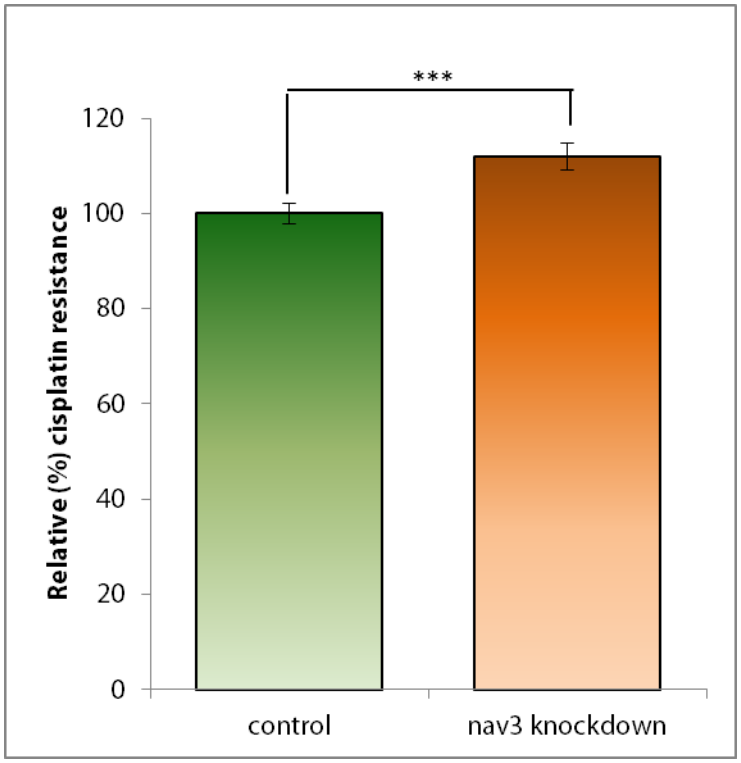

FIGURE 3.20: TRANSIENT NAV3 KNOCKDOWN RAISES CISPLATIN RESISTANCE LEVELS IN A2780

A2780 cells in 96-well plates were subjected to transient knockdown of NAV3 by treatment with 50nM siRNA alongside appropriate controls for 24 hours. They were then treated with 20μM cisplatin for 3 hours. After 48 hours, cell viability as measured by MTT was quantified as a percentage of untreated cells in each group and then normalised to control. Error bars show SEM of 20 biological replicates. The results show an increase in resistance by about 10% (students t-test, p-value < 0.001)

Similar results were obtained from the other cell lines (Figure 3.21). NAV3 knockdown in OVCAR-5 and IGROV-1 showed a marginal but significant (5-10%) increase in resistance (p-value < 0.01 and <0.05 respectively) while in the OVCAR-8 cell line, there was a substantial (almost 40%) increase in resistance in the cells in which NAV3 was knocked down as compared to the control (p-value < 0.01). Therefore NAV3 knockdown increases cisplatin resistance in ovarian cancer cell lines.

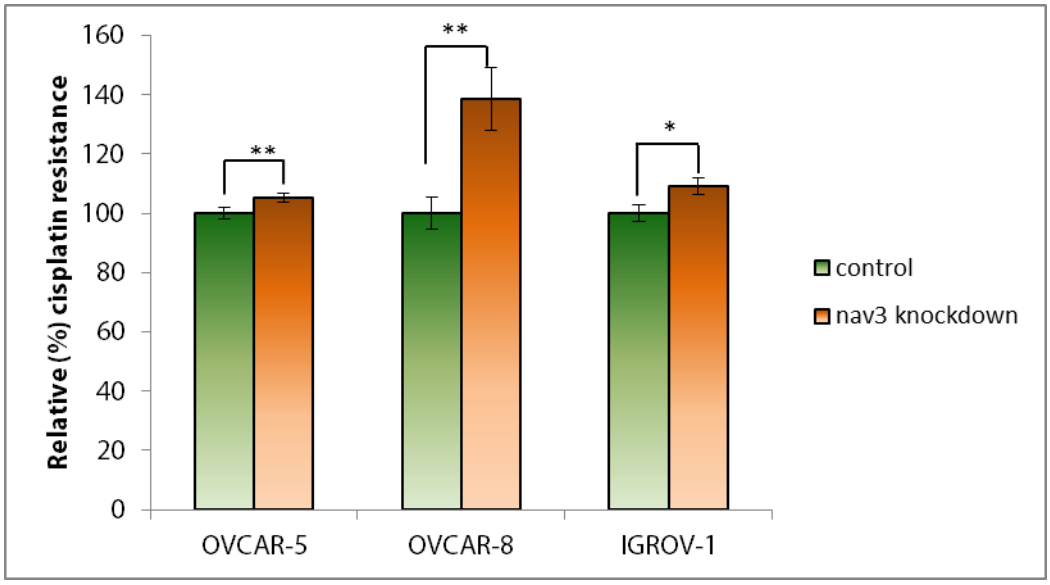

FIGURE 3.21: NAV3 KNOCKDOWN INCREASES CISPLATIN RESISTANCE IN OVARIAN CANCER CELL LINES

NAV3 was transiently knocked down in OVCAR-5, OVCAR-8 and IGROV-1 cells and response to cisplatin quantified by MTT assay. Cells were seeded in 96-well plates and transfected with 50nM NAV3 knockdown siRNA. 24 hours later, half the wells in each group were treated with cisplatin (50µM for OVCAR-5 and IGROV1 and 80µM for OVCAR-8) for 3 hours. Cell viability was assessed by MTT assay after 48 hours. Error bars show SEM of at least 12 biological replicates. All three cell lines showed an increase in resistance. (* - p-value <0.05; ** - p-value<0.01)

3.3.6.2. NAV3 LEVELS LOWER IN CP70

NAV3 protein expression levels were compared in A2780 and CP70 by western blotting (Section 2.7). The results, shown in FIGURE *3.22*, confirm decreased levels of NAV3 in the resistant CP70 cell line.

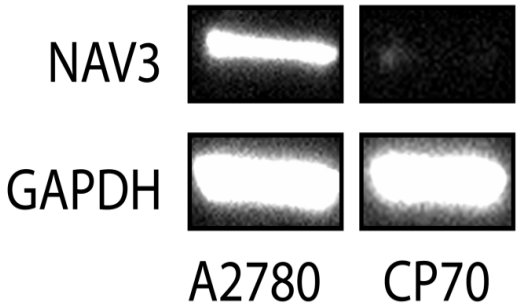

FIGURE 3.22: WESTERN BLOT SHOWS NAV3 LEVELS LOWER IN CP70 CELL LINE.

Protein was extracted from A2780 and CP70 cells and run on a 7.5% denaturing polyacrylamide gel, electrophoresed and transferred to a PVDF membrane. The membrane was blocked with 5% w/v Marvel in TBS-Tween, incubated with the primary antibody (1:10000 overnight at 4°C for NAV3 or 1:15000 for 1 hour at room temperature); then incubated with the HRP-labelled secondary antibody (1:5000 for 1 hour at room temperature) and washed. They were then immersed in ECL solution and digitally imaged. Results show lower levels of NAV3 in the resistant cell line. The original blots are shown in Appendix D: Figure D:4.

*3.3.7. NAV3 IS A DIRECT TARGET OF MIR-21**

Further experiments were carried out in order to validate that NAV3 is a direct target of miR-21*.

*3.3.7.1. NAV3 PROTEIN LEVELS ARE LOWER IN MIR-21*MIMIC TREATED CELLS*

NAV3 protein levels were quantified by western blotting (Section 2.7). Total protein was extracted from miR-21* mimic treated and negative control treated A2780 cells. Western blotting results for NAV3 performed on this protein are shown in FIGURE *3.23*. NAV3 protein levels are markedly decreased in cells transfected with miR-21* mimics suggesting that NAV3 is indeed a target of miR-21*.

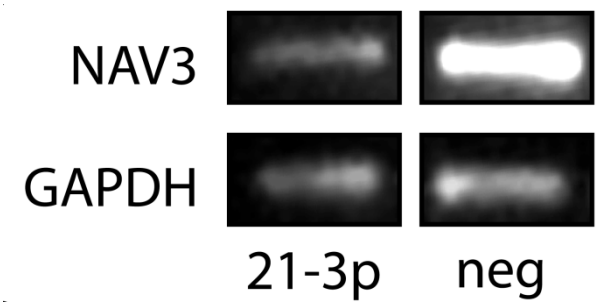

FIGURE 3.23: WESTERN BLOTTING SHOWS A DECREASE IN NAV3 LEVELS ON TREATMENT WITH MIR-21* MIMICS

Total protein was extracted from A2780 cells, 24 hours after transfection with 5 nM miR-21* mimics or negative control miRNA. Protein was extracted and run on a 7.5% denaturing polyacrylamide gel, electrophoresed and transferred to a PVDF membrane. The membrane was blocked with 5% Marvel in TBS-Tween, incubated with the primary antibody (1:10000 overnight at 4°C for NAV3 or 1:15000 for 1 hour at room temperature); then incubated with the HRP-labelled secondary antibody (1:5000 for 1 hour at room temperature) and washed. They were then immersed in ECL solution and digitally imaged. Results show lower levels of NAV3 in the resistant cell line. Levels are markedly lower in miR-21* mimic treated cells as compared to the control. The original blots are shown in Appendix D: Figure D.3.

*3.3.7.2. LUCIFERASE ASSAY SHOWS NAV3 IS A DIRECT TARGET OF MICRORNA-21**

In order to show that NAV3 is a direct target of microRNA-21*, luciferase assay (Section 3.2.1) was performed. A2780 cells stably expressing NAV3-3' UTR luciferase construct (Lentiviral vector pLSGNAV3 3′UTR-RenSP) were selected with puromycin. These cells were then treated with miR-21* mimic or negative

control and luciferase expression levels determined by the luciferase assay. Results (Figure 3.24) indicated that levels of luciferase expression decreased by 15% when treated with miR-21* mimics as compared to control miRNA transfection (p-value<0.001).

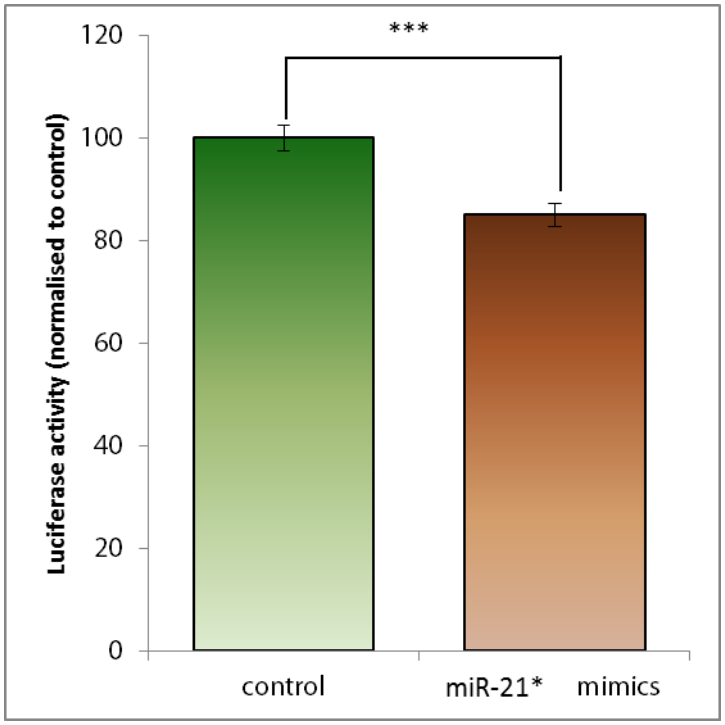

FIGURE 3.24: LUCIFERASE ASSAY INDICATES THAT NAV3 IS A DIRECT TARGET OF MIR-21*

A2780 cells stably expressing NAV3-3' UTR luciferase construct were transfected with 5nM miR-21* mimics or negative control microRNA. 24 hours later luciferase expression was determined by the luciferase assay. Results are presented normalised to the control; error bars show SEM of 6 biological replicates; there is decrease in luciferase expression by 15% on treatment with miR-21* mimics as compared to the control (p-value < 0.001).

3.3.8. MIR-21 AND PROLIFERATION/ APOPTOSIS*

To assess the effect of miR-21* on proliferation and apoptosis, Apo-Live Glo assay was performed. A2780 cells were treated with 5nM miR-21* mimics or control for 24 hours prior to treatment with cisplatin. 48 hours later, both proliferation and apoptosis were quantified by the Apo-Live Glo assay. Apoptosis was quantified as percentage of cisplatin untreated cells and normalised to the control. As seen in Figure 3.25, there is no significant change in apoptosis on miR-21* mimic treatment.

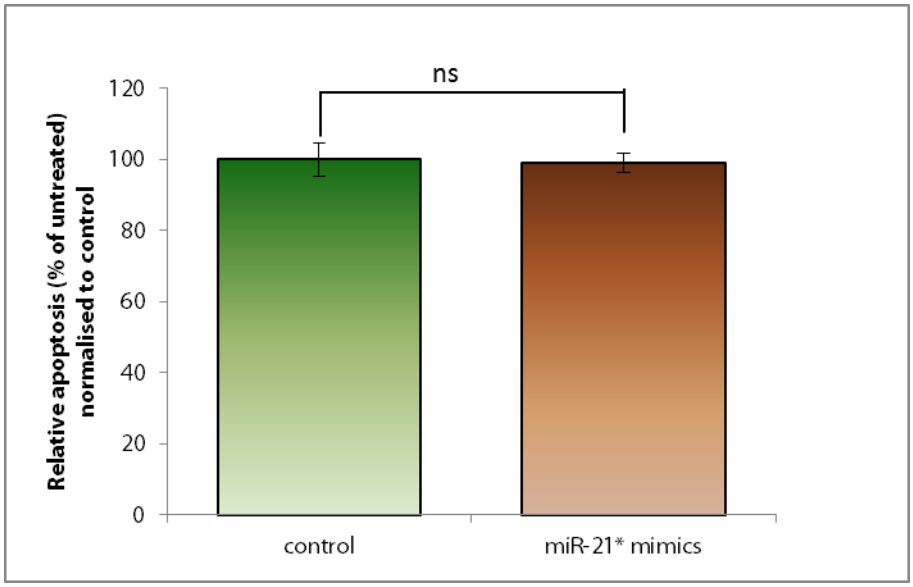

FIGURE 3.25: MIR-21* MIMIC TREATMENT DOES NOT CHANGE APOPTOSIS IN A2780 CELLS

A2780 cells seeded in 96-well plates were treated with 5nM miR-21* mimic or negative control; 24 hours later, half the wells in each group were treated with 20μM cisplatin. Proliferation and apoptosis were assessed 48 hours later by the Apo-Live Glo assay from Promega. Apoptosis was quantified as percentage of untreated cells normalised to control. There appears to be no significant change in apoptosis in miR-21* mimic treated cells.

When proliferation was compared between miR-21* mimic treated cells and control treated cells, there appears to be no significant change; however when the proliferation is compared between cisplatin treated cells, those treated with miR-21* mimics show a significant increase in proliferation as compared to control treated cells (student's t-test p-value =0.0092) (Figure 3.26).

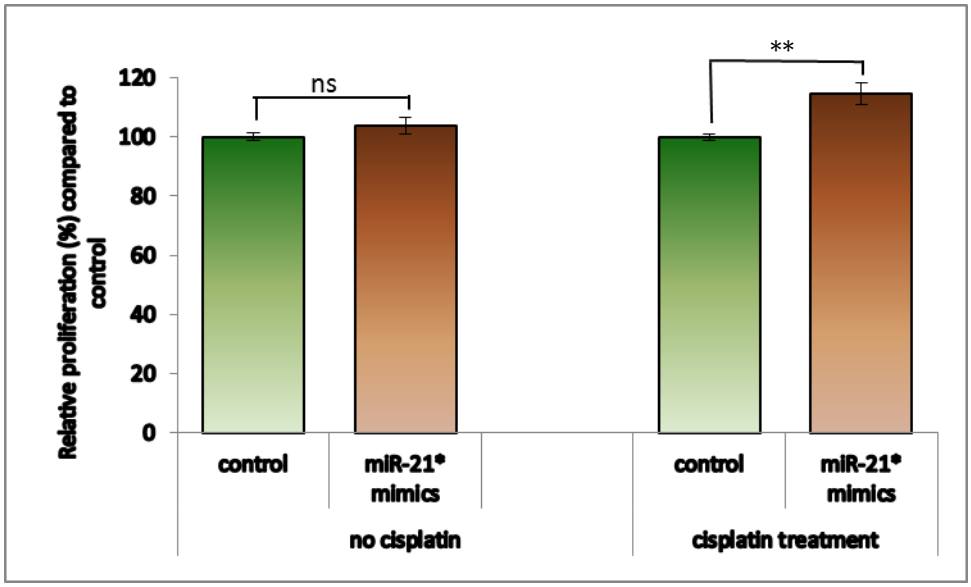

FIGURE 3.26: MIR-21* MIMIC INCREASE PROLIFERATION ON CISPLATIN TREATMENT
A2780 cells seeded in 96-well plates were treated with 5nM miR-21* mimic or negative control; 24 hours later, half the wells in each group were treated with 20μM cisplatin. Proliferation and apoptosis were assessed 48 hours later by the Apo-Live Glo assay from Promega. Proliferation was normalised to control; error bars show SEM of 6 biological replicates. There appears to be no significant change in proliferation in miR-21* mimic treated cells as compared to controls; however on cisplatin treatment, there is a significant increase in proliferation (students t-test p-value =0.0092) in the miR-21* mimic pretreated cells.

The same trend is seen from the MTT assay (combined data shown in Fig 3.4) in A2780 cells treated with miR-21* mimic or control with or without cisplatin treatment. In fact, there appears to be a slight decrease in cell viability on treatment with miR-21* mimic (student's t-test p-value <0.01) with no cisplatin treatment (Figure 3.27). However, on cisplatin treatment, cell viability is markedly increased (student's t-test p-value < 0.001) in the cells pre-treated with miR-21* mimics as compared to control.

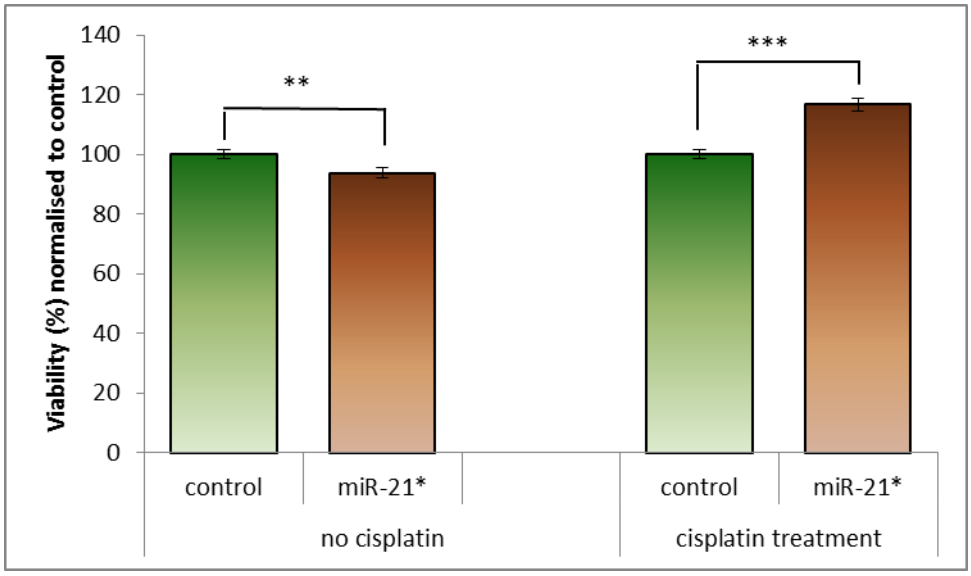

FIGURE 3.27: MIR-21* MIMICS INCREASE RESISTANCE ON CISPLATIN TREATMENT
A2780 cells were treated with 5 nM miR-21* mimics or negative control; 24 hours later, half the wells in each group were treated with 20μM cisplatin. Cell viability was assessed by the MTT assay 48 hours later. Viability was normalised to control; error bars show SEM of 64 biological replicates. There is a slight decrease in viability (student's t-test p-value < 0.01) in miR-21* mimic treated cells as compared to controls; however on cisplatin treatment, there is a significant increase in viability (student's t-test p-value < 0.001) in the miR-21* mimic pretreated cells.

This effect was verified in NAV3 knockdown in A2780 cells. Transient NAV3 knockdown was achieved using 50nM siRNA-NAV3 in A2780 cells alongside negative control treatment with or without further cisplatin treatment. There is no significant change in viable cells on NAV3 knockdown by siRNA (Figure 3.28); however on cisplatin treatment, NAV3 knockdown in A2780 cells appears to increase viability by about 20% (student's t-test p-value < 0.001).

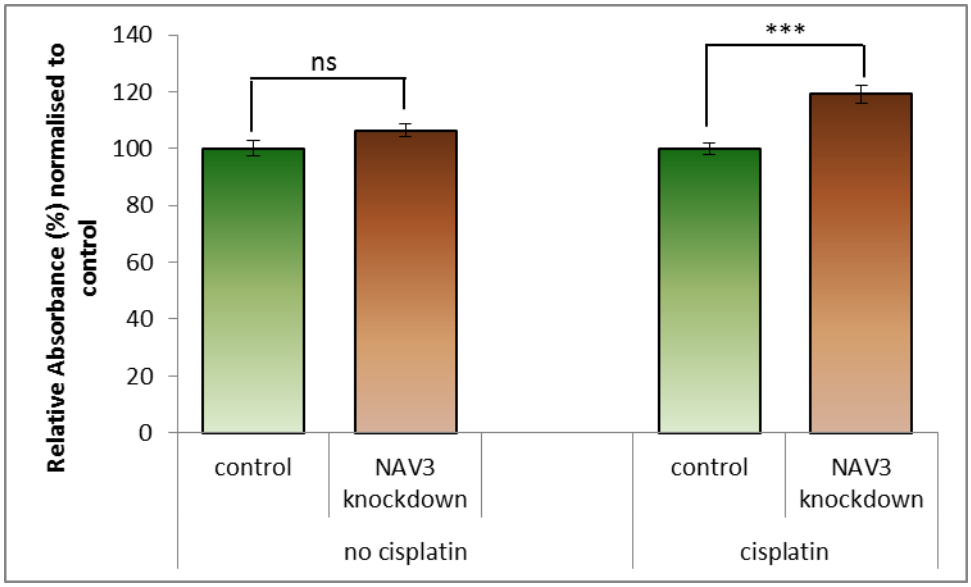

FIGURE 3.28: NAV3 KNOCKDOWN INCREASES RESISTANCE ON CISPLATIN TREATMENT IN A2780 CELLS

A2780 cells were treated with 50nM NAV3-siRNA or negative control; 24 hours later, half the wells in each group were treated with 20μM cisplatin. Cell viability was assessed by the MTT assay 48 hours later. Viability was normalised to control; error bars show SEM of 20 biological replicates. There is no significant change in viability in the cells with NAV3 knockdown as compared to controls; however on cisplatin treatment, there is a significant increase in viability (student's t-test p-value < 0.001) in the cells with transient NAV3 knockdown.

3.4 DISCUSSION

MicroRNAs are shown to regulate various aspects of cancer including metastasis, growth and drug resistance. This study used A2780, an ovarian adenocarcinoma cell line and its cisplatin resistant derivatives, MCP1 and CP70 to study the effect of microRNAs on drug resistance and to identify microRNAs that have thus far not been shown to have an effect on drug resistance. Initially, in order to identify microRNAs that might possibly be involved in cisplatin resistance, microRNAs differentially expressed between the sensitive and resistant cell lines were identified by miRNA microarray. 46 microRNAs were significantly deregulated; of these 8 microRNAs were significantly up regulated in both the resistant cell lines while 7 microRNAs were concordantly down regulated. Some of these microRNAs such as miR-17, miR-19a and miR-222 have also previously been shown to be similarly deregulated in published studies profiling microRNA expression in cisplatin resistant ovarian cancer cells (van Jaarsveld et al., 2013) increasing the confidence in the validity of the microarray. Other microRNAs showing differential expression include some microRNAs that are often shown to be deregulated in ovarian cancers such as

the miR-200 family (Muralidhar and Barbolina, 2015), let-7 family (Wang et al., 2012) and miR-214 (Penna et al., 2015).

One of the microRNAs which showed an increased expression in CP70 cells was miR-21*. MiR-21* was of special interest as it is a passenger strand microRNA. As discussed in section 3.1, studies have shown miR-21* to be deregulated in breast cancer, cervical cancers and squamous cell carcinoma of skin; however no studies have linked miR-21* with an active role in ovarian cancer thus far.

MicroRNA-21* was further investigated. The functional significance of miR-21* in cisplatin resistance was established by gain of function experiments with increasing levels of miR-21* shown to increase cisplatin resistance in ovarian cancer cell lines; this was further corroborated by loss of function experiments with inhibiting levels of miR-21* decreasing cisplatin resistance in four of the five tested cell lines. Though OVCAR-8 showed an increase in cisplatin resistance on treatment with miR-21* mimic, the converse supposition that a decrease in functional miR-21* would increase cisplatin sensitivity could not be shown. This could possibly be due to a number of factors including inefficiency of transfection in this particular cell line, cell line variation, the possibility of irreversibly altered genes or epigenetic modifications being involved in the resistance pathway, or the dominance or positive bias of a different pathway all of which points to the multifactorial and complex nature of cisplatin resistance.

MicroRNAs achieve their phenotypic effects by posttranscriptional modifications of target genes leading to a decrease in their protein levels and a possible decrease in mRNA levels. To this end, it is expected that an increase in levels of the microRNA, either endogenously or through artificial means would decrease the protein, and possibly mRNA levels of the target genes providing a method to identify genes of interest. A multipronged approach was taken to elucidate the pathways involved in miR-21*'s modulation of cisplatin resistance and to ascertain possible targets of miR-21* involved in cisplatin resistance:

- Genes down regulated by miR-21* were identified by microarray analysis
- Genes down regulated in CP70 and MCP1 cell lines which have a higher levels of miR-21* were determined by RNA sequencing

- Genes with predicted target sites for miR-21* were pinpointed using miRWalk, the online target prediction site
- Correlations between gene levels and cisplatin IC50 in the NCI panel of cell lines using data obtained from GEO dataset (GEO GDS1761 / 2618) were identified
- Analysis of gene expression levels in sensitive and resistant ovarian cancers from the TCGA database was used to corroborate possible involvement of the gene in cisplatin resistance

The results of the microarray analysis of gene expression in A2780s treated with miR-21* mimcs compared to those treated with control microRNAs have been presented in this chapter. Of the top 30 genes identified by microarray to be downregulated in miR-21* treated A2780s, RASSF6 has previously been identified as a tumour suppressor belonging to the RASSF family (Allen et al., 2007) affecting apoptosis and the cell cycle by modulating MDM2 and p53 (Iwasa et al., 2013); low expression of RASSF6 been shown previously to be associated with poor survival in pancreatic cancer (Ye et al., 2015) and treatment resistance in nasopharyngeal carcinoma (Liang et al., 2014) and to be frequently epigenetically inactivated in childhood leukaemias (Hesson et al., 2009). It has been shown that DSPP (one of the small integrin family of binding proteins) has previously been linked to breast and lung cancer but not with ovarian cancer (Fisher et al., 2004). There are a number of olfactory receptors and keratin associated protein family members on the list of deregulated genes; however none of these have been associated with ovarian cancers. This shows that a number of the genes pinpointed by the microarray have previously been associated with cancers and the list includes validated and novel targets of miR-21*.

In published literature, miR-21* has been shown to target human methionine adenosyltransferases 2A and 2B (Lo et al., 2013), CALM1 (Doberstein et al., 2014), HDAC8 (histone deacetylase-8) (Yan et al., 2015), knockdown SORB2 (sorbin and SH3 domain containing protein 2) and PDLIM5 (PDZ and LIM domain 5) (Bang et al., 2014). Of these genes, only PDLIM5 showed a marginal decrease (fold change 0.81, p-value = 0.02) in miR-21* treated A2780s according to the microarray.

However there was no significant difference between the expression of PDLIM5 in A2780s and CP70s.

As the main interest of the project was cisplatin resistance, the focus was to find genes that were concordantly changed in the cisplatin resistant derivatives of the A2780 cell line, the CP70 and MCP1. Hence genes were narrowed down based on the consistency of deregulation in these cell lines and in miR-21* treated A2780s. In a bid to check clinical relevance and widen the scope of the study, the expression of the genes in the TCGA RNA sequencing data in sensitive and resistant ovarian cancer cell lines as well as expression level correlations with IC50 of cell lines in the NCI60 panel were also taken into account.

Initially, PDIA4 was identified as a possible target for miR-21*'s effect on cisplatin resistance. PDIA4 belongs to the family of protein disulphide isomerases associated with the endoplasmic reticulum protein processing pathway forming native disulphide bonds and is an ER chaperone protein (Jessop et al., 2007, Satoh et al., 2005, Meunier et al., 2002). PDIA4 was induced by ER stress inducers tunicamycin and thrapsigargin exhibiting temporal changes (Mintz et al., 2008). In inflammatory bowel mucosa, levels of PDIA4 was found to be increased (Bogaert et al., 2011). PDIA4 was found to be downregulated in pseudopapillary tumour of the pancreas, a low grade malignant tumour (Zhu et al., 2014); on the other hand, PDIA4 was overexpressed in oesophageal squamous cell carcinoma (Pawar et al., 2011). Interestingly, PDIA4 was found to be over-expressed in cisplatin resistant lung adenocarcinoma; PDIA4 inactivation was found to restore the apoptotic pathway (Tufo et al., 2014). However, on investigation in the A2780 cell line, though there is possible evidence for the involvement of PDIs in cisplatin resistance, specific knockdown of PDIA4 failed to elicit a cisplatin resistant phenotype. Hence, it could not be established that PDIA4 had any functional relevance in cisplatin resistance in the A2780 cell lines. However, given the robust negative correlation between PDIA4 and cisplatin IC50 in the NCI60 ovarian cancer panel and survival and PDIA4 expression levels in the TCGA ovarian cancer data, PDIA4 is worth exploring in the context of other cell lines. Nevertheless, PDIA4 was not of further interest in the context of this study.

Therefore genes of interest were re-evaluated based on the multiple criteria described above and NAV3, which was down regulated in both the resistant cell lines and in A2780 cells with increased miR-21* levels and had two predicted target sites for miR-21*, was identified as a potential target for miR-21* in causing a cisplatin resistant phenotype.

NAV3 is one of the mammalian microtubule plus end tracking proteins, +TIPS, which are involved in many cellular processes including mitosis, cell migration and neurite extension (van Haren et al., 2009, Stringham and Schmidt, 2009). NAV3 has been shown to stabilise and enhance the growth of microtubules and is necessary for properly oriented chemotaxis (Cohen-Dvashi et al., 2015). It also has an actin-binding domain indicating a possibility that it might have effects on the cytoskeleton and is thought to be a component of the neuron specific nuclear pore complex (Coy et al., 2002). NAV3 is involved in axon guidance and is expressed in the adult brain as well as activated T cells, placenta, colon and some cancers (Coy et al., 2002, Maes et al., 2002). NAV3 was found to be increased in brain tissue of patients with Alzheimer's disease; it has been suggested that this is due to a decrease in miR-29a (Satoh, 2010, Shioya et al., 2010). NAV3 was also shown to be a target of miR-29c in a mouse model of Alzheimer's disease (Zong et al., 2015). It often shows expression changes in neural tumours (Coy et al., 2002), NAV3 amplification is associated with a favourable prognosis while loss of NAV3 leads to an unfavourable prognosis. Possible targets for NAV3 include IL23R (interleukin 23 receptor) and GnRHR (gonadotropin releasing hormone receptor) (Carlsson et al., 2013). Overexpression of MECP2_e1 (methyl CpG-binding protein 2 gene) associated with Rett syndrome, a progressive neurodevelopmental disorder, is associated with overexpression of various genes including NAV3 (Orlic-Milacic et al., 2014).

Recent studies have shown that other tumours may be associated with NAV3 expression changes; it is a "hill" gene in the Wood study on the topography of breast and colorectal cancer (Wood et al., 2007). Colorectal cancer and adenomas are often associated with NAV3 copy number changes; NAV3 silencing induces up-regulation of IL23R which correlates strongly with lymph node metastases and Duke's staging in colorectal cancer (Carlsson et al., 2012). NAV3 copy number changes were detected in 20-25% of basal cell and squamous cell carcinomas; loss of NAV3 was

shown to be associated with decrease in CECR1 (a protein active in sites of inflammation during hypoxia and tumour growth) and an increase in IRF8 which is involved in immune regulation (Maliniemi et al., 2011). NAV3 deletion was also found in at least 50% of patients with cutaneous T cell lymphoma (Karenko et al., 2005, Hahtola et al., 2008, Ranki et al., 2011); NAV3 silencing was shown to enhance the expression of interleukin 2 (Karenko et al., 2005). The expression of NAV3 was decreased in adrenocortical carcinomas as compared to adrenocortical adenomas (Soon et al., 2009). Loss of NAV3 has been shown to increase invasiveness, and possibly inhibit apoptosis, in breast cancer cells; this also correlates with shorter survival of breast cancer patients (Cohen-Dvashi et al., 2015). Duale et al. have also shown its possible induction by p53 after cisplatin treatment in testicular germ cell tumours (Duale et al., 2007). The literature therefore indicates that NAV3 is a tumour suppressor in some cancers, notably colorectal cancer and neural tumours; however, thus far, there is no proven role for NAV3 in ovarian cancer. NAV3 has previously been shown to be regulated by miR-29a and miR-29c in Alzheimer's disease (Shioya et al., 2010, Zong et al., 2015). MiR-21 has also been shown to target NAV3 in hepatocellular carcinoma (Wang et al., 2015). NAV3 has not been validated as a target of miR-21* previously.

The association between NAV3 and cisplatin resistance was explored by specific knockdown using NAV3-siRNA; this elicited cisplatin resistance in a panel of ovarian cancer cell lines. Moreover analysis of levels of NAV3 by western blot confirmed a decrease in the resistant cell lines. Corroboration that it was a target of miR-21* was obtained by a decrease in NAV3 levels on treatment with miR-21* and luciferase assays. Hence, the link has been established that miR-21* could decrease NAV3 levels and increase cisplatin resistance, knockdown of NAV3 levels could also produce a cisplatin resistant phenotype.

To further explore the mechanism of action of miR-21*, proliferation and apoptosis levels were quantified. There appears to be no change in apoptosis on treatment with miR-21* mimics. MiR-21* mimic treatment of cells by itself does not appear to induce an increase in proliferation; in fact there appears to be slight reduction in cell viability in miR-21* mimic treated A2780s. However, on cisplatin treatment, there is a significant increase in proliferation in miR-21* pre-treated cells. This indicates that

miR-21* acts as a driver of proliferation on induction by cisplatin. The effect was phenocopied by NAV3 knockdown; NAV3 knockdown by itself did not induce any changes in proliferation; however on cisplatin treatment, there is a significant increase in proliferation. These indicate that miR-21* drives proliferation on cisplatin treatment. One of the limitations of the project is that the actual mechanism of action of miR-21* and NAV3 has not been identified. Another limitation is that most of the experiments have been carried out *in vitro*. Other considerations include the fact that the effect of knockdown of gene function by the siRNAs was not confirmed in all the cell lines; also the efficacy of the drug 16F16 on PDIA4 was not quantified. There is much scope for future research into the actual mechanism by which miR-21* and NAV3 modify response to cisplatin and if this is the same in all the cell lines and tumours. More of the genes identified as being of interest could be followed up; pathway analysis could also be used to identify the actual mechanism. It would be interesting to study if this effect on cisplatin resistance is also present to other drugs such as paclitaxel as well as in other types of cancers. Another avenue to explore would include studying the levels in blood of patients with ovarian cancer to identify if this could distinguish some patients who are resistant, thereby predicting chemotherapy response.

Despite the limitations, the results indicate that miR-21* increases cisplatin resistance in ovarian cancer cell lines possibly by knockdown of NAV3 gene and protein levels. These novel findings contribute to the research in the field of the role of microRNAs in cisplatin resistance in ovarian cancer.

Chapter 4 MicroRNA-31 increases cisplatin resistance in ovarian cancer cells

4.1 Introduction

MiRNA microarray of total RNA from A2780 (cisplatin sensitive ovarian cancer cell line) and CP70 (cisplatin resistant derivative of the A2780) cell lines indicated deregulation in the expression of about 50 microRNAs between the two cell lines (described in Section 3.3.2). MicroRNA-31 showed the highest difference in expression levels between A2780s and CP70s on the miRNA microarray with 50-fold change between the two lines. This result was verified by qRT-PCR by Dr. Ryan Pink; the CP70s showed a significant increase in miR-31 expression while the MCP1 showed a small increase (Pink et al., 2015).

MiR-31 is a pleiotropic microRNA, often deregulated in cancers; however the associations are very complex and not clearly understood. High levels of miR-31 are present in some cancers such as lung (Edmonds et al., 2015, Meng et al., 2015), oesophageal (Zhang et al., 2011, Liu et al., 2013b, Saad et al., 2013) and colorectal (Schee et al., 2012, Yang et al., 2013b, Nosho et al., 2014) implicating miR-31 as an oncomiR; in fact, in lung (Meng et al., 2013, Edmonds et al., 2015), oesophageal (Zhang et al., 2011, Wu et al., 2013) and colorectal cancers (Schee et al., 2012, Wang et al., 2014a), high levels of miR-31 are associated with poor prognosis. However, in other cancers such as prostate (Bhatnagar et al., 2010, Lin et al., 2013), glioblastoma (Hua et al., 2012, Visani et al., 2014), breast (Korner et al., 2013, Ouyang et al., 2014) and leukemia (Yamagishi et al., 2012, Rokah et al., 2012) decreased levels of miR-31 are found; this suggests a tumour suppressing function with correlation of low miR-31 levels with poor prognosis in pancreatic cancer (Ma et al., 2013) and bladder (Segersten et al., 2014). Moreover, a study by Creighton et al suggests that miR-31 acts as a tumour suppressor in ovarian cancer cells with dysfunctional p53 signalling but not in cells with normal p53 (Creighton et al., 2010). Further to this, research into oesophageal cell lines with inactive p53 indicated that the tumour suppressive effect of miR-31 was only present if p21 was inactivated as well (Ning et al., 2014). These studies demonstrate that miR-31 can act through various targets and achieve different phenotypic effects in a tissue

specific manner; moreover, this effect can be modulated by mutations in other genes. The levels of miR-31 itself has been shown to be affected by various factors – the host gene MIR31 is located at chromosome 9p21.3, a frequently deleted location in cancers. Epigenetic modification also appears to play a role with EZH2 – subunit of the polycomb repressive complex 2 (Asangani et al., 2012, Yamagishi et al., 2012), androgen receptor (Lin et al., 2013) and EMSY (Vire et al., 2014) thought to affect methylation (Vrba et al., 2013) in the promoter region.

MiR-31 appears to modulate invasiveness and migration of cancer cells increasing the motility in lung (Meng et al., 2013), colorectal cancer (Cottonham et al., 2010, Nosho et al., 2014), oesophageal (Zhang et al., 2011) and cutaneous squamous cell carcinomas (Xu et al., 2012) while decreasing the invasion and motility in mesothelioma (Ivanov et al., 2010), glioblastoma (Hua et al., 2012), ovarian (Li et al., 2012a) and melanoma (Greenberg et al., 2011, Asangani et al., 2012). There also appears to be a variable effect on growth; proliferation being increased by higher levels of miR-31 in lung (Xi et al., 2010, Meng et al., 2013, Edmonds et al., 2015), colorectal cancers (Nosho et al., 2014, Xu et al., 2013) and oesophageal (Zhang et al., 2011) with a decrease in proliferation being observed in mesothelioma (Ivanov et al., 2010), medulloblastoma (Jin et al., 2014), leukemias (Yamagishi et al., 2012) and melanoma (Greenberg et al., 2011).

The effect of miR-31 on response to chemotherapy is also complex. Increased levels of miR-31 increased resistance to chemotherapeutic agents such as 5 fluorouracil (Wang et al., 2010) in lung cancer. In contrast, studies show increased levels of miR-31 associated with increased sensitivity to doxorubicin (Korner et al., 2013) in breast cancer; docetaxel (Bhatnagar et al., 2010, Zhang et al., 2014) in prostate cancer and paclitaxel (Mitamura et al., 2013) in ovarian cancers. The response to cisplatin is also modulated differentially as miR-31 increases sensitivity to cisplatin in prostate cancer (Bhatnagar et al., 2010) but induces cisplatin resistance in lung cancer (Dong et al., 2014); Chan et al found that only one isoform of miR-31 increased sensitivity to cisplatin in breast cancer cells (Chan et al., 2013). This suggests that the effects of miR-31 depend on numerous variables including tissue type, cell type and genetic profile.

In ovarian cancers, a study by Creighton et al (2010) suggests that miR-31 acts as a tumour suppressor in ovarian cancer cells with dysfunctional p53 signalling but not in cells with normal p53 (Creighton et al., 2010). Overexpression of miR-31 decreases colony formation in ovarian cancer cells (Mitra et al., 2012, Ibrahim et al., 2015); this might help to reprogram it ovarian fibroblast as as cancer associated fibroblasts (Mitra et al., 2012). MiR-31 also decreases the invasion and motility in ovarian cancers Ibrahim (Li et al., 2012). Studies show increased levels of miR-31 associated with increased sensitivity to paclitaxel (Mitamura et al., 2013) in ovarian cancers; it has been suggested that one of the gene targets involved in this modulation is Stathmin1 (Hassan et al., 2015).

Hence, miR-31 was further investigated and its potential involvement with cisplatin resistance and possible gene targets studied.

4.2 RESULTS

4.2.1. MICRORNA-31 MIMICS INCREASE RESISTANCE IN OVARIAN CANCER CELL LINES

Gain of function experiments were performed in ovarian cancer cell lines to confirm if microRNA-31 has a functional role in the modulation of cisplatin resistance in ovarian cancer cell lines. miR-31 mimic was confirmed by qPCR to increase levels of miR-31 in A2780 cells (Pink et al., 2015). A2780 cells were transfected with microRNA-31 mimic alongside a negative control and then treated with 20 μM cisplatin. Cell viability was quantified 48 hours after treatment by MTT assay; results (Figure 4.1) showed a 25% increase in survival of cells treated with microRNA-31 mimic than the control (student's t-test, p-value < 0.001). Cisplatin dose response curves (Figure 4.1b) show that the IC50 (GraphPad Prism) shifted significantly from 48.71 μM to 122.4 μM (p-value 0.0007). These results indicate that miR-31 transfection increases cisplatin resistance in A2780 cells.

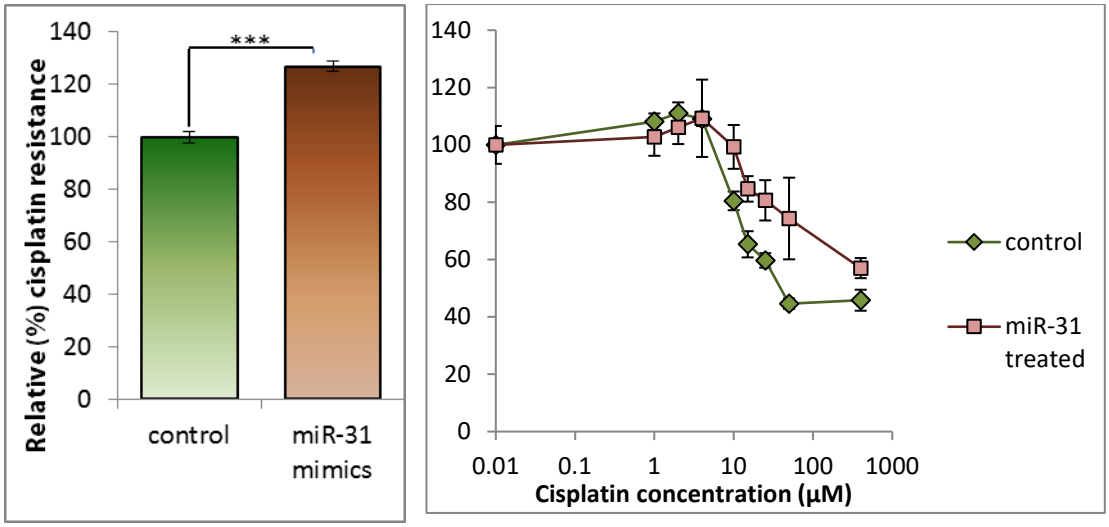

FIGURE 4.1: MICRORNA-31 RAISES RESISTANCE TO CISPLATIN IN A2780 CELLS
(a) A2780 cells seeded in 96-well plates were treated with 5 nM microRNA-31 mimics or control and then subjected to cisplatin treatment at 20 μM for 3 hours. The percentage of cell viability (as measured by the MTT assay) 48 hours after cisplatin treatment as compared to that of untreated cells in each group was then normalised to control and compared by the student's t-test. The results show an increase in resistance after treatment with microRNA-31 mimics by about 25% (student's t-test, p-value < 0.001). Each group has at least 20 biological replicates; error bars show standard error of mean. (b) A2780 cells transfected with miR-31 mimic or control miRNA were subjected to cisplatin treatment at various concentrations and cell viability quantified by MTT assay was normalised to untreated cells in each group. Each point is plotted from at least 3 biological replicates; error bars show standard error of mean. The IC50 (GraphPad Prism) shifted significantly from 48.71 μM to 122.4 μM (p-value 0.0007).

MiR-31 mimic treatment prior to cisplatin treatment also cisplatin resistance in MCP1 cell line (Figure 4.2) by 50% (student's t-test, p-value <0.001). Similarly in OVCAR-5 and IGROV-1 cell lines, miR-31 mimic treated cells were about 15% more resistant to cisplatin treatment at 20μM than control treated cells (student's t-test, p-value < 0.01 for OVCAR-5 and p-value <0.05 for IGROV1). However, the OVCAR-8 cell line showed no significant change in cisplatin resistance after treatment with miR-31 mimics.

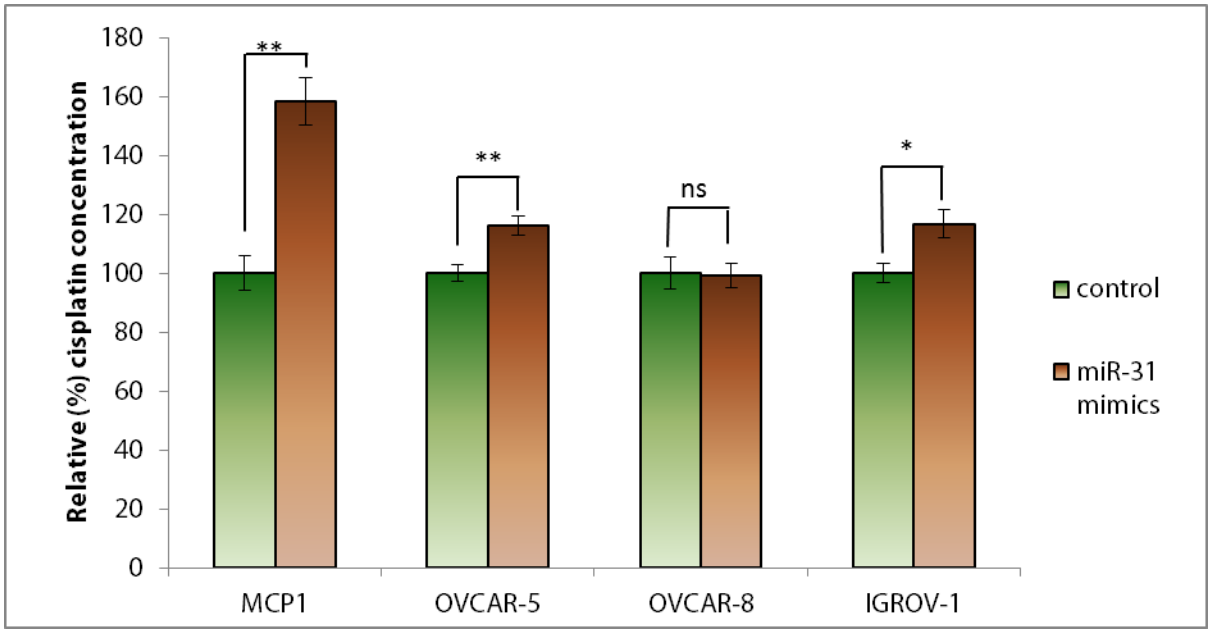

FIGURE 4.2: MICRORNA-31 RAISES RESISTANCE TO CISPLATIN IN OVARIAN CANCER CELLS

Cells seeded in 96-well plates were treated with 5nM microRNA-31 mimics or control and subjected to cisplatin treatment after 24 hours at the optimal concentration for each cell line. The percentage of cell viability (as measured by the MTT assay) after cisplatin treatment was then normalised to control and compared by the student's t-test. Error bars show SEM from at least 9 biological replicates. The results show significant increase in cisplatin resistance after treatment with microRNA-31 mimics in MCP1, OVCAR-5 and IGROV-1 (student's t-test p-value - < 0.01, < 0.01 and < 0.05 respectively)

4.2.2. IDENTIFICATION OF TARGET GENES

4.2.3.1. Gene Expression levels in A2780, MCP1 and CP70 by RNA Sequencing

Total RNA was extracted from A2780, MCP1 and CP70 and sent to Source BioScience to be sequenced using an Illumina Genome Analyser IIx. The single-end 38 base-pair reads were aligned using Illumina's pipeline software. Transcript abundance was estimated using Cufflinks and results presented as reads per kilobase of transcript per million mapped reads. Relative levels of transcripts between samples were compared using DESeq. 142 genes were upregulated more than twofold in both the resistant cell lines while 304 genes were significantly downregulated with a fold change of more than 2 in the two resistant cell lines. The top 50 upregulated or downregulated genes in the CP70s and their fold change and p-value is given in Appendix C, Tables C-1 and C-2.

4.2.3.2. DAVID analysis of the genes

The concordantly deregulated genes were further analysed using the online functional annotation tool in DAVID (Huang da et al., 2009a, Huang da et al., 2009b). Enriched molecular functions in this list of genes were identified using this tool. The top 15 GO (gene ontology) terms are shown in Table 4-1 with their corresponding p-values.

TABLE 4-1: ENRICHED GO TERMS IN SIGNIFICANTLY AND CONCORDANTLY DEREGULATED GENES

GO Term Molecular Function	Gene Count	P-Value
GO:0043167~ion binding	132	0.0005
GO:0022836~gated channel activity	19	0.0006
GO:0005509~calcium ion binding	39	0.0009
GO:0019838~growth factor binding	10	0.0010
GO:0004714~transmembrane receptor protein tyrosine kinase activity	8	0.0012
GO:0046872~metal ion binding	126	0.0019
GO:0043169~cation binding	126	0.0027
GO:0005216~ion channel activity	20	0.0028
GO:0015171~amino acid transmembrane transporter activity	7	0.0030
GO:0008289~lipid binding	22	0.0032
GO:0031420~alkali metal ion binding	14	0.0038
GO:0022838~substrate specific channel activity	20	0.0039
GO:0015267~channel activity	20	0.0057
GO:0022803~passive transmembrane transporter activity	20	0.0058
GO:0046873~metal ion transmembrane transporter activity	17	0.0064

The top 3 enriched molecular functions GO terms were “ion binding”, “gated channel activity” and “calcium ion binding” with significant p-values <0.001. The gene KCNMA1 was represented in all three of these groups. Moreover, KCNMA1 was present in 11 out of the top 15 GO categories. The product of KCNMA1 forms the alpha subunit of the large calcium-activated potassium conductance channel. KCNMA1 is in the top 50 of significantly and concordantly downregulated genes. When the genes were analysed based on KEGG pathways, 11 pathways were highlighted with significant p-values (Table 4-2).

TABLE 4-2: KEGG PATHWAYS ENRICHED IN THE CONCORDANTLY DEREGULATED GENES

KEGG pathway	Count	P-Value
hsa04360:Axon guidance	14	2.18E-04
hsa04510:Focal adhesion	17	6.33E-04
hsa04540:Gap junction	9	0.0073
hsa04270:Vascular smooth muscle contraction	10	0.0093
hsa04512:ECM-receptor interaction	8	0.0174
hsa04020:Calcium signaling pathway	12	0.02500
hsa04666:Fc gamma R-mediated phagocytosis	8	0.0319
hsa04370:VEGF signaling pathway	7	0.0322
hsa04080:Neuroactive ligand-receptor interaction	15	0.0341
hsa04664:Fc epsilon RI signaling pathway	7	0.0381
hsa04960:Aldosterone-regulated sodium reabsorption	5	0.0411

It is worth noting that the calcium signalling pathway is one of the pathways in which the list of genes was significantly enriched (p-value 0.025). The calcium signalling pathway is shown in Figure 4.3. Though KCNMA1 is not present on the list of genes in the pathway, the channel may be activated by calcium signalling and would be involved in the subsequent outcomes of calcium signalling. Hence the KCNMA1 gene was further investigated.

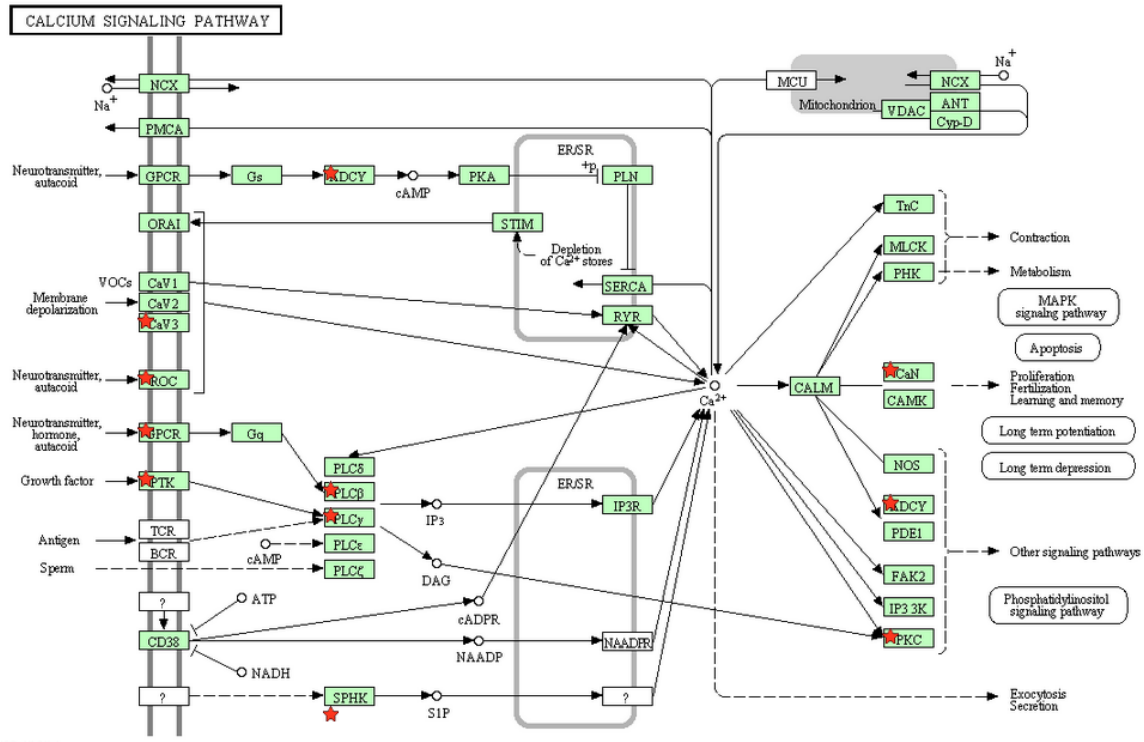

FIGURE 4.3: CALCIUM SIGNALLING PATHWAY

The Calcium signalling pathway as highlighted by the DAVID functional tool is shown here. The pathway shows the interaction of various genes in calcium signalling and levels of calcium affecting various pathways including MAPK signalling pathway and apoptosis. Red stars indicate genes that were present in the list of genes with differential expression in CP70 as compared to A2780.

4.2.3. KCNMA1 KNOCKDOWN INCREASES CISPLATIN RESISTANCE

In order to assess if KCNMA1 modulates resistance, loss of function experiments were performed. Three shRNAs (sequences shown in Section 2.5) were designed with sequences targeting different areas of the KCNMA1 mRNA. KCNMA knockdown in A2780s was validated by qPCR by Dr. Ryan Pink. A2780s were transfected with each of these shRNAs to transiently knockdown KCNMA1 alongside a scrambled control shRNA. Cells were then cisplatin treated and viability quantified by the MTT assay. Results presented are a percentage of viability after cisplatin treatment normalised to control. Figure 4.4 shows an increase in cisplatin resistance of between 20% and 30% in sets transfected with each of the three KCNMA1 knockdown shRNA as compared to the control. The most potent of the three shRNAs is the KCNMA1 shRNA 2 with the group showing an increase of about 30% as compared to the control (student's t-test, p-value = 0.00036).

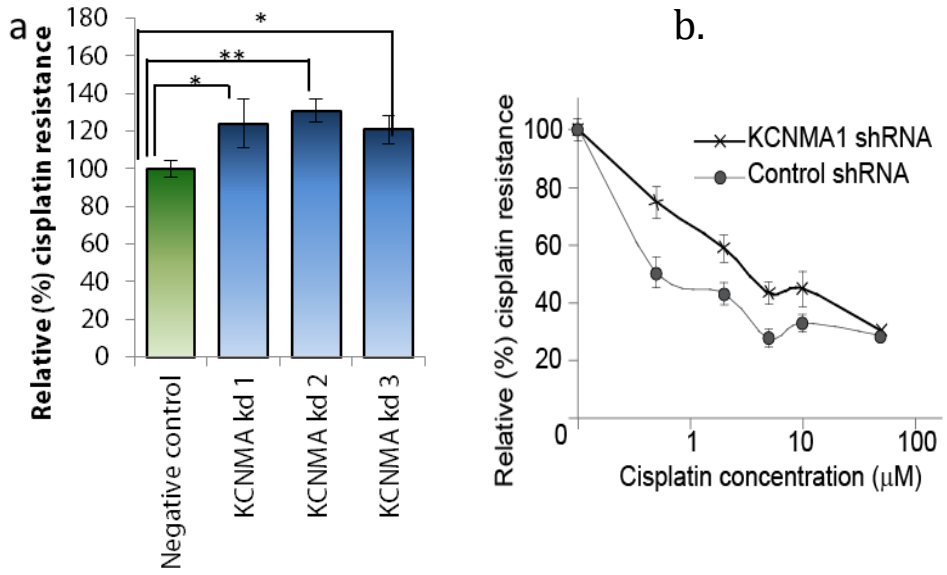

FIGURE 4.4: KCNMA1 KNOCKDOWN IN A2780 INCREASES CISPLATIN RESISTANCE
(a) Transient KCNMA knockdown was achieved using 3 shRNAs designed to target different regions on the KCNMA1 gene. A2780 cells were split into 4 groups; each group was transfected with 50 ng of one of the shRNAs and one group was treated with the scrambled control 24 hours before treatment with 20 μM cisplatin. Viability was assessed by the MTT assay 48 hours later and is presented as percentage of survival after cisplatin resistance normalised to control. Error bars show SEM from 5 biological replicates. All three shRNAs increase resistance to cisplatin as compared to control (student's t-test, pvalues = 0.049, 0.00036 and 0.014 for KCNMA kd 1, 2 and 3 respectively). (b) A2780 cells were treated with 150 ng/well of all shRNAs pooled together and 24 hours later treated with varying concentrations of cisplatin. MTT assay was used to assess viability after 48 hours and cisplatin dose response curve plotted with the absorbance data normalised to cisplatin untreated cells in each group. Error bars show SEM of 5 biological replicates. The IC50 (GraphPad PRISM) changes significantly from 10.54 μM in the control treated cells to 24.25 μM in the KCNMA knockdown group (p-value <0.0001)

In subsequent experiments, the three shRNAs were pooled and then transfected into the cells to give maximum probability of knocking down KCNMA1. The cisplatin dose response curve plotted by treating A2780 cells with 150ng of pooled KCNMA1 knockdown shRNA alongside control and then with varying concentrations of cisplatin between 0 and 50 μM is shown in Figure 4.4b. There is a clear increase in resistance of A2780 cells on knockdown of KCNMA1 at most of the concentrations of cisplatin tested. KCNMA1 was also transiently knocked down in a panel of ovarian cancer cell lines which were then treated with cisplatin at optimum concentrations. The MTT assay (Section 2.2) was used to quantify viability; results are presented as a percentage of viability after cisplatin resistance normalised to control. While A2780 and OVCAR-5 show a modest but significant increase (student's t-test, p-value < 0.05 and < 0.001 respectively) in cell viability after

cisplatin treatment of about 20%, MCP1 show a more pronounced increase of nearly 50% in cisplatin resistance. On the other hand, the IGROV1 shows no significant difference in cisplatin resistance after KCNMA knockdown. KCNMA1 knockdown has thus been shown to increase cisplatin resistance in a panel of ovarian cancer cell lines.

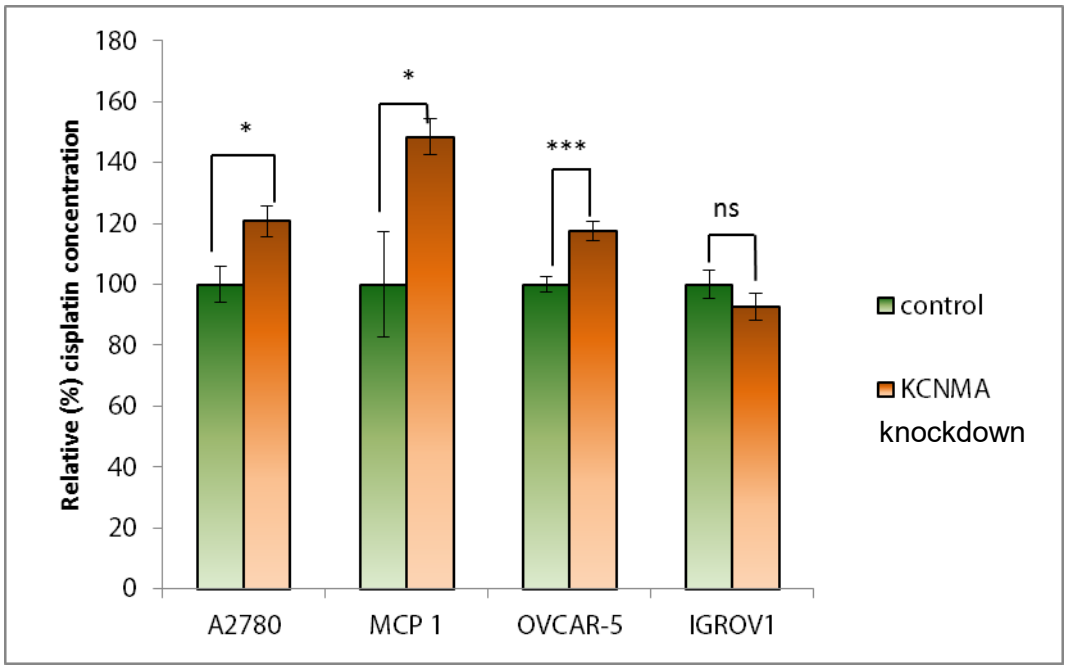

FIGURE 4.5: KCNMA KNOCKDOWN INCREASES CISPLATIN RESISTANCE IN OVARIAN CANCER CELL LINES

KCNMA1 was transiently knocked down in A2780, MCP1, OVCAR-5 and IGROV1 cell lines seeded in 96-well plates using 150ng pooled shRNAs per well alongside scrambled control; 24 hours later cells were treated with optimum concentrations of cisplatin (A2780 - 20µM, OVCAR5 and IGROV1 - 50µM and MCP1 - 80µM). MTT assay was used to quantify viability 48 hours later. Results are presented as percentage of viability after cisplatin treatment normalised to control. Error bars represent SEM of at least 6 biological replicates. There is a significant increase in survival of cells on cisplatin treatment after KCNMA1 knockdown in A2780, MCP1 and OVCAR-5 cell lines (student's t-test, p-value <0.05, <0.05 and <0.001 respectively). IGROV1 cells showed no change in cisplatin resistance.

4.2.4. KCNMA1 LEVELS LOWER IN CISPLATIN RESISTANT CELL LINES

KCNMA protein levels in the different cell lines were assessed by western blot. Approximately 20 µg of protein from each cell line was denatured, electrophoresed and transferred onto PVDF membrane. This was incubated with primary antibody (Section 2.7) (anti-KCNMA1 or anti-GAPDH) and secondary antibody, stained,

imaged and analysed. Images are shown in Figure 4.6. The levels of KCNMA1 in CP70 and OVCAR-5 are very low compared to the A2780 cell line. There is a more modest but clear difference in levels in the MCP1 cell line as compared to the A2780 cell line.

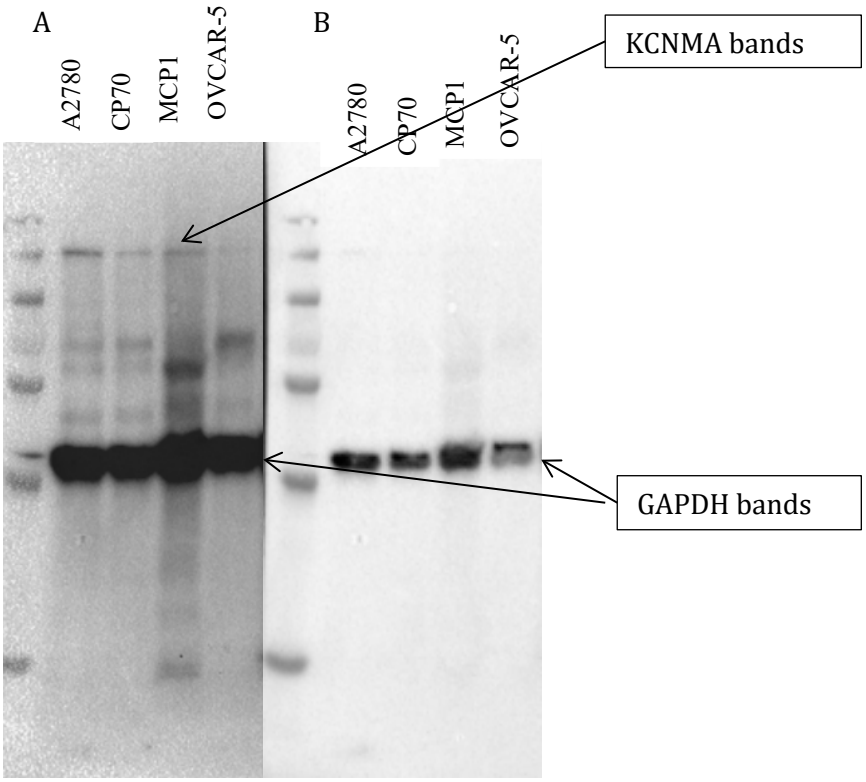

FIGURE 4.6: KCNMA PROTEIN LEVELS IN OVARIAN CANCER CELL LINES
KCNMA protein levels were assessed by Western Blotting. Approximately 20 µg of protein from each cell line was denatured, electrophoresed on a polyacrylamide gel and transferred onto PVDF membrane. The membrane was blocked with 5% w/v BSA in TBS with Tween, incubated with primary antibody (1in 500 KCNMA1) at 4°C overnight and then with secondary antibody for 1 hour at room temperature. The membranes were immersed in ECL solution and digitally imaged. The procedure was repeated with GAPDH primary antibody (1in 15000). The KCNMA1 band was identified at 130 kDa and the GAPDH at 37kDa. Three biological replicates were done (the originals of all the blots are shown in Appendix D; Figure D.1) . KCNMA1 protein levels are lower in CP70, MCP1 and OVCAR-5 than in the A2780 cell line.

4.2.5. MICRORNA-31 MIMICS DECREASE KCNMA1 PROTEIN LEVELS

The interaction between MiR-31 and KCNMA1 was then analysed. Using miRWalk, the online miRNA target prediction tool, which also provides predictions from 7 other prediction sites, predicted target sites for miR-31 on KCNMA1 were analysed.

Only one program predicts that KCNMA1 is a direct target of miR-31. Hence, KCNMA1 is probably an indirect target of miR-31. The interaction was examined by estimating KCNMA1 protein levels on transfection with miR-31 mimics. MiR-31 was transfected into A2780 cells along with negative control. Total protein was extracted from the cells 24 hours after transfection and protein levels assessed by Western Blotting using GAPDH as control. Results show a marked decrease in KCNMA1 protein levels on transfection with microRNA-31 as compared to control.

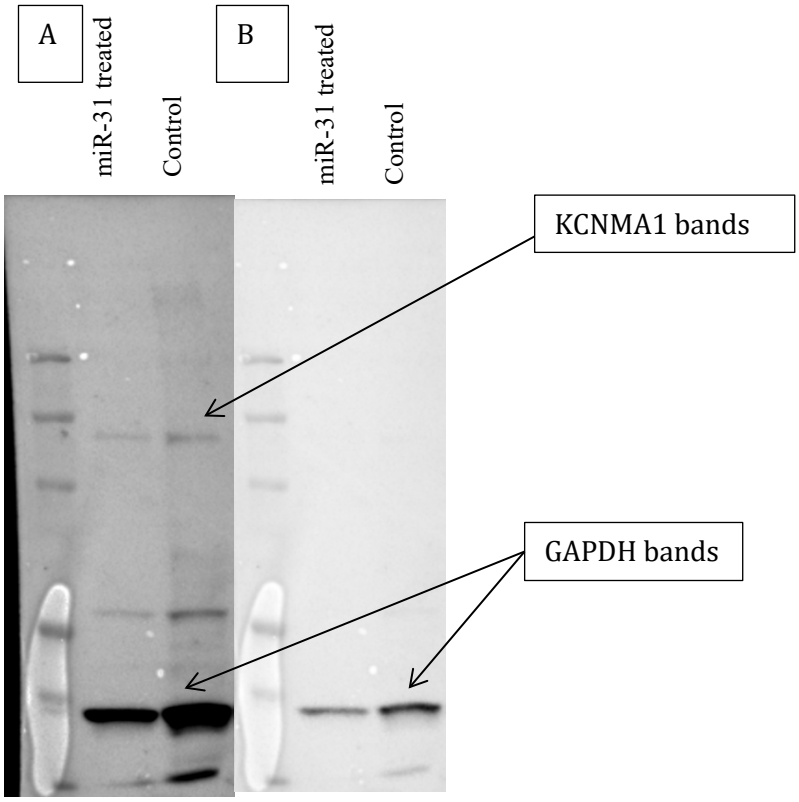

FIGURE 4.7: MICRORNA-31 TRANSFECTION DECREASES KCNMA1 PROTEIN LEVELS
A2780 cells were transfected with 5nM miR-31 or negative control. Total protein was then extracted from the two groups. Approximately 20 µg of protein was denatured, electrophoresed on a polyacrylamide gel and transferred onto PVDF membrane. The membrane was blocked with 5% w/v BSA in TBS with Tween, incubated with primary antibody (1in 500 KCNMA1) at 4°C overnight and then with secondary antibody for 1 hour at room temperature. The blots were immersed in ECL solution and digitally imaged. The procedure was repeated with GAPDH primary antibody (1in 15000). The KCNMA1 band was identified at 130 kDa and the GAPDH at 37kDa. Three biological replicates were done (Original blots are shown in Appendix D: Figure D.2). KCNMA1 protein levels are lower in A2780 cells treated with microRNA-31 mimic.

4.2.6. BLOCKING OF THE BK CHANNEL INCREASES RESISTANCE IN OVARIAN CANCER CELL LINES

The product of the KCNMA1 gene is the alpha subunit of the large calcium activated potassium channel. In order to evaluate if these channels are involved in cisplatin

resistance, paxilline, a pharmacological inhibitor of these potassium channels (Sanchez and McManus, 1996) was utilised. Paxilline has been used in cell culture to block BKCa channels at concentrations upto 20μM (Oeggerli et al., 2012, Bednarczyk et al., 2013a, Bednarczyk et al., 2013b, Li et al., 2014d). For these experiments, the concentration of 10μM employed by Bednarczyck et al (2013a) was used as Cheng et al (2016) have shown that concentrations of 25μM or above inhibit proliferation. A2780 cells were pre-treated with 10 μM paxilline for 30 minutes before cisplatin treatment at 20 μM. An MTT assay was used to quantify cell viability after 28 hours. Results (Figure 4.8) show a significant increase in cisplatin resistance (student's t-test, p-value <0.001). The test was repeated with the MCP1 and the OVCAR-5 cell lines. While the MCP1 cell line showed a significant increase in survival after cisplatin treatment in the paxilline pre-treated group as compared to the control (student's t-test, p-value <0.001), the OVCAR-5 showed no significant increase in resistance. These results appear to indicate that paxilline increases resistance to cisplatin.

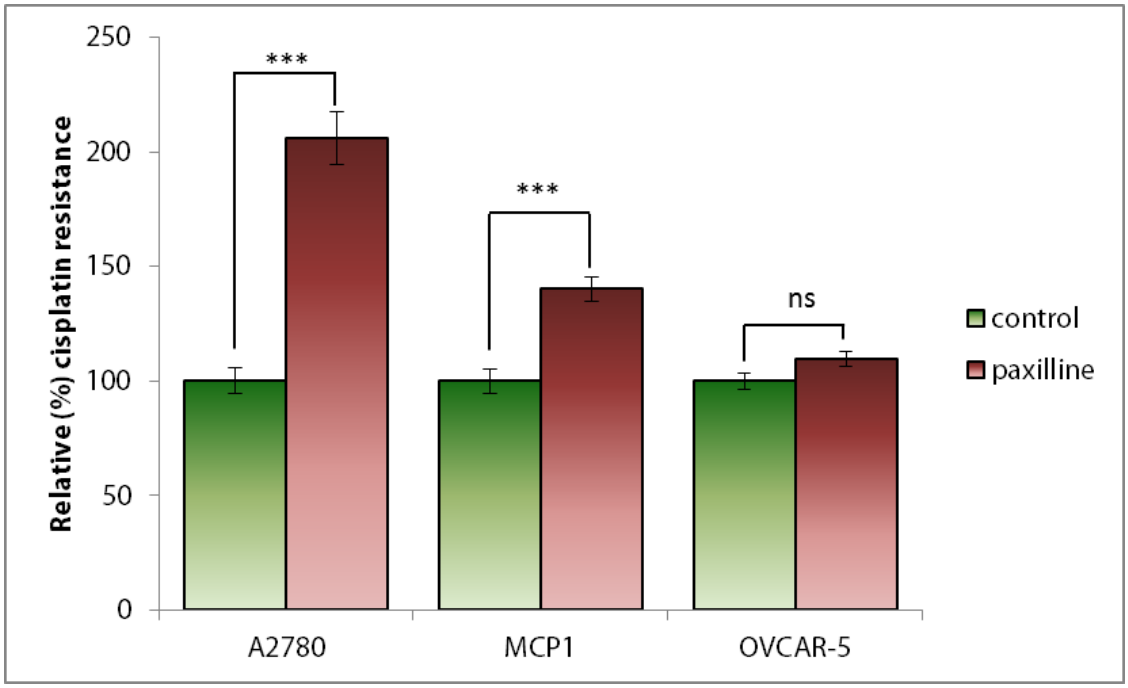

FIGURE 4.8: PAXILLINE INCREASES RESISTANCE TO CISPLATIN IN A2780 AND MCP1
A2780, MCP1 and OVCAR-5 cells were pre-treated with paxilline at 10 μM for 30 minutes prior to cisplatin treatment (A2780 – 20 μM, MCP1-80 μM, OVCAR-5 – 50 μM) for 3 hours. Viability was assessed by MTT assay 48 hours later; results are presented as percentage of viability after cisplatin treatment normalised to control. Error bars represent SEM from at least 5 biological replicates. Results show a substantial increase in resistance to cisplatin in the A2780 and MCP1 cells treated with paxilline (student's t-test, p-value < 0.001). However OVCAR-5 cells show no significant change in cisplatin resistance after paxilline treatment.

4.3 DISCUSSION

MicroRNA-31 was identified by microarray to be higher in the cisplatin resistant CP70 cells than in the cisplatin sensitive parent A2780 cells. Moreover, microRNA-31, with a 50-fold increased expression in CP70s, exhibited the highest differential expression between the cell lines, indicating the possibility of its involvement in modulating cisplatin resistance. There was also a modest increase in its expression in MCP1, the other cisplatin resistant derivative of A2780 cells.

Microarray results indicating an increase in miR-31 levels in CP70 were confirmed by qPCR Figure1A in Samuel et al. (2015); levels of miR-31 were almost undetectable in the A2780 cell line while there was a much higher level in the CP70 cell line. Gain of function experiments indicated an increase in cisplatin resistance in A2780, MCP1, OVCAR-5 and IGROV-1 cell lines, but the OVCAR-8 cell line did

not show any change in cisplatin resistance. As discussed in section 4.1, this could be due to inhibition or modulation of the downstream pathways within these cells which might override modulation by miR-31.

The increase in levels of miR-31 in the resistant phenotype CP70 as compared to the parent line A2780 taken together with the gain of function experiments showing an increase in cisplatin resistance indicate a strong probability of the involvement of miR-31 in cisplatin resistance. In the published literature, miR-31 increases sensitivity to cisplatin in prostate cancer (Bhatnagar et al., 2010) and breast cancer (Chan et al., 2013) but induces cisplatin resistance in lung cancer (Dong et al., 2014). The above presented results agree with that of Dong et al (2014). However, the results are contrary to the study by Mitamura et al (2013) who showed that miR-31 overexpression increased the sensitivity of ovarian cancer cells to paclitaxel suggesting that the mechanisms involved in the resistance to the two drugs are different and involve different pathways. The next step was to identify a possible mechanism or target through which miR-31 modulates the cisplatin resistant phenotype.

Numerous targets have been proposed for miR-31; one bioinformatics study predicted 1000 cancer-related targets for miR-31 affecting 163 pathways (Gao et al., 2014). Pathways shown to be affected by miR-31 include the oncogenic NF-κB pathway through induction by *NIK* (Yamagishi et al., 2015, Yamagishi et al., 2012, Asangani et al., 2012) or *PKCϵ* (Korner et al., 2013); RAS pathway by inhibition of *RASA1* (Edmonds et al., 2015), Wnt signalling pathways through *DKK-1* and *DACT-3* (Xi et al., 2010), p53 signalling through *STK40* (Creighton et al., 2010) and the MAPK pathway by inhibition of *SPRED1* and *SPRED2* (Edmonds et al., 2015) or *KSR2* (Zhang et al., 2011). Other validated targets of miR-31 include oncogenes such as *SRC* and *MET* (Asangani et al., 2012, Mitamura et al., 2013) as well as tumour suppressors such as *LATS2* and *PPP2R2A* (Liu et al., 2010), *EMP1* (Zhang et al., 2011), *ARID1A* (Wang et al., 2014b) and *RhoBTB1* (Xu et al., 2013); cell cycle regulators such as *E2F1*, *E2F2* (Creighton et al., 2010), *MCM2* (Lin et al., 2013, Jin et al., 2014) and *E2F6* (Bhatnagar et al., 2010) and genes involved in EMT or motility such as *TIAM1* (Cottonham et al., 2010), *SATB2* (Aprelikova et al., 2010), radixin (Hua et al., 2012) and *RGS4* (Zhang et al., 2011). DICER, the enzyme

involved in miRNA processing, was shown to be targeted by one isoform of miR-31 (Chan et al., 2013).

In order to research the possible mechanism of action of miR-31 along with its possible gene targets, gene expression levels determined by RNA sequencing of total RNA samples from A2780 and the resistant cell lines CP70 and MCP1 were analysed. 142 genes were upregulated while 304 genes were downregulated significantly in both the resistant cell lines. The genes shown by RNA Sequencing to be downregulated in CP70s as compared to the A2780s include genes which often show mutations or epigenetic silencing in ovarian cancer such as BEX4 (Chien et al., 2005), GSTM3 (Lim et al., 2011) and HTRF1 (Cody et al., 2009) in addition to genes showing deregulation in other cancers such as CASP5 (Quaye et al., 2009, Notaridou et al., 2011), CRABP1(Miyake et al., 2011) and GRM8 (Kan et al., 2010). The genes downregulated in cisplatin resistant cells also included MLH1, a mismatch repair gene, deregulation of which has been associated with drug resistance (Plumb et al., 2000). Moreover a number of genes involved in the apoptotic pathway such as CRADD, CYB5A and CYP7B1 as well as CASP5 are downregulated in CP70s as compared to A2780s. This increases the confidence in the genes of interest identified as they overlap with genes identified previously as being deregulated in various cancers. Of the genes shown to be significantly different, RhoBTB1, a tumour suppressor has previously been validated as a target of miR-31 in colon cancer (Xu et al., 2013).

While most of these genes may not be related to cisplatin resistance, there is an increased likelihood that genes involved in cisplatin resistance are present among these. Concordant deregulation in both cell lines indicates an increased probability of the genes' involvement in pathways producing the different phenotype – in this case – cisplatin resistance.

To narrow down this list of genes, they were analysed using the DAVID online functional annotation tool which uses previously published literature and pathways to ascertain if the given list of genes is enriched in a particular pathway or molecular process. Pathways over-represented in the list included pathways with significant effects on cancer progression such as focal adhesion, ECM-receptor interaction and

VEGF signalling while the gene ontology terms that were enriched included 'ion binding' and 'gated channel' as well as 'transmembrane transporter activity'. While the gene ontology (GO) terms indicated a significant enrichment of genes involved in 'calcium ion binding' and 'gated channel activity'; KEGG pathway analysis indicated the calcium signalling pathway as one of the significantly enriched pathways. Deregulation of calcium homeostasis has been associated with drug resistance (Padar et al., 2004, Solar and Sytkowski, 2011) and has been linked to a possible role in apoptosis (Al-Bahlani et al., 2011). Consequently, genes linked with calcium ion binding and calcium signalling were further examined.

Of the genes involved in calcium ion signalling, KCNMA1 was represented in 11 of the top 15 GO term categories showing a significant enrichment. Moreover, it was one of the genes with the highest fold change between CP70 and A2780 and the second highest in the calcium ion binding category. The KCNMA1 gene is located at chr 10q22; the protein product of the KCNMA1 gene forms the alpha pore forming subunit of the large calcium activated potassium channel, the BK_{Ca} channel. Higher levels of KCNMA1 expression was noted in some cancers such as breast (Oeggerli et al., 2012), prostate (Bloch et al., 2007), melanoma (Mazar et al., 2010) and glioblastoma (Weaver et al., 2006); in contrast, KCNMA1 levels were shown to be lower in prostate cancer (Altintas et al., 2013). KCNMA1 has been shown to be higher in breast cancer metastasis to the brain than to the other organs or in the primary tumour (Khaitan et al., 2009) and to be associated with higher proliferation and poor prognosis in breast cancer (Oeggerli et al., 2012). KCNMA1 has been shown to be associated with selenium resistance (Savas et al., 2010) and platinum resistance (Ziliak et al., 2012); expression levels were lower in a group of ovarian cancers resistant to chemotherapy (Bell D, 2011). There is evidence that epigenetic changes are associated with KCNMA1 in cisplatin resistant CP70 cells (Zeller et al., 2012). This suggests that the KCNMA1 gene may possibly be involved in cisplatin resistance. Hence KCNMA was selected for further scrutiny.

Of online miRNA target prediction programs in miRWalk, only one predicts KCNMA1 as a target of miRNA-31. Hence, it is probable that KCNMA1 is an indirect target. As the initial step, its functional activity in resistance was ascertained by transient knockdown using shRNAs; this was shown to increase resistance to

cisplatin treatment in A2780, MCP1 and OVCAR-5 suggesting a role in modulation of cisplatin resistance. These results are in agreement with published literature showing a decrease in expression of KCNMA1 is associated with cisplatin resistance (Bell D, 2011, Ziliak et al., 2012). However, in the IGROV-1, there was no change in cisplatin resistance – this could indicate a different mechanism of action of miR-31 in this particular cell line. There is the possibility that as KCNMA1 is postulated to be an indirect target, there could be inhibition or irreversible modulation of an intermediary mechanism leading to a different pathway coming into play. Moreover, the efficiency of the knockdown was not validated in the IGROV1 cell line – inefficient knockdown could possibly be a reason for this apparent lack of response. As IGROV-1 did not show an increase in cisplatin resistance on KCNMA1 knockdown, it was excluded from further analysis.

Further corroboration of the RNA sequencing data was subsequently obtained by western blot quantification of KCNMA1 protein; a higher level of KCNMA1 was found in A2780 than in the other three cell lines. These results, together with loss of function experiments by knockdown of KCNMA1 leading to cisplatin resistance, point to an involvement of KCNMA1 in cisplatin resistance.

As the protein expression levels of KCNMA1 are lower in the MCP1 and CP70 correlating with the higher levels of miR-31 in these cell lines, there is an indication that KCNMA1 could possibly be a target of miR-31. That the MCP1 KCNMA1 band appears to be stronger than that of CP70 further lends credence to the theory that miR-31 targets KCNMA1 as CP70 has many fold higher expression levels of miR-31 than A2780 and shows a larger decrease in levels of KCNMA1 while MCP1 shows only a modest increase in levels of miR-31 as compared to A2780 and shows a corresponding decrease in KCNMA1 levels.

Further confirmation is obtained from the results of the western blot showing a significant decrease in the levels of KCNMA1 in A2780 cells treated with miR-31 mimic as compared to control treated cells. Taken together, the higher levels of KCNMA1 in A2780 and the decrease in levels on transfection with miR-31 mimic indicates that KCNMA1 is a target – direct or indirect – of miR-31.

KCNMA1 gene forms the α-subunit of the large calcium activated potassium conductance (BKCa) channel; four α-subunits are integrated together to form the gated channel. The BKCa channel is expressed on the plasma membrane of most cells; it is also expressed on the membranes of mitochondria, endoplasmic reticulum, nucleus and Golgi apparatus (Singh et al., 2012). These channels are activated by either membrane depolarisation or intracellular calcium levels. Studies have varyingly shown that blocking BKCa channels can inhibit cell proliferation (Bloch et al., 2007, Oeggerli et al., 2012, Bury et al., 2013, Gackiere et al., 2013) or increase the rate of proliferation (Cambien et al., 2008). It has been suggested that BKCa channel activity may be involved in apoptosis in response to various stimuli including cisplatin (Liang et al., 2005, Ma et al., 2012). In ovarian cancer cells, stimulation of the BKCa channel activity leads to increased apoptosis and decreased proliferation (Han et al., 2008). BKCa channel activity has also been linked to paroptosis (Bury et al., 2013), another form of programmed cell death, and to the immune response to tumours due to its ability to regulate cytokine production from tumour cells (Cambien et al., 2008, Mound et al., 2013).

It was then investigated if the large calcium activated potassium channel itself is involved in cisplatin resistance. Blocking of this channel was achieved by using paxilline (Singh et al., 2012), a pharmacological inhibitor of this channel; this increased cisplatin resistance in A2780 and MCP1 cells. This seems to indicate an involvement of the actual channel in cisplatin resistance at least in some cell lines. Hence, it could be argued that knockdown of KCNMA causes an increase in cisplatin resistance probably by knockdown of the large calcium activated potassium channel possibly leading to modulation of apoptosis.

As a part of the project Dr. Carter analysed the correlation between levels of miR-31 and KCNMA1 with the IC50 for cisplatin in the cell lines in the NCI60 panel using publically available datasets. The results (Pink et al., 2015) show a positive correlation between levels of miR-31 and IC50 with an inverse correlation for KCNMA1 indicating that increased miR-31 and decreased KCNMA1 are associated with increased resistance to cisplatin.

The actual mechanism by which miR-31, KCNMA1 and the BKCa channel increases cisplatin resistance has not been identified. Moreover most of the experiments were carried out on ovarian cancer cell lines *in vitro.* The knockdown of genes using siRNA has not been quantified in all the cell lines; similarly, the efficiency of paxilline in blocking the BKCa channel has not been validated. Moreover most of the cisplatin resistance data relies on the MTT assay for quantification though the initial experiments were counterchecked with the sulfurhodamine assay. Based on these, there are many interesting questions that could be followed up such as the actual effect of the BKCa channel on cisplatin as well as investigating the effect on response to other chemotherapeutic drugs. *In vivo* experiments might also be done to confirm the effect of miR-31 and knockdown of KCNMA1 on cisplatin resistance. Another avenue to explore would be to check if there is a possibility that these levels are reflected in the serum of patients or in biopsies from ovarian cancer patients and if these could then be used to predict response to chemotherapy and may even be used to tailor the use of drugs to each patient's profile.

Thus, in this chapter, miR-31 is shown to increase resistance in ovarian cancer cells, KCNMA1 is shown to be a possible indirect target of miR-31, KCNMA1 and the large calcium activated potassium channel are shown to be involved in modulating cisplatin resistance. This effect of miR-31 on cisplatin resistance in ovarian cancer cells is a novel finding as well as the possibility that KCNMA1 could be an indirect target of miR-31.

Chapter 5 THE ROLE OF EVS IN CISPLATIN SENSITIVITY

5.1 INTRODUCTION

Thus far, the role of microRNAs in cisplatin resistance has been investigated. Interestingly, it was demonstrated that microRNAs and mRNAs can be transferred between cells by means of EVs (Valadi et al., 2007). EVs are membrane bound vesicles which have been shown to act as a means of intercellular communication as reviewed by (Fevrier and Raposo, 2004). In a study using mouse dendritic cells, it has been shown that the microRNAs transferred via EVs are functional once internalised into the recipient cell and can repress target mRNAs (Montecalvo et al., 2012, Stoorvogel, 2012). EVs were also shown to mediate transfer of drug resistance from resistant cells to sensitive cells probably through transfer of p-glycoprotein (Lv et al., 2014).

Another interesting aspect of EVs is their possible role in bystander effect. Bystander effect has been described in irradiated cells where cells that have not been directly irradiated show signs of radiation damage (Al-Mayah et al., 2012, Jella et al., 2014, Marin et al., 2015, Xu et al., 2015b). The bystander effect has been shown to lead on to the adaptive response where cells undergoing bystander effect may become resistant to irradiation (Buonanno et al., 2015, Marin et al., 2015). A similar bystander-effect-induced increase in DNA damage and a pro-survival effect has been described in mouse fibroblasts treated with bleomycin (Savu et al., 2015). It is, therefore, possible, that there is a similar bystander and adaptive response in cells upon cisplatin treatment. It can be hypothesised that, when treated with cisplatin, cells that are ‘stressed’ can then transmit the ‘stress’ through ‘stress induced EVs’ to neighbouring cells causing bystander effect and a consequent adaptive response making the cells more resistant to cisplatin. Therefore it is possible that, if EV uptake could be inhibited, the cells may be more sensitive to cisplatin. This possibility was investigated further in a series of experiments.

In this chapter, results are presented regarding investigations into

a. transfer of cisplatin resistance between cells through EVs and
b. effects of inhibition of EV uptake on cisplatin resistance in cell lines *in vitro*, and
c. effects of an EV inhibitor – heparin – on response to cisplatin in a mouse model of ovarian cancer.

5.2 MATERIALS AND METHODS

5.2.1. EV EXTRACTION

CP70 cells were grown in T175 flasks till 80% confluent. They were then conditioned in EV cleared media for 24 hours. This media was then centrifuged at 300 g for 5 minutes and then at 16,500 g for 20 minutes to remove cells and debris. The media was then filtered through a 0.22 µm filter before being ultracentrifuged at 120,000 g for 90 minutes at 4°C. The supernatant was discarded; pellets resuspended in fresh media and transferred onto A2780 cells that had been seeded 24 hours previously in 96 well plates at an equivalence factor of 20:1.

5.2.2. DRUG TREATMENTS:

Heparin (Sigma), an EV uptake inhibitor, was diluted to a concentration of 10 mg/ml in deionised distilled water, filtered through 0.22 µm filters and stored at -20°C; it was diluted in media and added to cells at a final concentration of 10 µg/ml. Amiloride (5-(N-ethyl-N-isopropyl) amiloride or EIPA) inhibits uptake of EVs by blocking macropinocytosis; it was stored in DMSO at a concentration of 108 mM and added to cells at 50 µM concentration. Dynasore, a dynamin-2 inhibitor shown to inhibit clathrin and caveolin dependent endocytosis, was diluted in DMSO to a concentration of 31 mM, stored at -20°C and added to cells at a concentration of 50 µM. All three drugs were added to cells 30 minutes before treatment with cisplatin for three hours. An MTT assay was carried out 48 hours after cisplatin treatment. Guggulsterone, an inducer of EV release, was stored in DMSO at a concentration of 32mM at -20°C and diluted in media and added to cells at a final concentration of 50 µM. Stock solution of bexarotene, another inducer of EV release, in DMSO was stored at - 20°C; cells were treated at 5 µM final concentration.

5.2.3. XENOGRAFT

Xenograft experiments were carried out at the animal testing facility at Queen's University, Belfast by Dr. Helen McCarthy; A2780s for this experiment were kindly provided by Dr Fiona Furlong, Queen's University, Belfast. $5X10^6$ A2780 cells in matrigel were implanted subcutaneously into the flanks of BALB-C SCID mice. Animals were monitored regularly and body weights were measured three times a week. Tumour volume was calculated as {[$\sqrt[3]{}$(L*B*D)]/2}^3 where L, B and D are the dimensions of the tumour. Treatment was started when the tumour measured 100 mm^3. Twenty four mice were then divided into four treatment groups (1) cisplatin 5mg/kg once weekly i.p. (2) heparin only – 10 mg/kg every day i.p. (3) combination group – cisplatin 5mg/kg once weekly i.p. and heparin 10 mg/kg once daily i.p. and (4) control group. Tumour volume was monitored three times a week; when the tumour quadrupled in size, the animal was sacrificed. Any mice that lost 20% of body weight during the experiment were removed from the experiment as the treatment is deemed too toxic.

5.3 RESULTS

5.3.1. TRANSFER OF RESISTANCE BETWEEN CELL LINES

In order to investigate if the property of resistance can be transferred between cell lines by transferring EVs released from one cell line onto another cell line, EVs released by CP70 (cisplatin resistant cell line) were transferred onto A2780 (cisplatin sensitive cell line) and the response to cisplatin analysed. CP70s were grown in T75 flasks till 80% confluent and then grown for 24 hours in media that had been pre-cleared of EVs by ultracentrifugation at 120 000 g for 16 hours. EVs were extracted by ultracentrifugation (Section 5.2.1) from this media. The method of EV extraction has been used in various papers to isolate EVs (Raposo et al., 1996, Fevrier and Raposo, 2004, Christianson et al., 2013). The EVs have also been validated by visualisation through Transmission electron microscopy, western blotting for HSP70, GAPDH as well as the absence of cytochrome C and GM130 as well as particle counting and sizing using a Nanosight (Malvern instruments) (Mulcahy L.A., 2016). The EV pellet was resuspended in fresh media and added to A2780 cells

in 96-well plates alongside PBS treated controls. After 24 hours, half the wells in each group were treated with cisplatin at 20 μM for 3 hours. The survival was quantified by MTT assay 48 hours later. The results (Figure 5.1) show an increase in cell survival after cisplatin treatment of CP70 EV treated A2780s by 15% as compared to the control (p-value <0.05). The results show that addition of EVs from the cisplatin resistant cell line CP70 makes A2780 cells more resistant to cisplatin.

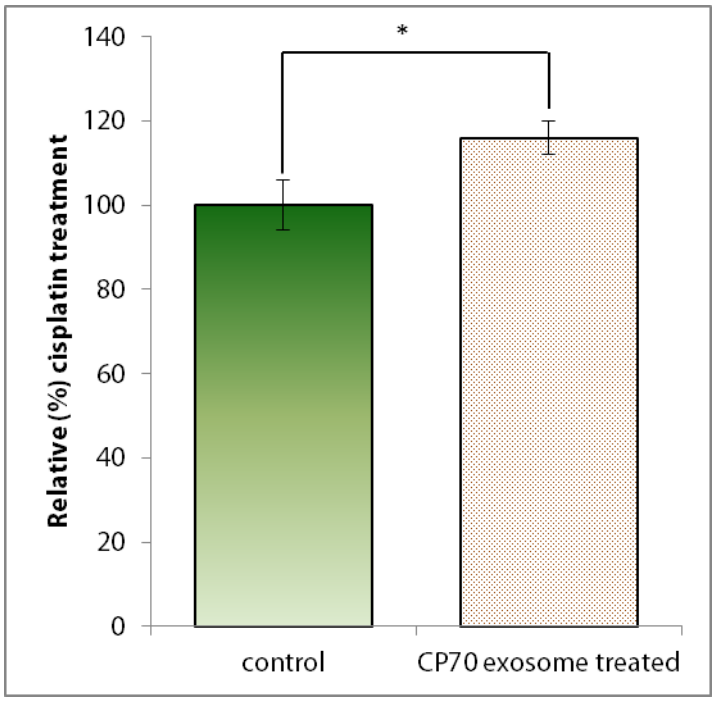

FIGURE 5.1: EVS FROM CP70S INCREASE CISPLATIN RESISTANCE IN A2780
A2780 cells in 96 well plates were treated with CP70 EVs or PBS (control) and then subjected to cisplatin treatment at 20μM. The percentage of cell viability (as measured by the MTT assay) 48 hours after cisplatin treatment as compared to that of untreated cells in each group was then normalised to control and compared by the Student's t-test. Results show that EVs from CP70 appear to raise the resistance of A2780 cells to cisplatin with an increase in survival of about 15% (p-value < 0.05)

5.3.2. EV UPTAKE INHIBITION BY HEPARIN AND CISPLATIN RESISTANCE

Addition of EVs from the cisplatin resistant cell line CP70 has been shown to increase cisplatin resistance in A2780 cells. As described in section 5.1, it is possible that cells stressed by cisplatin can release EVs which cause a bystander effect and a subsequent adaptive response in the other cells that take up the EVs. Consequently, inhibition of the uptake of these EVs may possibly make cancer cells more sensitive to cisplatin. In order to test this possibility, heparin, an inhibitor of EV uptake was utilised. Christianson et al. (2013) showed that treatment with 10μg/ml of heparin

decreased uptake of EVs by 50%; hence this concentration was used for the experiments. A2780 cells were treated with heparin at 10 µg/ml for 10 minutes before and during cisplatin treatment for 3 hours at various concentrations alongside control cells; cells were maintained in media containing heparin for 48 hours after cisplatin treatment to prevent uptake of any stress EVs. Results (Figure 5.2 and Table 5-1) show a significant decrease in cell viability in the heparin treated cells at concentrations between 5 and 100 µM cisplatin.

Table 5-1 also shows p-values comparing the normalised absorbance values of control cells and heparin treated cells at each concentration of cisplatin. IC50 (GraphPad PRISM) was shown to change significantly from 31.31µM to 21.24µM (p-value < 0.0001). Treatment with heparin alone, however appears to increase cell viability (Appendix B, Figure B-6). This indicates that the heparin treatment decreases resistance to cisplatin.

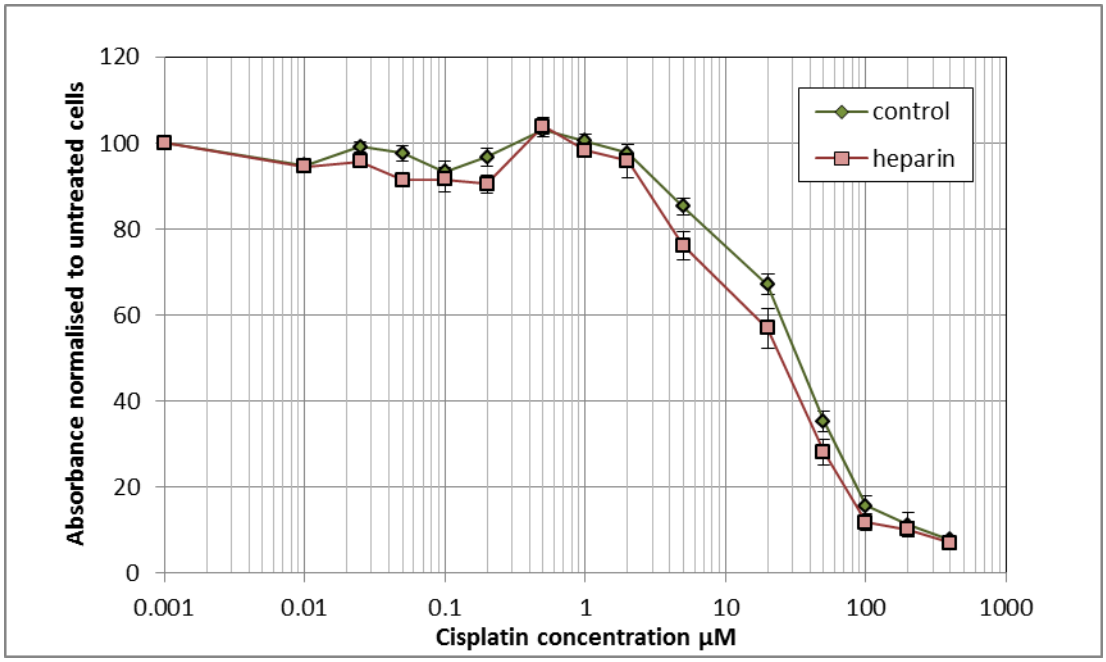

FIGURE 5.2: HEPARIN PRE-TREATMENT DECREASES CISPLATIN RESISTANCE IN A2780 CELLS

A2780 cells in 96-well plates were pre-treated with heparin at 10µg/ml for 10 minutes before addition of cisplatin at varying concentrations for 3 hours; cells in heparin treated group were also treated with heparin for 48 hours after cisplatin treatment. Viability was assessed by MTT assay and normalised to untreated cells in each group. Error bars show SEM of at least 9 biological repeats. IC50 (GraphPad PRISM) was shown to change significantly from 31.31µM to 21.24µM (p-value < 0.0001).

Table 5-1: Cisplatin concentration curves of heparin treated cells vs control and p-values

Cisplatin concentration (μM)	Control (%) of untreated	Heparin (%) of untreated	Student's t-test p-value
400	7.76	7.01	ns
200	11.08	10.12	ns
100	15.59	11.79	0.00052
50	35.25	28.07	0.033862
20	67.12	56.95	0.002967
5	85.20	76.20	0.021385
2	97.70	95.81	ns
1	100.50	98.32	ns
0.5	103.02	103.88	ns
0.2	96.80	90.52	0.013962
0.1	93.25	91.55	ns
0.05	97.63	91.42	0.043288
0.025	99.17	95.74	ns
0.01	94.80	94.54	ns
0	100.00	100.00	na

This experiment was repeated in two other ovarian cancer cell lines – CP70 and IGROV-1. The results shown in Figure 5.3 and Figure 5.4 respectively and Table 5-2 indicate a decrease in viability in the heparin treated cells in both CP70 and IGROV-1 as compared to the control cells. The viability as a percentage of untreated cells is shown in the table for each group and concentration. Student's t-test was used to compare the heparin treated cells to control at each cisplatin concentration tested. The p-values are shown in Table 5-2. IC50 (GraphPad PRISM) in CP70 cells was shown to change significantly from 146.8 μM to 118.9 μM (p-value = 0.0006). In IGROV1 cell line, IC50 changed significantly from 60.42 μM to 52.62 μM (p-value <0.0001). These clearly demonstrate a decrease in viability in the heparin treated cells when compared with the control cells.

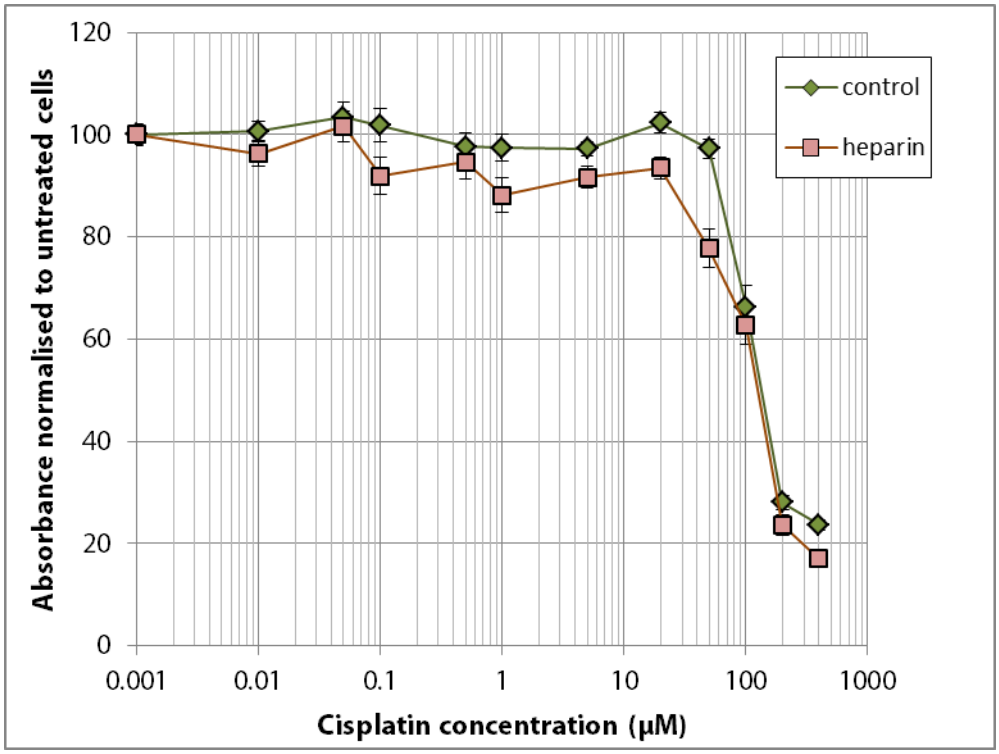

FIGURE 5.3: HEPARIN TREATMENT DECREASES CISPLATIN RESISTANCE IN CP70 CELLS
CP70 cells in 96 well plates were treated with 10µg/ml heparin prior to cisplatin treatment for 3 hours at varying concentrations; cells in heparin treatment group were also treated with heparin for 48 hours after cisplatin treatment. Viability was assessed by MTT assay 48 hours after cisplatin treatment. Absorbance levels were normalised to cisplatin untreated cells in each group. Error bars show SEM of 9 biological replicates. IC50 (GraphPad PRISM) was shown to change significantly from 146.8 µM to 118.9 µM (p-value = 0.0006).

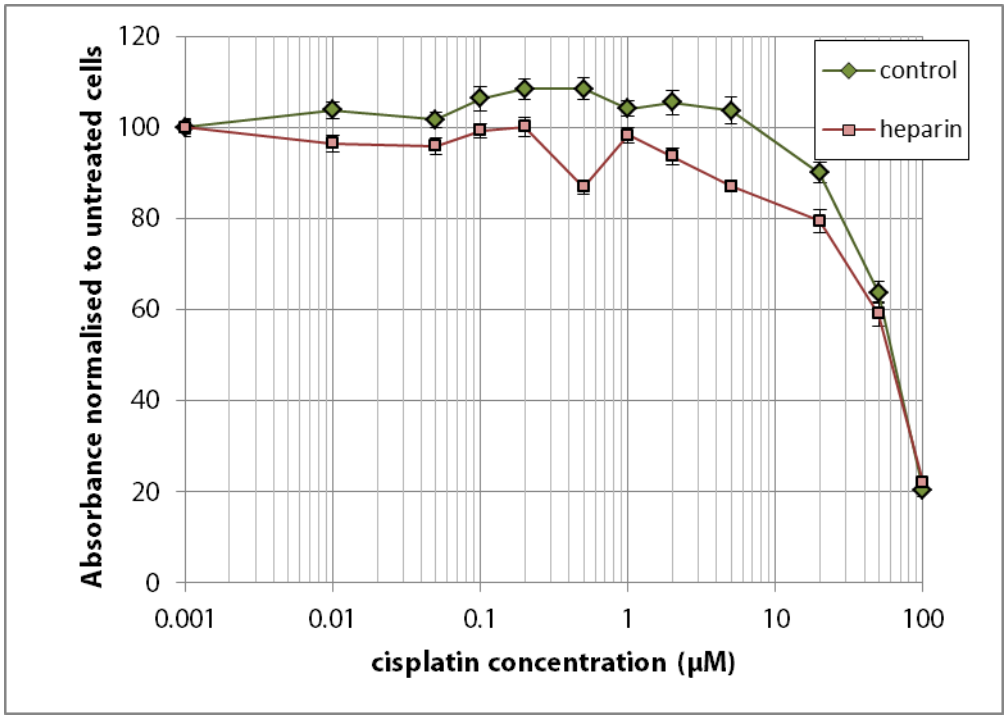

FIGURE 5.4: HEPARIN TREATMENT DECREASES RESISTANCE TO CISPLATIN IN IGROV-1 CELLS

IGROV-1 cells were treated with 10μg/ml heparin prior to cisplatin treatment for 3 hours at varying concentrations; cells in heparin treatment group were also treated with heparin for 48 hours after cisplatin treatment. Viability was assessed by the MTT assay 48 hours after cisplatin treatment; results are presented as absorbance values normalised to cisplatin untreated cells in each group. Error bars show SEM of 9 biological replicates. IC50 changed significantly from 60.42 μM to 52.62 μM (p-value <0.0001).

TABLE 5-2: HEPARIN TREATMENT DECREASES CISPLATIN RESISTANCE IN CP70 AND IGROV-1 OVARIAN CANCER CELL LINES

	CP70			IGROV-1		
Cisplatin concentration (μM)	Control (%) of untreated	Heparin (%) of untreated	Student's t-test p-value	Control (%) of untreated	Heparin (%) of untreated	Student's t-test p-value
400	23.64	16.97	7.22E-05	-	-	-
200	28.04	23.61	ns	-	-	-
100	66.29	62.65	ns	20.26	21.98	ns
50	97.20	77.74	0.000376	63.81	59.10	ns
20	102.30	93.47	0.0067	90.08	79.51	0.006207
5	97.29	91.61	0.037116	103.71	87.16	0.000258
1	97.43	88.16	0.042851	104.19	98.40	0.027748
0.5	97.64	94.59	ns	108.57	86.98	1.68E-06
0.1	101.71	91.87	0.060381	106.37	99.35	0.036079
0.05	103.36	101.64	ns	101.70	96.04	0.040101
0.01	100.60	96.31	ns	103.86	96.58	0.01169
0.000	100.00	100.00	na	100	100	na

5.3.3. OTHER INHIBITORS OF EV UPTAKE AND CISPLATIN RESISTANCE

In order to assess the effect of extracellular vesicle uptake inhibition by other methods, dynasore and amiloride were used. Dynasore is a dynamin-2 inhibitor shown to inhibit clathrin and caveolin dependent endocytosis. Amiloride (5-(N-Ethyl-N-isopropyl) amiloride or EIPA) on the other hand inhibits uptake of EVs by blocking macropinocytosis. In published literature amiloride and dynasore have been used at 50μM to inhibit EV uptake (Escrevente et al., 2011, Gong et al., 2015). The same concentrations were used in these experiments.

A2780 cells were treated with 50μM dynasore or 50μM amiloride for 30 minutes prior to cisplatin treatment at various concentrations for 3 hours and after cisplatin treatment. 48 hours later, viability was assessed by MTT assay. Results are shown in Figure 5.5, error bars show SEM of 9 biological replicates. It can be clearly seen that dynasore and amiloride decrease survival significantly at concentrations of cisplatin 5, 10 and 20 μM. The absorbance values were normalised to untreated cells in each group. IC50 (GraphPad PRISM) was shown to change significantly from 22.17μM in the control to 12.73μM in amiloride (p value <0.0001) and 12.48μM in dynasore (p-value <0.0001). They were also compared at each concentration with control by the student's t-test. The student's t-test p-values are shown in Table 5-3. However there is no significant difference between dynasore treated cells and amiloride treated cells. These results show that dynasore and amiloride, inhibitors of EV uptake, can sensitise A2780 cells to cisplatin.

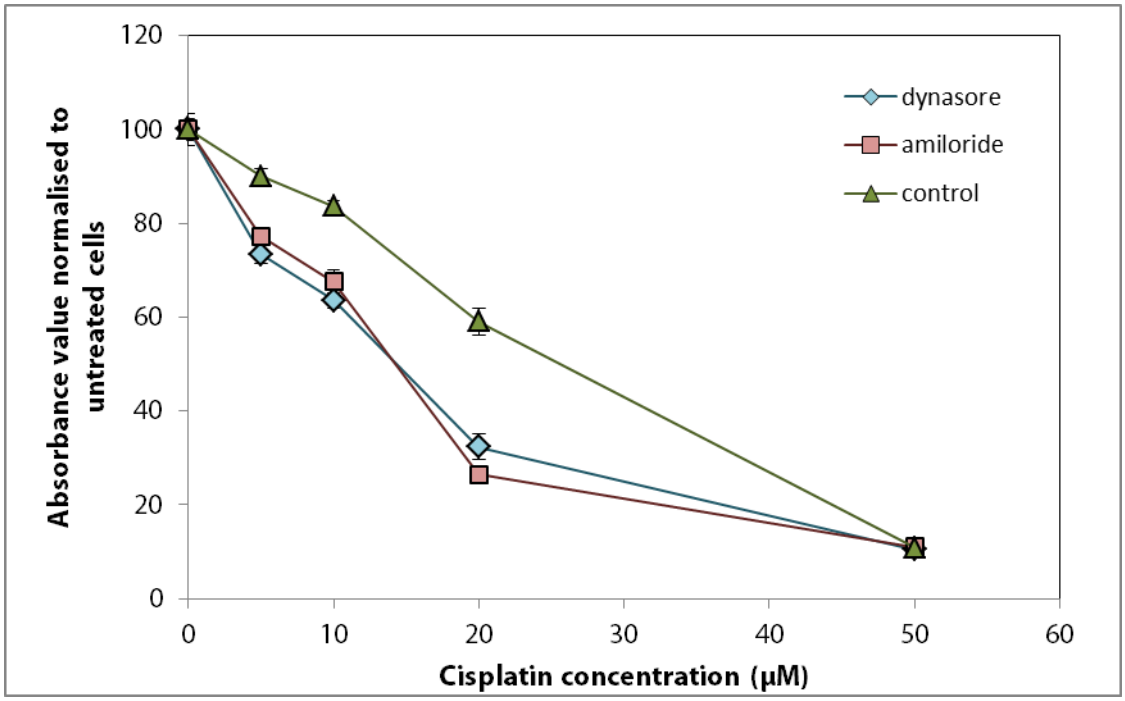

FIGURE 5.5: DYNASORE AND AMILORIDE SENSITISE A2780 CELLS TO CISPLATIN
A2780 cells were treated with 50µM dynasore or 50µM amiloride alongside control for 30 minutes prior to cisplatin treatment for 3hours at varying concentrations. MTT assay was used to assess viability 48 hours later. Error bars show SEM of 9 replicates. IC50 (GraphPad PRISM) was shown to change significantly from 22.17µM in the control to 12.73µM in amiloride (p value <0.0001) and 12.48µM in dynasore (p-value <0.0001)

TABLE 5-3: EFFECT OF DYNASORE AND AMILORIDE TREATMENT ON CISPLATIN RESISTANCE - NORMALISED ABSORBANCE VALUES AND STUDENT'S T-TEST P-VALUES

cisplatin concentration (µM)	control	dynasore	student's t-test p-value dynasore v control	amiloride	student's t-test p-value amiloride v control
50	10.89	10.45	ns	11.04	ns
20	59.04	32.37	5.03E-06	26.48	8.09E-07
10	83.60	63.57	1.19E-07	67.55	0.000117
5	90.01	73.36	4.91E-06	77.18	3.23E-05
0	100.00	100.00	na	100.00	na

5.3.4. THE EFFECT OF GUGGULSTERONE AND BEXAROTENE ON CISPLATIN SENSITIVITY

It has been reported by Kong et al that guggulsterone and bexarotene increase sensitivity to cisplatin by causing release of EVs containing ABCG2 drug transporter; thus rendering the cell less efficient at exporting the drug (Kong et al.,

2015). We hypothesised that if guggulsterone and bexarotene could sensitise cells to doxorubicin by secretion of ABCG2 within the EVs, then inhibiting the reuptake of EVs with heparin would act synergistically. Two possible scenarios exist with regard to synthesis and release of stress induced EVs – one is that the cell has a store of "pre-made" and ready to use "stress EVs", the release of which is triggered by the stress; the other is that upon induction by the stress; the payload of the EVs changes and "stress EVs" are newly synthesised. If the stress EVs are pre-stored and the release is induced by guggulsterone and bexarotene, there would be no "stress EVs" left to be released; therefore the cells would be more sensitive, but adding heparin after cisplatin treatment would make no difference as all the "stress EVs" would have been released. However if the "stress EVs" are synthesised and released as and when the stress occurs, though guggulsterone and bexarotene would induce release of the EVs before cisplatin treatment, more "stress EVs" would be synthesised and released during and after cisplatin treatment containing information to cause a bystander effect and an adaptive response. The addition of heparin during and after the stress will increase the sensitivity of the cells by inhibiting the uptake of the EVs released during and after cisplatin treatment.

Based on this, an experiment was set up with the following aims: 1. To check if guggulsterone and bexarotene did indeed induce sensitivity to cisplatin, 2. If heparin acts synergistically with guggulsterone and bexarotene and 3. to investigate if heparin is most effective if added before or after cisplatin treatment. Guggulsterone and bexarotene were used at the concentrations of 50 μM and 5 μM respectively as used by Kong et al. A2780 cells seeded in 96 well plates were divided into 6 groups for treatment as shown in Table 5-4; in each group, wells were treated with 0, 5, 10, 20 or 50 μM cisplatin. Viability was quantified 48 hours later by MTT assay.

TABLE 5-4: THE EFFECT OF GUGGULSTERONE, BEXAROTENE AND HEPARIN ON CISPLATIN SENSITIVITY - TREATMENT GROUPS

Group	Guggulsterone 50µM 24 hours before cisplatin treatment	Bexarotene 5 µM 24 hours before cisplatin treatment	Heparin 10µg/ml, 24 hours before cisplatin treatment	Cisplatin treatment, 0, 5, 10, 20 and 50 µM for three hours	Heparin 10µg/ml during cisplatin treatment	Heparin 10µg/ml for 48 hours after cisplatin treatment
ctrl	–	–	–	+	–	–
G+b+pre-hep+post-hep	+	+	+	+	+	+
G+b+pre-hep	+	+	+	+	–	–
G+b+post-hep	+	+	–	+	+	+
G+b	+	+	–	+	–	–
hep	–	–	–	+	+	+

Results are presented in figures 5.6 to 5.8. Guggulsterone and bexarotene (Figure 5.6 and Table 5-4) sensitise cells to cisplatin by about 10% compared to control at a concentration of 20 µM (student's t-test, p-value <0.05); however the shift of the IC50 from 47.42 to 75.76 (GraphPad PRISM) is not significant. Heparin significantly changes the IC50 from 47.42 to 28.67 (p-value <0.0001). The difference in IC50 between the heparin group and the guggulsterone/ bexarotene treated group is also significant (p-value = 0.004).

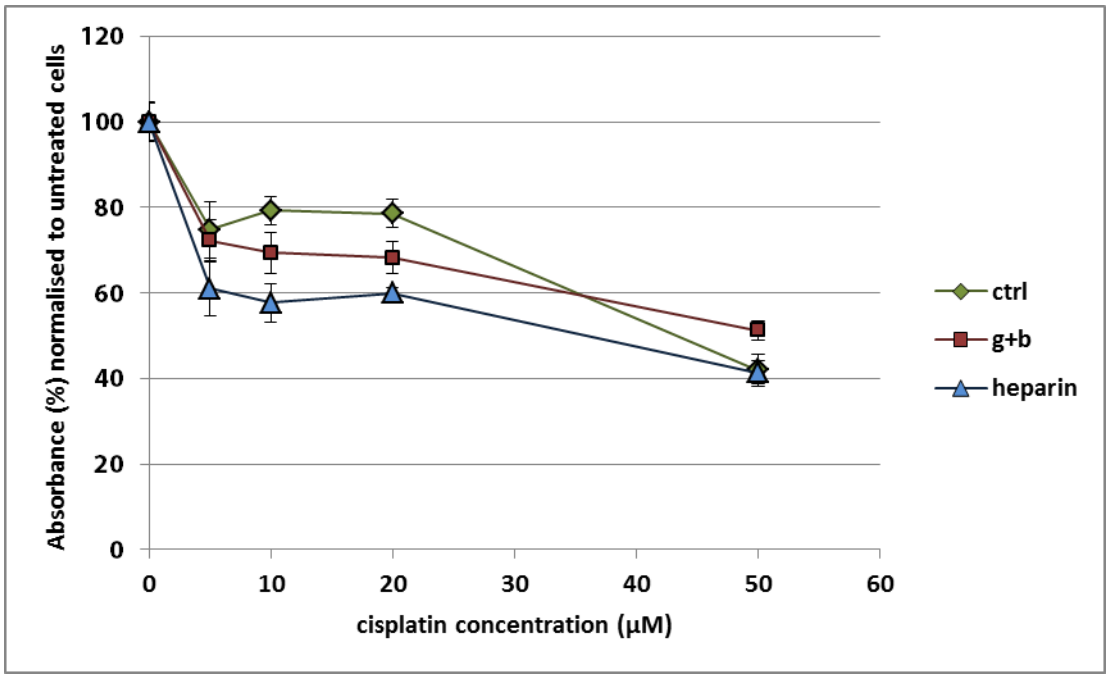

FIGURE 5.6: GUGGULSTERONE AND BEXAROTENE SENSITISE A2780 TO CISPLATIN
A2780 cells were treated with control/ guggulsterone/ bexarotene for 24 hours or heparin during and after cisplatin treatment groups. Guggulsterone was used at a final concentration of 50 µM and bexarotene at a final concentration of 5 µM; heparin at 10 µg/ml and cisplatin at 4 different concentrations for 3 hours. Viablility was assessed by MTT assay 48 hours after cisplatin treatment. Error bars show SEM of 9 biological replicates. Results show that guggulsterone and bexarotene appear to sensitise cells to cisplatin only at concentrations less than 20 µM as compared by students t-test (see Table 5-5); however the IC50 appears to have increased from 47.42 to 75.76 and the difference is not significant. The group treated with heparin is more sensitive to cisplatin; the IC50 (GraphPad PRISM) changes significantly from 47.42 to 28.67 (p-value <0.001). The difference in IC50 between heparin group and guggulsterone and bexarotene group is also significant (p-value = 0.004).

In order to determine if heparin acts synergistically with guggulsterone/ bexarotene, the response to cisplatin of compared between the group treated with heparin and guggulsterone/ bexarotene with that of either heparin or guggulsterone/ bexarotene (Figure 5.7). As compared to control the IC50 shifted significantly from 47.42 to 27.78 (p-value = 0.004); the IC50 is also significantly different from the guggulsterone/ bexarotene alone group (p-value = 0.01)but almost similar to the heparin alone group (28.67 and 27.78). This suggests that though the treatment of all three drugs significantly sensitises the cells to cisplatin, the effect is comparable to the heparin only group indicating a lack of synergistic effect.

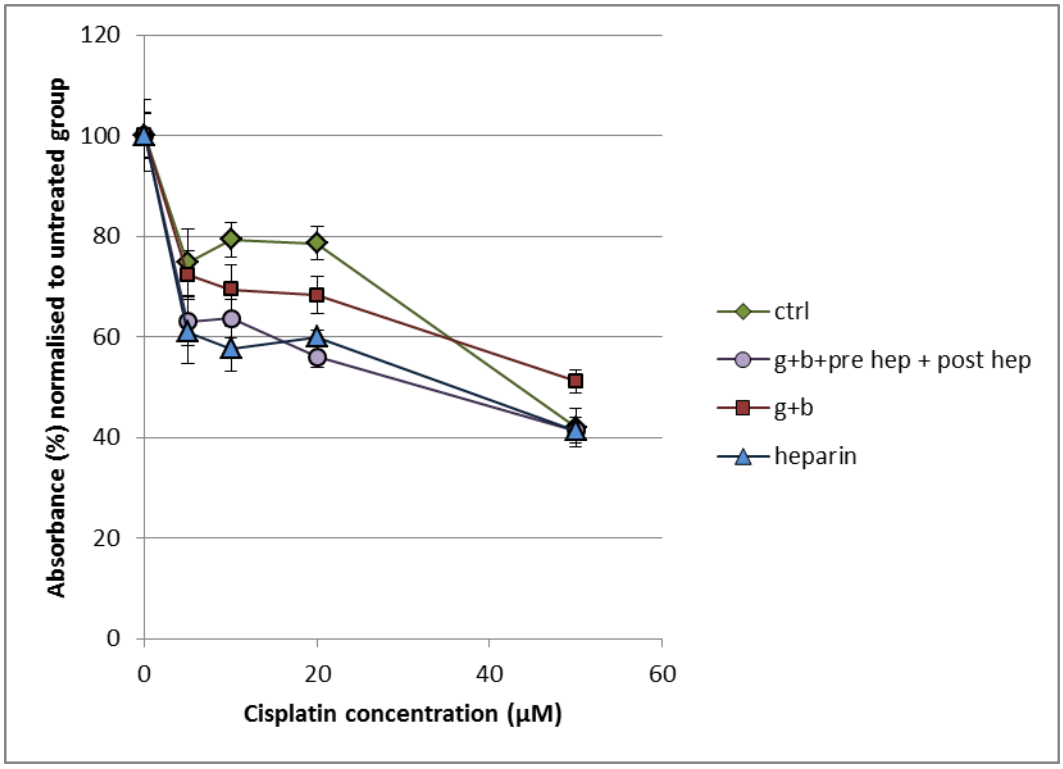

FIGURE 5.7: NO SYNERGY EXISTS BETWEEN HEPARIN AND GUGGULSTERONE/ BEXAROTENE ON RESPONSE TO CISPLATIN IN A2780 CELLS

A2780 cells were treated with control/ guggulsterone/ bexarotene for 24 hours/ heparin during and after cisplatin treatment/ aombination of all the three drugs. Guggulsterone was used at a final concentration of 50 µM and bexarotene at a final concentration of 5 µM; heparin at 10 µg/ml and cisplatin at 4 different concentrations for 3 hours. Viablility was assessed by MTT assay 48 hours after cisplatin treatment. Error bars show SEM of 9 biological replicates. The group treated with all the three drugs is more sensitive to cisplatin (IC50 shift to 27.78 from 47.42 in control (p-value = 0.004) or 75.76 in the guggulsterone/ bexarotene only group (p-value = 0.01); however the effect is comparable to heparin (IC50 28.67).

Comparing the groups treated with guggulsterone/ bexarotene/ heparin prior to treatment with cisplatin and guggulsterone/ bexarotene/ heparin during and after treatment with cisplatin shows a difference in IC50 with the heparin before group showing an IC50 of 86.18 and the heparin post treatment group recording an IC50 of 34.49; this difference is significant (p-value = 0.016).

These results indicate that 1. heparin has no synergistic effect with guggulsterone/ bexarotene and 2. Heparin is effective if given during and after cisplatin treatment.

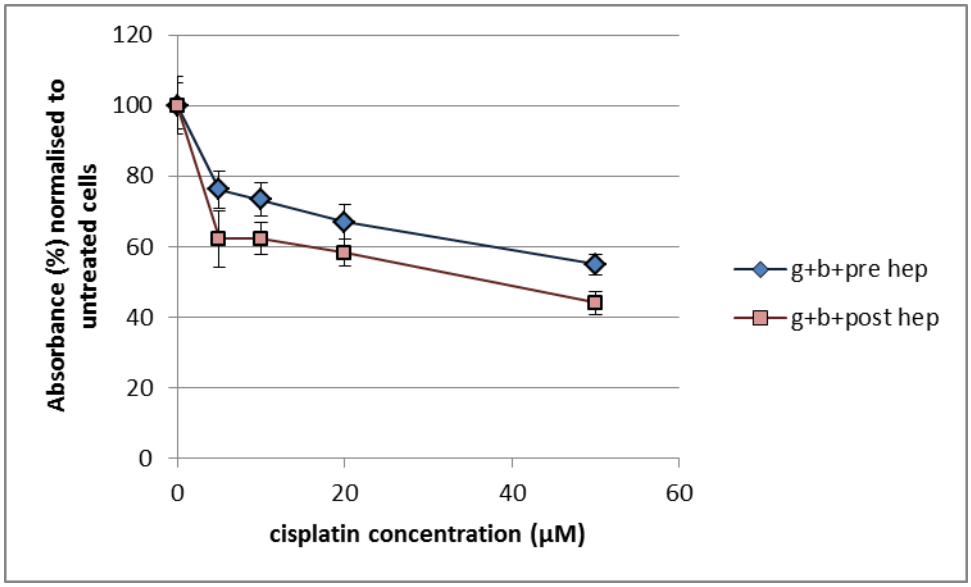

FIGURE 5.8: TREATMENT WITH HEPARIN AFTER CISPLATIN TREATMENT IS MORE EFFECTIVE THAN PRE-TREATMENT

A2780 cells were treated with guggulsterone/ bexarotene/heparin for 24 hours prior to cisplatin treatment or guggulsterone/ bexarotene for 24 hours prior to cisplatin treatment and heparin during and after cisplatin treatment. Guggulsterone was used at a final concentration of 50 µM and bexarotene at a final concentration of 5 µM; heparin at 10 µg/ml and cisplatin at 4 different concentrations for 3 hours. Viablility was assessed by MTT assay 48 hours after cisplatin treatment. Error bars show SEM of 9 biological replicates. The IC50 of the pre hep group is 86.18 while that of the post hep group is significantly different (p-value = 0.016) at 34.49

TABLE 5-5: A2780 CELLS TREATED WITH GUGGULSTERONE AND BEXAROTENE WITH/WITHOUT HEPARIN AND VARYING CONCENTRATIONS OF CISPLATIN PAIRWISE COMPARISON OF POINTS BY STUDENTS T-TEST P-VALUES

cisplatin conc	Control (%) of untreated	gug+bex (%) of untreated	p-value vs control	Heparin (%) of untreated	p-value vs control	gug+bex+h1 (%) of untreated	p-value vs control	gug+bex+h2 (%) of untreated	p-value vs control	gug+bex+h1+h2 (%) of untreated	p-value vs control
50	42.0	51.2	0.05	41.4	0.91	54.9	0.02	44.0	0.69	41.4	0.89
20	78.6	68.3	0.05	60.0	0.00	67.0	0.08	58.3	0.00	56.1	0.00
10	79.3	69.4	0.12	57.7	0.00	73.3	0.32	62.3	0.01	63.6	0.01
5	74.8	72.3	0.76	61.0	0.16	76.1	0.88	62.2	0.25	63.0	0.18
0	100	100		100		100		100		100	

- Significantly Increased cisplatin sensitivity compared to control
- Decreased cisplatin sensitivity compared to control

5.3.5. CISPLATIN AND HEPARIN EFFECTS ON A2780 XENOGRAFT IN MICE

Heparin appears to significantly increase sensitivity of cells to cisplatin *in vitro.* In order to assess the effect of heparin on cisplatin response *in vivo*, A2780s were injected subcutaneously into the flanks of nude BALB-C SCID mice; when the tumours were 100mm^3 in volume these were then divided into four treatment groups - control (no treatment), heparin only, cisplatin only or heparin and cisplatin. Tumour volume was assessed regularly; the animal was sacrificed when the tumour quadrupled in size. All animal studies were conducted by Dr Helen McCarthy at Queens University, Belfast; A2780s for this experiment were kindly provided by Dr Fiona Furlong, Queens University, Belfast. The survival is shown in Figure 5.9 and Table 5-6 shows median survival for each group. This shows that as expected, the cisplatin treated mice (11 days) appear to survive longer than control mice (7 days) (log-rank test p-value 0.0059). However, unexpectedly, the group with the combination treatment appear to have shorter median survival (8 days) than the cisplatin alone group (11 days) (log-rank test p-value – 0.076); moreover the heparin only treatment group (5 days) also has a shorter median survival than the control group (7 days) (log rank test p-value – 0.015). Tumour volumes (Figure 5.10) and tumour doubling time calculated by non-linear regression analysis (shown in Table 5-6 and Figure 5.11) show similar trends with cisplatin treated group doubling time being almost twice as long as that of the control group or the heparin treated group. Again, the group treated with the combination appears to have a shorter doubling time (3.81 ± 0.98) than the cisplatin treated group (4.94 ± 1.51) while the difference in doubling time between the heparin treated group and the control is minimal. However, only the difference in tumour doubling time between the cisplatin treatment group and control group was significant (student's t-test p-value 0.018). Hence the *in vivo* experiment appears to show that heparin does not increase the sensitivity of A2780 tumours in mice to cisplatin, there was no decrease in tumour growth or increase in survival.

TABLE 5-6: MEDIAN SURVIVAL AND DOUBLING TIMES

Treatment group	median survival (days)	Log-rank (Mantel –Cox test)		Tumour doubling time (days)	Students t-test	
		P-value vs control	P-value vs Cisplatin	mean ± standard deviation	p-value vs control	p-value vs cisplatin
control	7			2.87 ± 0.65		
cisplatin	11	0.00586		4.94 ± 1.51	0.018	
heparin	5	0.0152		2.54 ± 0.53	0.345	
Cisplatin + heparin	8	0.076	0.14	3.81 ± 0.98	0.108	0.170

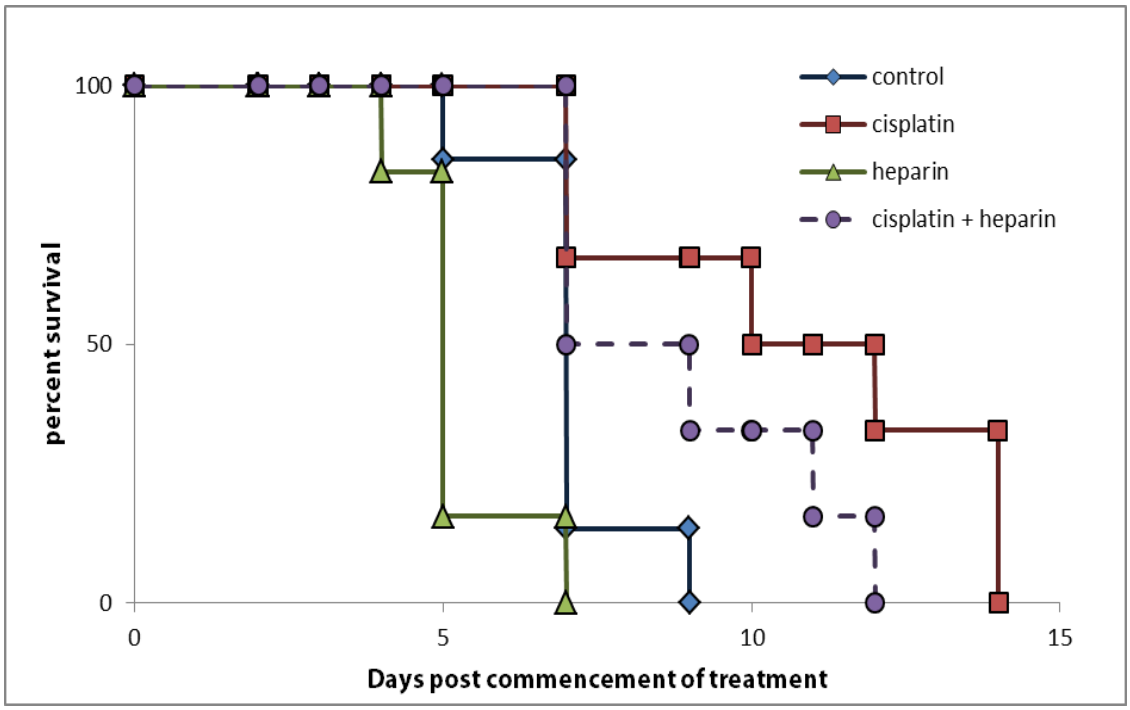

FIGURE 5.9: SURVIVAL CURVES OF THE FOUR TREATMENT GROUPS OF MICE
A2780s were injected subcutaneously into the flanks of nude BALB-C SCID mice; when the tumours were 100mm^3 in volume 24 mice were divided into four treatment groups - control (no treatment), heparin only, cisplatin only or heparin and cisplatin. Tumour volume was assessed regularly; the animal was sacrificed when the tumour quadrupled in size. Survival times have been plotted and appear to show a decrease in survival with combination treatment.

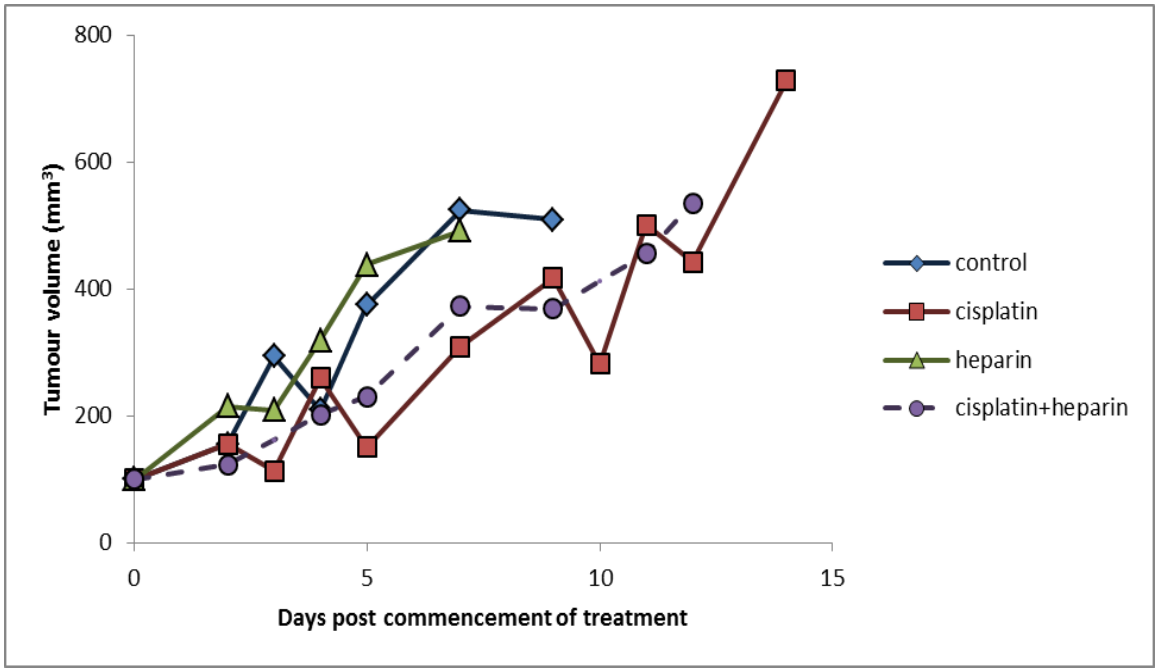

FIGURE 5.10: TUMOUR VOLUMES - MICE IN THE FOUR TREATMENT GROUPS
A2780s were injected subcutaneously into the flanks of nude BALB-C SCID mice; when the tumours were >100mm^3 in volume, the mice were treated with one of the following - control (no treatment), heparin only (10mg/kg od ip), cisplatin only (5mg/kg once weekly ip) or heparin (10mg/kg od ip) and cisplatin (5mg/kg once weekly ip). Tumour volume was assessed regularly; the animal was sacrificed when the tumour quadrupled in size. Tumour volumes have been normalised and plotted against time. The graph indicated no increase in time taken for the tumour to quadruple in the combination treatment group as compared to the cisplatin treated group.

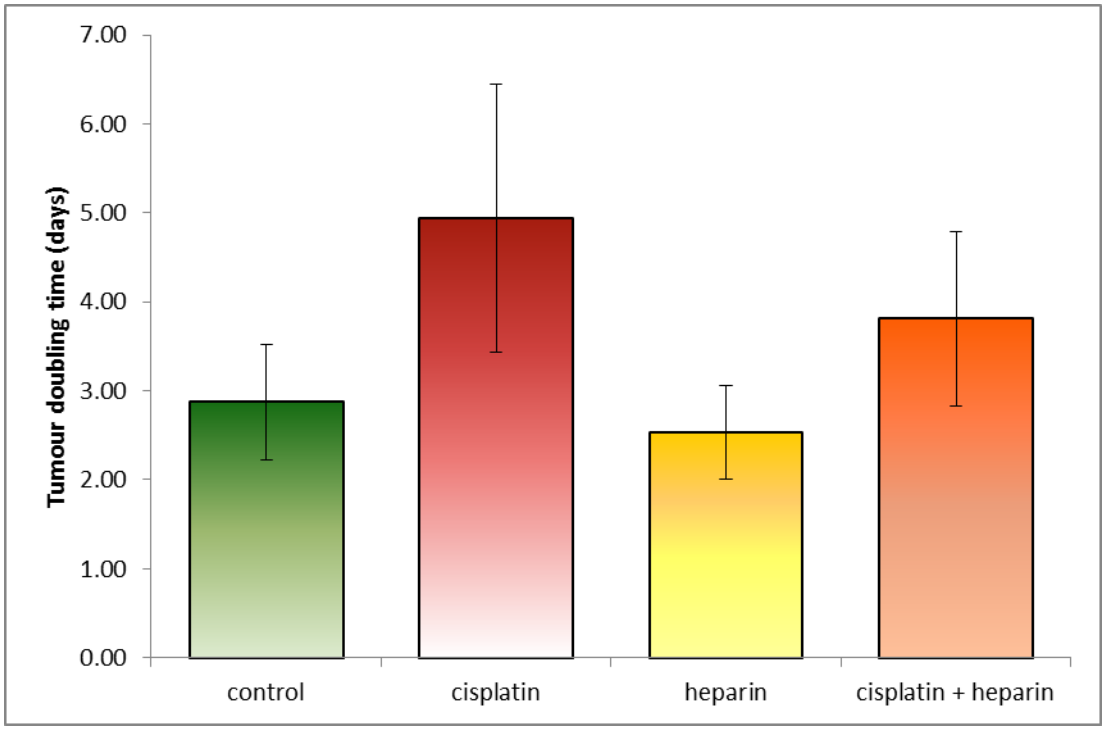

FIGURE 5.11: TUMOUR DOUBLING TIME
A2780s were injected subcutaneously into the flanks of nude BALB-C SCID mice; when the tumours were >100mm^3 in volume, the mice were treated with one of the following - control (no treatment), heparin only (10mg/kg od ip), cisplatin only (5mg/kg once weekly ip) or heparin (10mg/kg od ip) and cisplatin (5mg/kg once weekly ip). Tumour doubling times were calculated by using nonlinear regression analysis. Error bars show standard deviation of at least 6 biological samples in each group. Tumour doubling time was increased in cisplatin treated group as compared to control group (students t-test p-value 0.018); combination treatment group appears to show a decrease in tumour doubling time as compared to cisplatin though this is not significant.

5.4 DISCUSSION

Extracellular vesicles have generated great attention over the last decade as a means of cell – cell communication including transfer of RNA and proteins to distant cells by virtue of their being transferred through body fluids including blood. They are involved in numerous physiological and pathological processes. As discussed in Section 1.5.6, in cancers, they have been shown to affect tumorigenesis including proliferation and metastasis, angiogenesis, tumour microenvironment, anti-tumoral immune response and chemotherapy resistance.

Evidence suggests that EVs may affect drug resistance through transfer of proteins such as drug transporters (Corcoran et al., 2012, Goler-Baron and Assaraf, 2012, Lv et al., 2014,), microRNAs (Jaiswal et al., 2012, Corcoran et al., 2014, Wei et al.,

2014), lncRNA (Takahashi et al., 2014c, Takahashi et al., 2014a). Moreover they have been shown to sequester drugs (Ifergan et al., 2009, Gong et al., 2013, Ma et al., 2014) or oncogenic microRNAs (Akao et al., 2014) away from the cells. The payload of EVs has not been fully characterised nor are the complex mechanisms through which EVs may affect drug resistance.

Initially, the aim of the project was to investigate if resistance could be transferred from the resistant CP70 to the sensitive A2780 ovarian cancer cell lines. It was demonstrated that transfer of EVs from CP70 to A2780 could increase the survival of cells after cisplatin treatment as compared to control. This raised the possibility that on cisplatin treatment, cells that are damaged could then transmit this 'stress signal' to other cells in the same population or flask via EVs causing a bystander effect and adaptive response as described in section 5.1. This could result in a higher proportion of cisplatin resistance. Blocking the uptake of these EVs should then make the cells more sensitive to cisplatin.

EVs can be taken up in many different ways including phagocytosis, clathrin or caveolin mediated endocytosis, heparan sulphate proteoglycan associated uptake, micropinocytosis or lipid raft mediated uptake as described in section 1.5.4. Different chemicals have been employed to decrease the uptake of EVs; in fact EV uptake cannot be inhibited completely by one method (Escrevente et al., 2011). Heparin competes for binding with heparan sulfate proteoglycans and thereby decreases the uptake of EVs; heparin treatment was shown in one study to inhibit the uptake of EVs by 50% (Christianson and Belting, 2014, Franzen et al., 2014). Other well-known inhibitors of EV uptake include dynasore, a dynamin inhibitor that blocks uptake of EVs by endocytosis and amiloride which inhibits uptake of EVs by macropinocytosis.

Experiments showed that heparin treated cells were significantly more sensitive to cisplatin than the control cells in A2780, CP70, and IGROV-1 cell lines. This appeared to confirm the hypothesis that cells transfer resistance within the population and blocking of EV uptake would decrease resistance by preventing this transfer. A recent study appeared to confirm this by showing that low molecular weight heparin tinzaparin antagonises cisplatin resistance of A2780 ovarian cancer

cells and that cell surface haparan sulfate proteoglycans were involved in the cisplatin resistance (Pfankuchen et al., 2015). Heparin was also shown to decrease invasion and migration in breast cancer cell lines MDA-MB-231 and MDA-MB-435 (Li et al., 2013) and in lung cancer cells (Zhong et al., 2015, Liao et al., 2015); the latter studies suggest that this is mediated through heparin cofactor II (HCII) and consequent disruption of the AkT – PI3K-mTOR pathway. Heparin was also shown to increase cytotoxicity caused by chemotherapeutic drugs in breast cancer cells; an effect on drug efflux transporters ABCG2 and ABCC1 was noted increasing levels of cytotoxic drugs within cells (Chen et al., 2014c). A xenograft trial which appears to confirm these results indicating that heparin decreases cisplatin resistance of lung cancer side population cells by decreasing ABCG2 expression (Niu et al., 2012). In another xenograft trial, both tinzaparin (a low molecular weight heparin) and a non-anticoagulant heparin – S-NACH decreased tumour growth and increased apoptosis in pancreatic cancer cells (Sudha et al., 2014); S-NACH also increased chemotherapy sensitivity in breast cancer xenograft (Phillips et al., 2011). Xenograft studies appear to suggest that heparin decreased tumour growth in lung cancer (Kim et al., 2015) and to confer sensitivity to gefitinib, a chemotherapeutic agent (Pan et al., 2015). Heparin is widely used as a treatment for deep vein thromboembolism in patients with cancers. In one retrospective clinical trial, addition of a low molecular weight heparin, dalteparin to combination chemotherapy improved progression free survival and overall survival in a cohort of 67 patients with small cell lung cancer (Altinbas et al., 2004). Another study in pancreatic cancer patients has suggested that the time to progression of cancer increased from 4 months in control group (cisplatin and gemcitabine- another chemotherapy drug) to 7 months in patients treated with heparin in addition to cisplatin and gemcitabine (Icli et al., 2007). Heparin use was associated with improved survival in docetaxel chemotherapy for prostate cancer (Park et al., 2015). In a study of 385 patients with advanced malignancy, there appeared to be improved survival associated with dalteparin treatment as compared to a placebo; this was more obvious in patients with a better prognosis (Kakkar et al., 2004). A metaanalysis by Lazo-Langner et al shows an association of heparin treatment with improved survival rates in cancer patients (Lazo-Langner et al., 2007). However other studies appear to suggest no benefit of adding heparin to

conventional chemotherapy (Sanford et al., 2014, Macbeth et al., 2015, Sideras et al., 2006).

Amiloride, another EV uptake inhibitor, has also been associated with modulatory effects on cancers. It has been shown to increase sensitivity to chemotherapeutic drug, erlotinib in pancreatic cancer (Zheng et al., 2015) while a combination of amiloride and guggulsterone suppressed growth in oesophageal cancer cells and xenografts (Guan et al., 2014). Dynasore appeared to inhibit metastasis and invasion by reducing dynamin2 (Gong et al., 2015); another study showed a decrease in the invasive capacity of U2OS cells induced by dynasore by disrupting the actin cytoskeleton (Yamada et al., 2009).

Pre-treatment of A2780 cells with amiloride or dynasore before treatment with cisplatin appears to mirror the effect of heparin by significantly sensitising them to cisplatin. This corroborates the evidence presented that blocking the uptake of EVs sensitises the cell to cisplatin.

A study by Kong et al (Kong et al., 2015) appeared to show that treatment of breast cancer cells with a combination of guggulsterone and bexarotene sensitised the cells to doxorubicin; this appeared to correlate with an increase in ceramide which stimulates the release of EVs and a depletion of BRCP2/ ABCG2 drug transporters; this appears to indicate a secretion of ABCG2 transporters within the EVs. There is previous evidence regarding the sequestration of drug transporters in EVs (Ifergan et al., 2005, Ifergan et al., 2009, Goler-Baron and Assaraf, 2011, Goler-Baron et al., 2012). Moreover, guggulsterone has been shown to reduce viability in oesophageal cancer cells (Yamada et al., 2014), while a combination of amiloride and guggulsterone reduced tumour growth in an oesophageal adenocarcinoma xenograft study (Guan et al., 2014).

We hypothesised that if guggulsterone and bexarotene could sensitise cells to doxorubicin by secretion of ABCG2 within the EVs, then inhibiting the reuptake of EVs with heparin would act synergistically. Two possible scenarios exist with regard to synthesis and release of stress induced EVs – one is that the cell has a store of "pre-made" and ready to use "stress EVs", the release of which is triggered by the stress; the other is that upon induction by the stress; the payload of the EVs changes

and stress EVs are newly synthesised. If the stress EVs are pre-stored and the release is induced by guggulsterone and bexarotene, there would be no stress EVs left to be released; therefore the cells would be more sensitive, but adding heparin after cisplatin treatment would make no difference as all the stress EVs would have been released. However if the stress EVs are synthesised and released as and when the stress occurs, though guggulsterone and bexarotene would induce release of the EVs before cisplatin treatment, more "stress EVs" would be synthesised and released during and after cisplatin treatment containing information to cause a bystander effect and an adaptive response. The addition of heparin during and after the stress will further increase the sensitivity of the cells by inhibiting the uptake of the EVs released during and after cisplatin treatment. Hence, A2780 cells were subjected to treatment with combinations of guggulsterone, bexarotene, heparin and cisplatin. The results showed that guggulsterone and bexarotene did indeed sensitise cells to cisplatin suggesting that induce the release of EVs prior to cisplatin treatment did sensitise cells to cisplatin. Heparin, if added during and after cisplatin treatment, increased this sensitivity suggesting that EVs released during and after cisplatin treatment have the potential to induce resistance. This suggests that the evidence is weighted towards stress EVs being made as and when the stress occurs. However, heparin treatment alone during and after cisplatin with no additional treatment with guggulsterone or bexarotene had a sensitising effect equivalent to combined treatment. A possible reason is that preventing the reuptake of EVs released during and after cisplatin treatment is sufficient to induce cisplatin sensitivity.

All the experiments thus far suggested that *in vitro*, modifying the uptake of EVs during and after cisplatin treatment decreases cancer cell viability; to determine the efficacy *in vivo*, a xenograft study was performed to establish if combining heparin treatment with conventional cisplatin based chemotherapy could induce an increase in sensitivity to cisplatin treatment. Mice grafted with A2780 tumours were divided into four treatment groups (1) control to establish a baseline for tumour growth with no treatment (2) cisplatin only treatment to provide the baseline for tumour growth with conventional therapy (3) heparin only to indicate any changes to tumour growth induced solely by heparin and (4) combined treatment to establish any synergistic effects upon treatment with heparin and cisplatin i.e. to show if heparin induced an

increased sensitivity to cisplatin. The results however, were unexpected. Cisplatin, as expected, did reduce tumour growth and increase survival as compared to control. However, surprisingly, adding heparin to cisplatin did not potentiate the effects of cisplatin. Instead it resulted in a decrease in the effect of cisplatin showing increased tumour growth and decreased survival than the cisplatin only treatment group, though this was not significant. Moreover, the heparin only treatment group also appeared to have a decreased survival as compared to the control group, although this was also not significant. These results suggest that *in vivo*, other effects of heparin appear to play a predominant role and can confound the effects of inhibiting EV uptake. One recent study studying microvesicles from pregnant women noted that microvesicles from patients who had been treated with low molecular weight heparin had a higher content of proteins such as Ang-2, VEGFR-3, angiostatin, TIMP-1 and TNF-α which are known to be involved in angiogenesis (Shomer et al., 2016). If angiogenesis were increased by heparin; tumour growth would be influenced by this, leading to increased tumour growth and consequent decrease in survival. This appears to be given credibility by the fact that heparin alone treatment shows a trend towards decreased survival and increased tumour growth as compared to control. Moreover, published literature appears to suggest that the survival benefit of using heparin along with conventional chemotherapy is not universal and that in some groups of patients with cancer, there is no significant increase in survival on treatment with heparin along with conventional chemotherapy (Sideras et al., 2006, Sanford et al., 2014, Macbeth et al., 2015). Therefore, the other *in vivo* effects of heparin and their influences on heparin's cisplatin-sensitising property would have to be further studied. There could be variations based on type of tumour, type of animal, routes of administration, dosage of drugs etc. Hence more experiments would have to be done before firm conclusions can be drawn.

This part of the project has started to explore the role of EVs on cisplatin resistance. However most of the experiments have been based on the MTT assay to quantify viability. Other methods of quantifying proliferation/ apoptosis or response to chemotherapy would help to validate this data. Moreover, the change has not been shown to be because of the direct effect of heparin, amiloride or dynasore on EVs. These experiments would need to be done to confirm that this is not the result of some other action of heparin or the other drugs on cells. Moreover, the actual effect

of the other drugs have not been tested *in vitro*. The pathways by which EVs might be able to induce the 'bystander effect' or the 'adaptation effect' have not been elucidated. These could be profitably explored in future studies. Moreover, EV uptake studies and assessment of the RNA/ protein content of the EVs would help us determine the possible mechanisms by which heparin, amiloride and dynasore cause their effects on cisplatin resistance. *In vitro* studies need to be done with other cell lines and amiloride to assess its effect on cisplatin response in the other cell lines. Possible future avenues to explore include xenograft studies with different doses of cisplatin possibly with a different cell line – IGROV-1 or SKOV-3. The other possibility is to use amiloride to avoid the angiogenic effects of heparin.

The main contributions from this chapter is evidence that transfer of EVs from the cisplatin resistant cell line CP70 to the cisplatin sensitive cell line A2780 increases cisplatin resistance in A2780s. EV uptake inhibitors heparin, amiloride and dynasore increase cisplatin sensitivity in A2780 ovarian cancer cells *in vitro*. Heparin has also been shown to increase cisplatin sensitivity in CP70, IGROV-1 and SKOV-3 cell lines. Heparin's effect has been mainly during and after cisplatin treatment, indicating the possibility that "stress induced EVs" are synthesised as and when the stress occurs. Xenograft studies however have indicated a decrease in cisplatin sensitivity on addition of heparin to the treatment regimen.

Chapter 6 DISCUSSION

Ovarian cancers have a very low 5-year survival rate of only 45%; one of the contributors to this low survival is resistance to platinum drugs which are the treatment of choice for chemotherapy. The causes of cisplatin resistance are complex and multifactorial and are the subject of investigations described in this thesis. Two aspects of the modulation of cisplatin resistance have been investigated in this project.

MicroRNAs have emerged as new modulators of various cellular processes in physiological and pathological conditions including cancer. As microRNA-target site interactions are imperfectly complementary, one microRNA can target many genes and one gene can be targeted by many microRNAs. This introduces a whole new layer of modulation opening up exciting possibilities where one microRNA can modify whole pathways.

The mechanisms of platinum resistance and microRNAs have not yet been exhaustively studied. Gaps in the knowledge exist that could be exploited in the quest for the improved survival of patients. Therefore, this project investigated cisplatin resistance in ovarian cancers to identify microRNAs involved in modulating the cisplatin resistant phenotype but have, thus far not shown to be involved in cisplatin resistance. The results from the first two chapters provide some insight into microRNAs thus far not described in cisplatin resistance in ovarian cancer cells.

Differences in microRNA expression between cisplatin sensitive ovarian adenocarcinoma cell line – A2780s, and its cisplatin resistant derivatives - MCP-1 and CP70 - were used to identify potential candidates for further investigation. MicroRNA-21* and microRNA-31 were identified as potentially of interest in the modulation of cisplatin resistance and further investigated. MicroRNA-21* was shown to increase resistance in a panel of ovarian cancer cells; NAV3 was validated as one of the targets of miR-21*. Knockdown of NAV3 was shown to increase resistance to cisplatin. MiR-21* was shown to be a driver of proliferation upon induction by cisplatin. Though general PDI inhibitors increased cisplatin resistance

in A2780 cells, specific knockdown of PDIA4 did not modulate cisplatin resistance in A2780 cell line.

These results provide evidence that miR-21* increases cisplatin resistance in ovarian cancer cells, possibly through targeting of NAV3. Firstly, the significance of miR-21*, the passenger strand of a well-known oncomiR, has largely been overshadowed by its more predominant guide strand. Therefore, this work adds to an ever growing body of work that indicates that passenger strands have significant effects which may be synergistic or antagonistic with the guide strand. The identification of NAV3 as a novel target of miR-21* is of significant importance as there is some evidence that loss of NAV3 may be associated with poor prognosis and increased invasiveness in various cancers. Deletions in the NAV3 gene do not account for all the copy number changes (Marty et al., 2008, Maliniemi et al., 2011). NAV3 has previously been shown to be a target of miR-29a (Satoh, 2010, Shioya et al., 2010) and miR-29c (Zong et al., 2015). This study has shown that NAV3 expression may also be modulated by miR-21*. Moreover, knockdown of NAV3 has been shown for the first time to be associated with cisplatin resistance in ovarian cancer cells.

MiR-31's role in cisplatin resistance has been described here. MiR-31 was shown to increase resistance; KCNMA1 was validated as a possible indirect target of miR-31; knockdown of KCNMA1 was shown to increase cisplatin resistance. MiR-31 is a pleiotropic miRNA, shown to have contradictory effects in different types of tumours. Even within the same type of tumour, the effects have been different depending on p53 expression and p21 expression. Hence, any description of the involvement of this miRNA adds to available knowledge which will help to clarify the ways by which this miRNA's effects are regulated. KCNMA1 has been shown to be involved in platinum resistance (Ziliak et al., 2012) in addition to its diverse effects in various cancers. This study adds to the knowledge about the regulation of this gene. Moreover, this project indicates the possibility of involvement of the BKCa channel in cisplatin resistance; this is corroborated by other studies linking the BKCa channel to apoptosis in response to stimuli including cisplatin (Ma et al., 2012).

These findings add to the understanding of the miRNA networks and regulation in cisplatin resistance. As discussed, a full understanding of miRNA regulation could lead to improved, and possibly personalised, therapy for ovarian cancers and improved survival of patients.

There is much scope for further research. In all probability, there are many more targets of miR-21* and miR-31. Identifying these may enable elucidation of the exact mechanisms involved in miR-21*'s and miR-31's modulation of cisplatin resistance and facilitate the categorisation into pre-, on-, post- or off-target mechanisms of action. The role of these miRNAs, especially miR-21*, in other tissue types are yet to be completely understood. Hence they could profitably be studied in other types of cancers as well as in specific characteristics of cancer behaviour such as invasion and metastasis. Previous evidence suggests that other microRNAs, for example miR-433 have been involved in chemoresistance to paclitaxel (Weiner-Gorzel et al., 2015); hence these microRNAs could also be investigated for involvement in chemoresistance to paclitaxel. Experimental evidence has been derived from cell line studies, there is, therefore, the scope for other models of study, possibly xenograft trials, to investigate the *in vivo* behaviour of these miRNAs in cisplatin resistance. Other microRNAs which were identified by the miRNA microarray could also be followed up.

The other aspect of cisplatin resistance investigated in this project has been the role of EVs in development of cisplatin resistance. EVs have emerged as an important mode of cell-to-cell communication, including communication with remote cells, especially as it has been described that functional RNA and protein can be conveyed between cells. The presence of bystander effect and adaptive response described with irradiation and bleomycin treatment raises the possibility of a similar response in cisplatin treatment. If this adaptive response occurred within a population of cells, it would result in the population having a higher resistance to cisplatin. As the adaptive and bystander effects can be mediated by EVs, the inference would be that reducing this communication would make the cells more sensitive to cisplatin.

Investigating this possibility, experimental results show that treatment with heparin/ amiloride/ dynasore, all of which reduce the uptake of EVs could increase the

sensitivity of A2780 cells to cisplatin. With heparin, this was confirmed in three other ovarian cancer cell lines too. This corroborates previous studies where ovarian cancer cells were rendered cisplatin sensitive by treatment with heparin (Pfankuchen et al., 2015). A study by Kong et al has shown that treatment with guggulsterone and bexarotene causes release of EVs associated with ABCG2 drug transporter; the authors postulate that this leads to increased intracellular concentration of cytotoxic drug making cells more sensitive (Kong et al., 2015). In the project described here, it was shown that, guggulsterone and bexarotene did indeed sensitise cells to cisplatin. However this did not further potentiate the sensitivity induced by heparin. As discussed before, this appears to endorse the view that "stress induced EVs" are synthesised *de novo.*

Once *in vitro* studies established a clear role for heparin in the modulation of cisplatin resistance, a xenograft study was performed to study the effect of a combination of cisplatin and heparin on tumour growth. Unexpectedly, this study indicated a decrease in survival and increase in tumour growth with the combined treatment compared with cisplatin alone. This increase in tumour growth, though not significant, was also mirrored in the heparin only treatment group as compared to the control group. There could be several reasons for this apparent contradiction of the *in vitro* findings – one of them being that the other effects of heparin, such as induction of angiogenesis, may increase the proliferation of the tumour *in vivo*. Moreover, most of the results obtained have relied on the MTT assay to assess the response to cisplatin. Despite this, there is scope for further investigation. Various possibilities include using a different cell line to induce tumours, possible changes in the dosage and route of administration of cisplatin and heparin, as well as investigating the effect of amiloride or dynasore on cisplatin sensitivity. More experiments could also be performed *in vitro* investigating the bystander effect and adaptive response and studying the RNA and protein content of these EVs, their uptake by recipient cells, and the induced responses in the recipient cells.

6.1. CONTRIBUTIONS:

The following have been the novel contributions from this work to the knowledge in the field of cisplatin resistance in ovarian cancers (Figure 6.1):

In this project, miR-21* has been shown to increase cisplatin resistance in ovarian cancer cells. NAV3 has been validated as a direct target of miR-21* while knockdown of NAV3 has been shown to increase cisplatin resistance. miR-21* and NAV3 knockdown were shown to increase resistance in ovarian cancer cells upon cisplatin treatment. PDI inhibitors were shown to increase cisplatin resistance but specific knockdown of PDIA4 had no effect.

MiR-31 increases cisplatin resistance in ovarian cancer cells; KCNMA is a possible indirect target of miR-31. KCNMA1 knockdown and blocking of the BK channels by paxilline increase cisplatin resistance in ovarian cancer cells.

Transfer of EVs from the cisplatin resistant cell line CP70 to the cisplatin sensitive cell line A2780 increases cisplatin resistance in A2780s. Heparin treatment is shown to sensitise cells to cisplatin in three ovarian cancer cell lines, while dynasore and amiloride, other EV uptake inhibitors also increase sensitivity to cisplatin in A2780 cells. Xenograft studies indicate a decrease in survival and increase in tumour growth when heparin is added to the conventional chemotherapy with cisplatin.

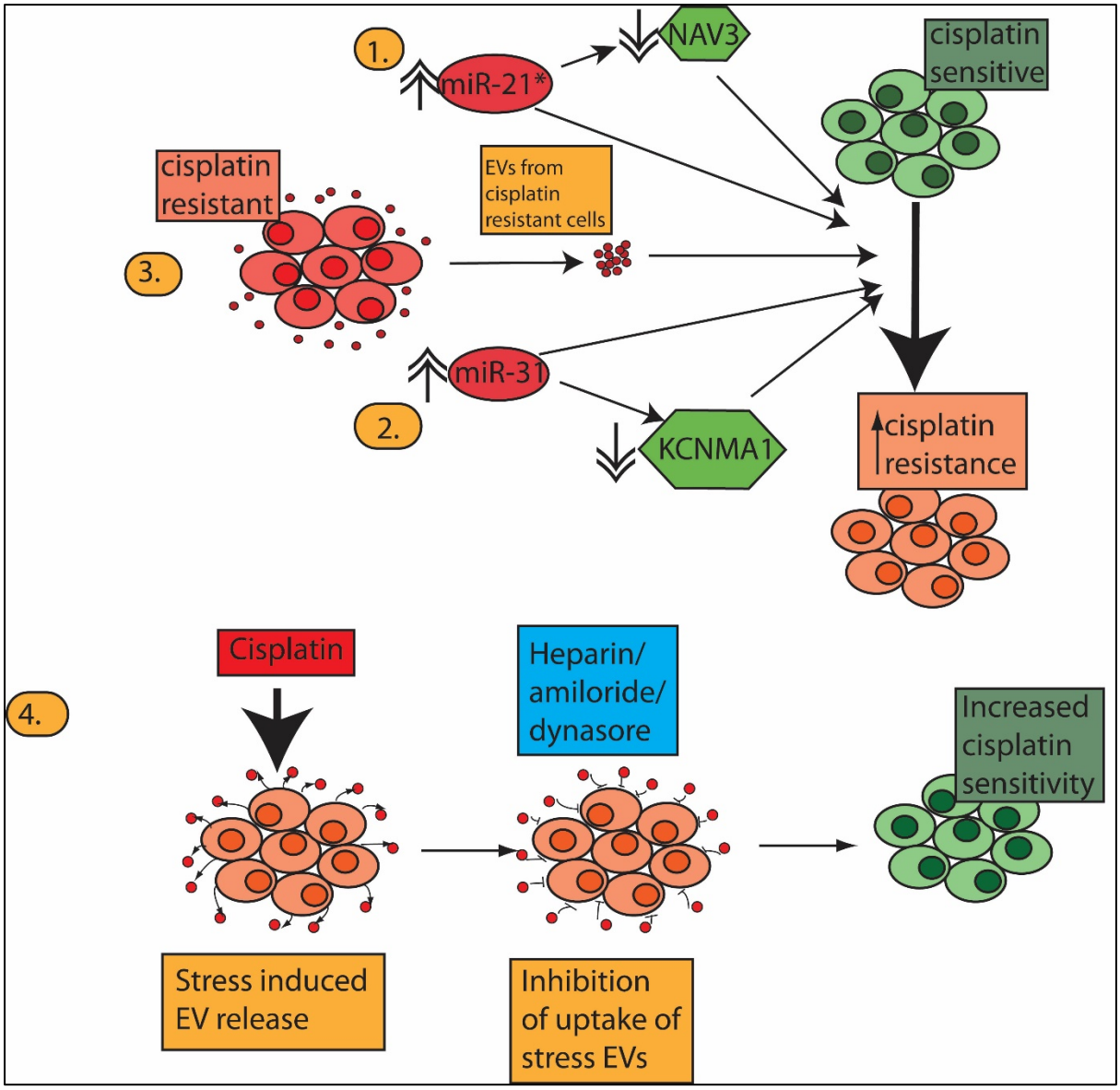

FIGURE 6.1: NOVEL CONTRIBUTIONS

Novel Contributions from this project include: 1. Increase in miR-21* decreases NAV3 expression causing increase in cisplatin resistance 2. Increase in miR-31 indirectly decreases KCNMA1 causing increase in cisplatin resistance 3. EVs isolated from cisplatin resistant cells if transferred on to cisplatin sensitive cells increases cisplatin resistance 4. Inhibition of uptake of EVs that have been released on stress by cisplatin increases cisplatin sensitivity.

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Appendices

Appendix A: Cell Growth Characteristics and Cisplatin response curves

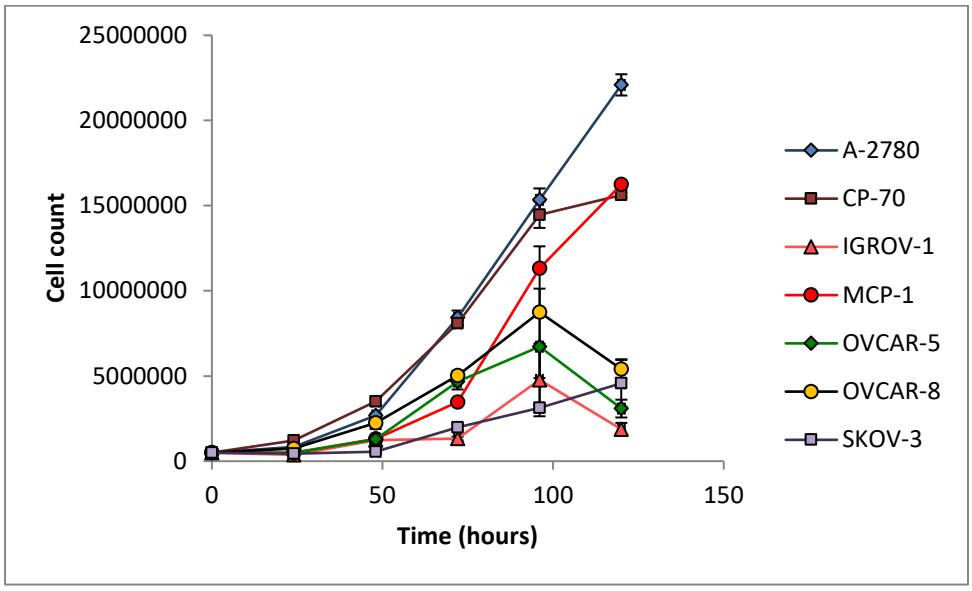

FIGURE A.1: CELL GROWTH CURVES

Cells were seeded in T25 flasks at a starting concentration of 500 000/ flask. Cells were counted at 24 hours, 48 hours, 72 hours, 96 hours and 120 hours using a hemocytometer. Each point represents the mean of 3 replicates; error bars indicate SEM. A2780 and CP70 appear to have similar characteristics and a faster doubling time than the other cell lines.

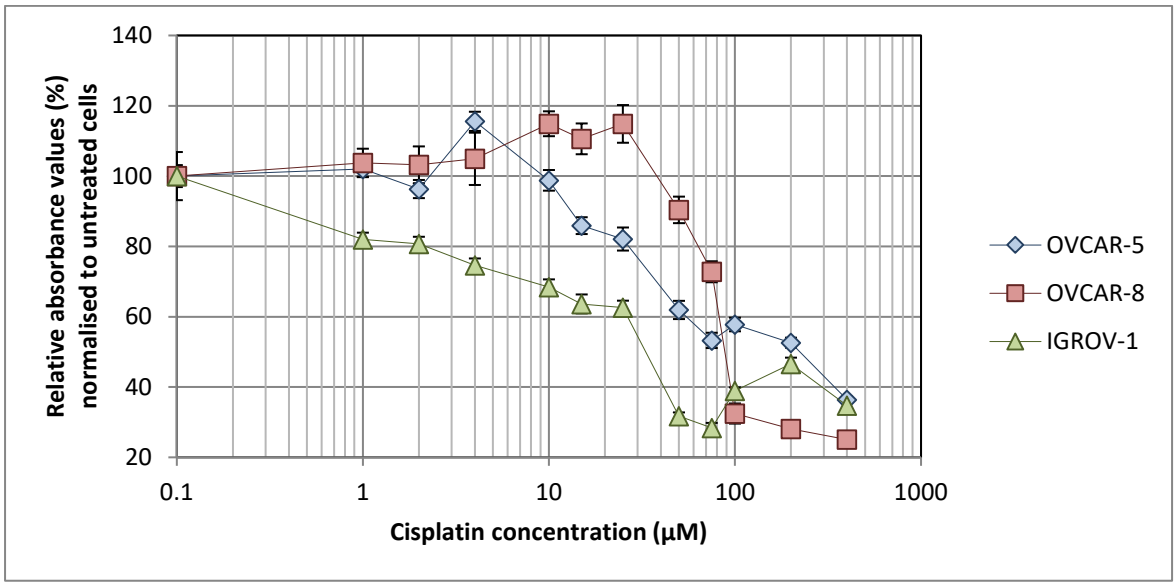

FIGURE A.2: CISPLATIN RESPONSE CURVES FOR OVCAR-5, OVCAR-8 AND IGROV-1

OVCAR-5, OVCAR-8 and IGROV-1 cells seeded at optimal concentrations were treated with increasing concentrations of cisplatin for 3 hours. Cell viability was assessed after 48 hours by the MTT assay. Absorbance was normalised to untreated cells in each group; error bars show SEM from at least 6 biological replicates for each point of the graph.

APPENDIX B: Effects of Different treatments on cell viability

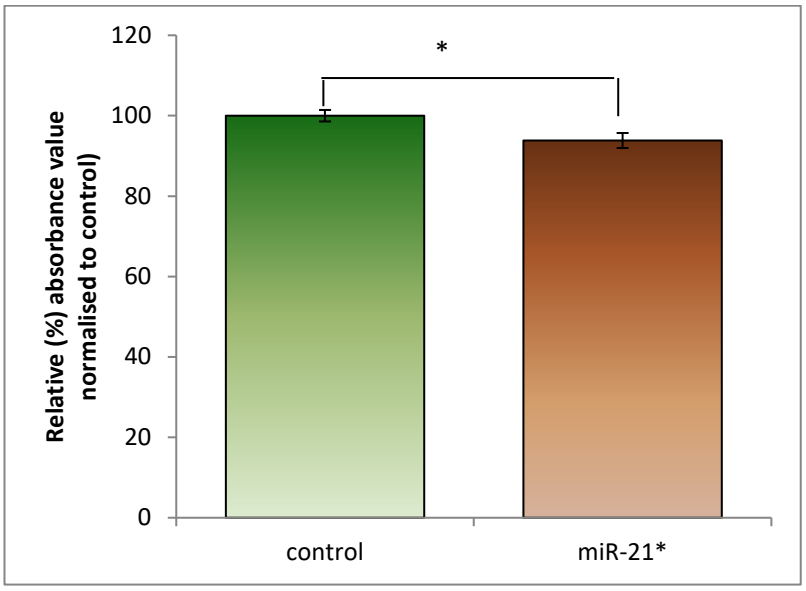

FIGURE B.1: EFFECT OF MIR-21* ON CELL VIABLILITY
A2780 cells in 96 well plates were treated with miR-21* mimics or control. The percentage of cell viability (measured by the MTT assay) was normalised to control and compared by the student's t-test; error bars show SEM of 50 biological replicates. The results show slight decrease in cell viability (p-value<0.01)

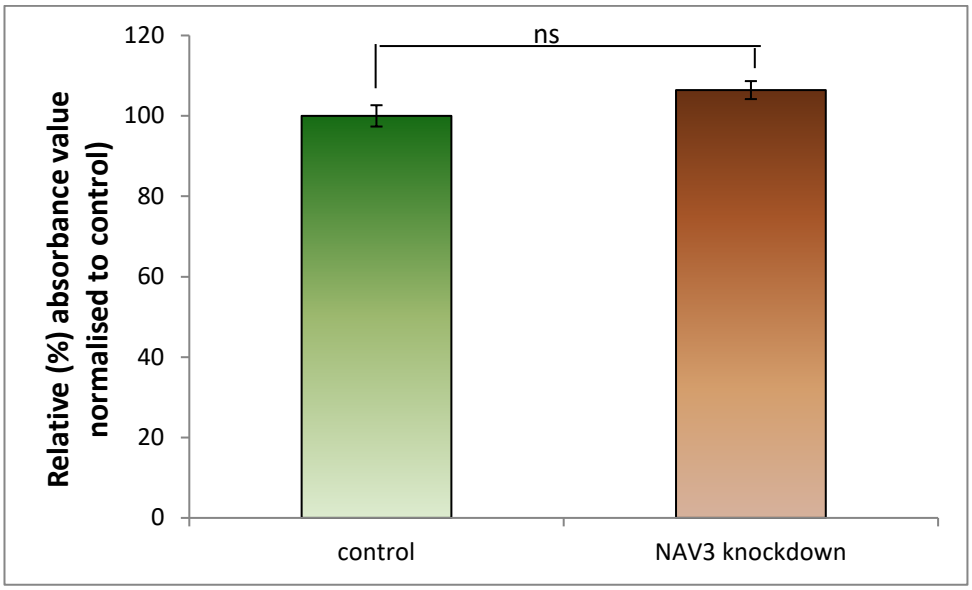

FIGURE B.2: EFFECT OF NAV3 KNOCKDOWN ON CELL VIABILITY
A2780 cells in 96 well plates were treated with NAV3 siRNA or control. The percentage of cell viability (measured by the MTT assay) was normalised to control and compared by the student's t-test; error bars show SEM of 16 biological replicates. The results show slight increase in cell viability (not significant)

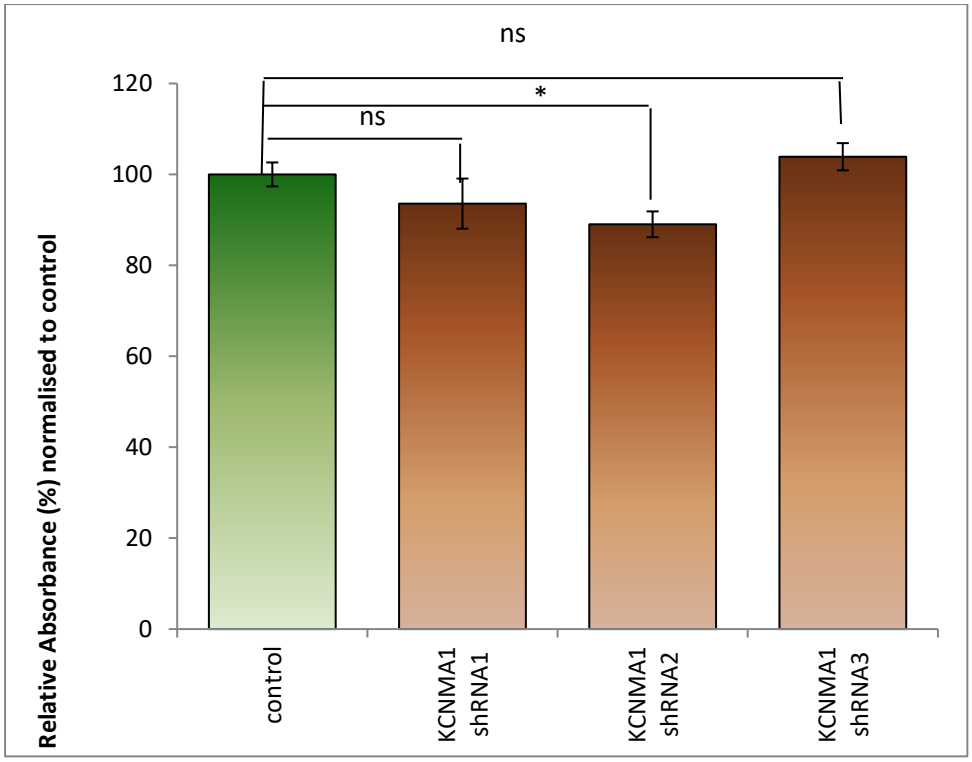

FIGURE B.3: EFFECT OF KCNMA1 KNOCKDOWN ON CELL VIABILITY
A2780 cells in 96 well plates were treated with KCNMA1 knockdown shRNAs or control. The percentage of cell viability (measured by the MTT assay) was normalised to control and compared by the student's t-test; error bars show SEM of 10 biological replicates. The results show slight decrease in cell viability in KCNMA1 shRNA2 (p-value < 0.05)

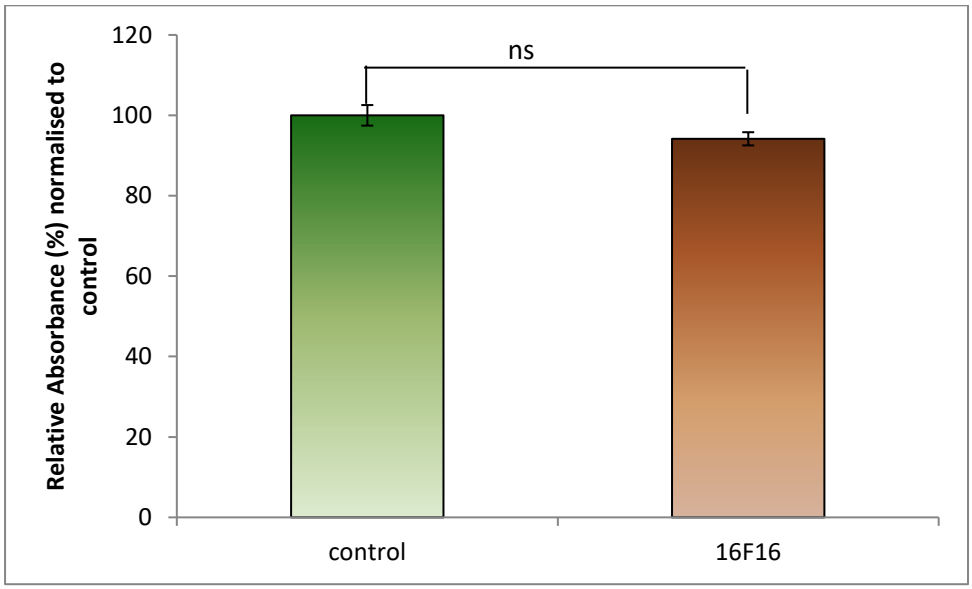

FIGURE B.4: EFFECT OF PDI INHIBITOR 16F16 ON CELL VIABILITY
A2780 cells in 96 well plates were treated with 16F16 at 2 μM for 24 hours; media was changed and MTT assay was done 48 hours later. The percentage of cell viability was normalised to control and compared by the student's t-test; error bars show SEM of 15 biological replicates. No significant change in cell viability is detected

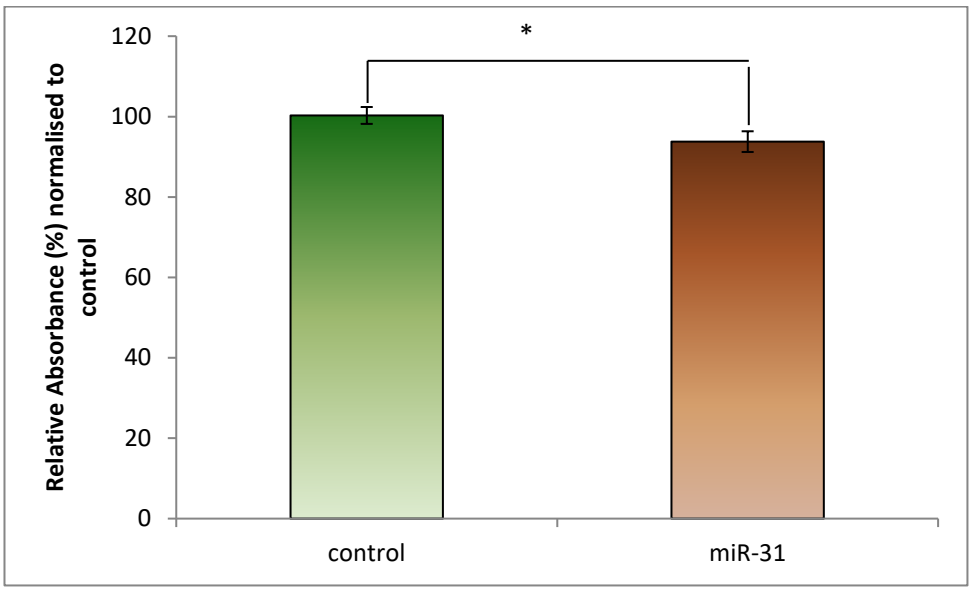

FIGURE B.5: EFFECT OF MIR-31 MIMICS ON CELL VIABILITY
A2780 cells in 96 well plates were treated with miR-31 mimics or control for 24 hours; media was changed and MTT assay was done 48 hours later. The percentage of cell viability was normalised to control and compared by the student's t-test; error bars show SEM of 20 biological replicates. There was a slight decrease in cell viability as compared to control (student's t-test p-value < 0.05)

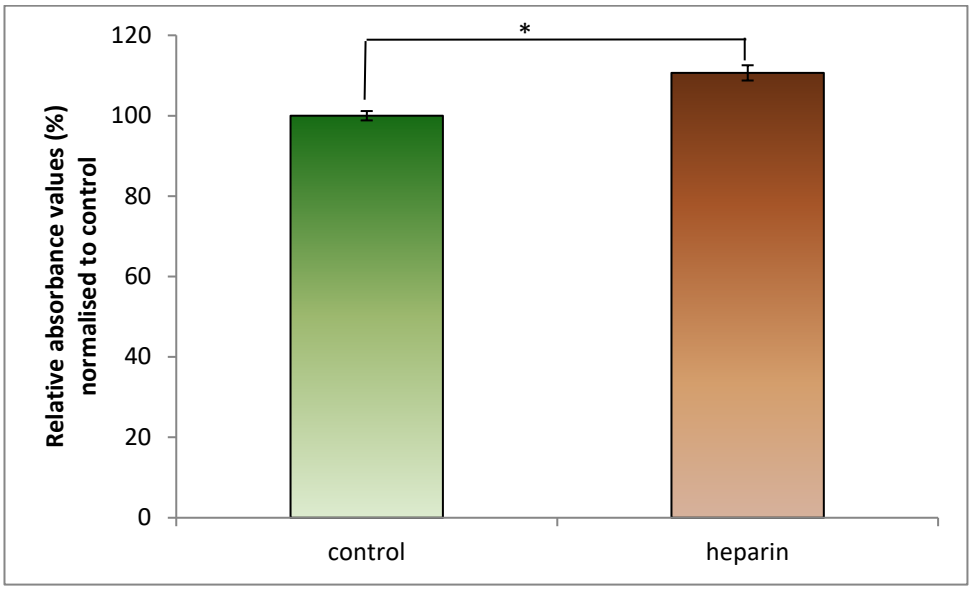

FIGURE B.6: EFFECT OF HEPARIN ON CELL VIABILITY
A2780 cells in 96 well plates were treated with heparin at 10µg/ml for 72hours. The percentage of cell viability (Assessed by MTT assay) was normalised to control and compared by the student's t-test; error bars show SEM of 20 biological replicates. There was a slight increase in cell viability as compared to control (student's t-test p-value < 0.05)

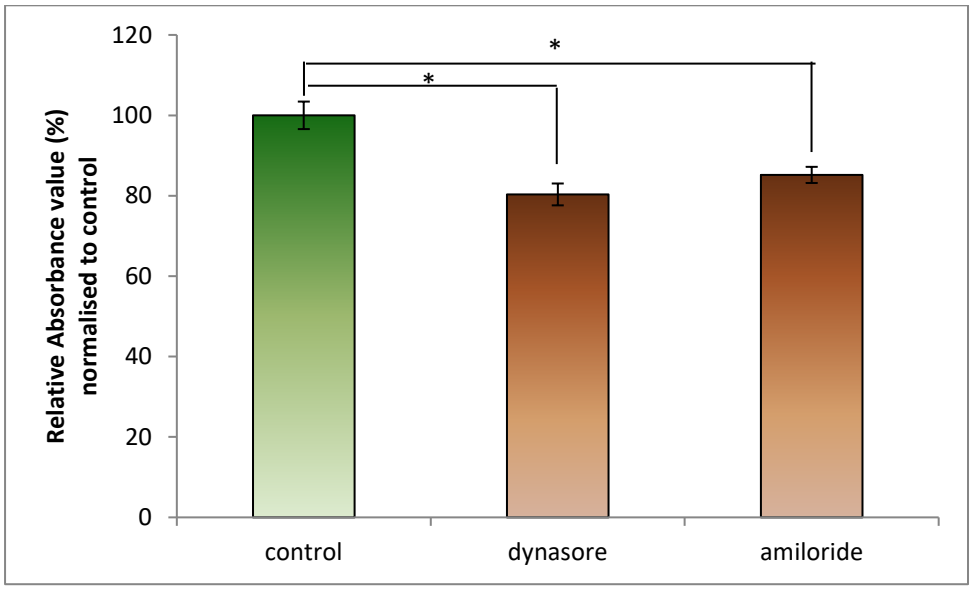

FIGURE B.7: EFFECT OF DYNASORE AND AMILORIDE ON CELL VIABILITY
A2780 cells in 96 well plates were treated with dynasore or amiloride for 72 hours. The percentage of cell viability was normalised to control and compared by the student's t-test; error bars show SEM of 9 biological replicates. There was a slight decrease in cell viability in both groups as compared to control (student's t-test p-value < 0.05)

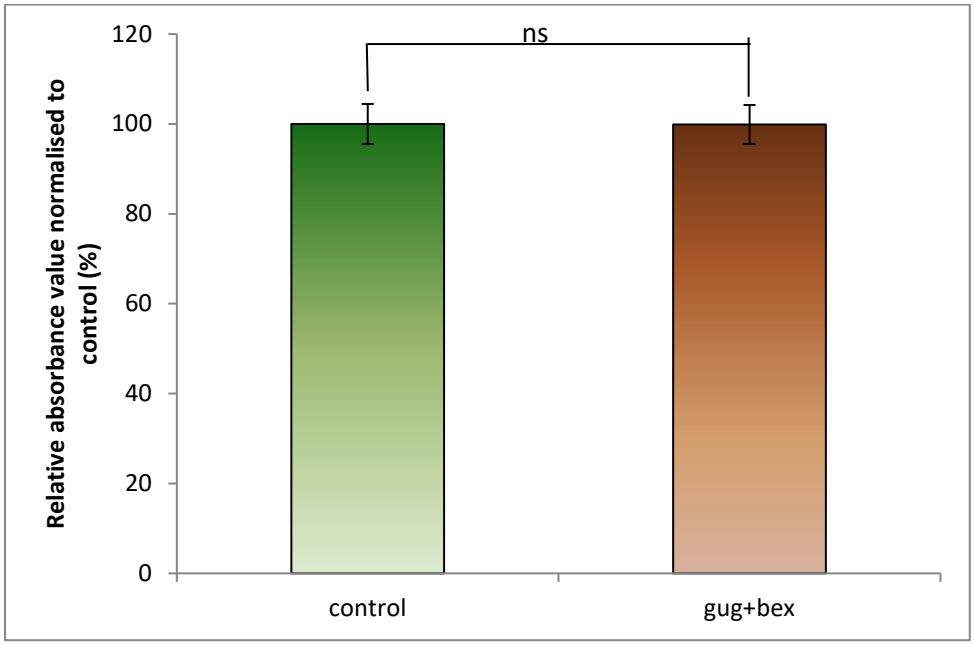

FIGURE B.8: EFFECT OF GUGGULSTERONE AND BEXAROTENE ON CELL VIABILITY
A2780 cells in 96 well plates were treated with Guggulsterone at 50 µM and bexarotene at 5 µM or control for 24 hours; media was changed and MTT assay was done 48 hours later. The percentage of cell viability was normalised to control and compared by the student's t-test; error bars show SEM of 9 biological replicates. There is no change in cell viability as compared to control (student's t-test p-value < 0.05)

Appendix C: Top De-regulated genes in CP70s as compared to A2780s

TABLE C-1: TOP 50 DOWN-REGULATED GENES IN CP70 BY RNA SEQUENCING AS COMPARED TO A2780 WITH FOLD CHANGE AND P-VALUE

Gene	Fold change CP70 v A2780	p-value
BEX4	**	5.36E-52
BTBD6	**	1.02E-09
C3orf67	**	5.58E-20
CASP5	**	2.08E-18
CHST9	**	1.44E-14
CRABP1	**	1.41E-85
CRADD	**	2.42E-14
CYB5A	**	3.24E-15
CYP7B1	**	3.52E-11
DENND2A	**	1E-10
EMILIN3	**	3.24E-47
EPM2AIP1	**	2.34E-63
ESRRG	**	3.55E-22
FAM107A	**	1.01E-17
FCER1A	**	1.1E-40
GRM8	**	5.38E-11
GSTM3	**	1.26E-16
HTATIP2	**	2.76E-19
HTR1F	**	5.19E-55
KCTD16	**	7.11E-15
KLF8	**	3.6E-30
MAPKAP1	**	4.97E-23
MBL2	**	3.28E-12
MDFI	**	8.7E-32
MEGF10	**	2.21E-13
MLH1	**	1.7E-92
NSUN7	**	1.81E-12
PPP2R2B	**	6.35E-44
RIN3	**	7.51E-31
RNF212	**	7.26E-53
ROCK2	**	1.49E-29
SCFD2	**	1.16E-14
SERTAD4	**	6.93E-10
SETBP1	**	3.92E-21
SLC7A3	**	8.29E-55
SNRPD1	**	1.73E-11
SORCS1	**	2.07E-19
TDRD1	**	1.11E-33

Table C-1 contd..

Gene	Fold change CP70 v A2780	p-value
UNC13C	**	2.12E-53
USP44	**	4.52E-19
FABP4	0.000144	2.3E-06
POU3F2	0.0002	7.29E-94
PDGFD	0.000375	1.36E-95
SV2A	0.000433	3.1E-115
NRK	0.000465	3.7E-113
KCNMA1	0.000589	1.2E-83
FREM1	0.001067	1.88E-78
PTH1R	0.00116	2.55E-46
ARMCX2	0.001233	1.64E-74
OLFML1	0.001258	1.73E-62

** - indicates genes where no transcripts were detected in CP70s while transcripts were quantified in A2780s

TABLE C-2: TOP 50 UP-REGULATED GENES IN CP70 AS COMPARED TO A2780

Gene	Fold change CP70 v A2780	p-value
LAYN	10000000	4.43E-16
PDLIM1	10000000	3.35E-26
ANXA1	1554.572	1E-196
LMX1B	782.7477	3.75E-44
MUSK	575.4211	4.23E-28
PRSS12	185.0256	3.02E-27
MTMR3	151.7484	0.00046
MYO6	137.4301	9.58E-83
AP1M2	101.5283	7.27E-05
SP100	90.21407	4.09E-58
LPAR1	86.62011	2.38E-98
FOXE1	77.90603	4.77E-59
ALX4	54.28959	2.09E-59
MARCKS	54.04807	3.84E-51
SOX8	52.58902	7.41E-33
MYEOV	49.0835	1.27E-20
SCNN1A	45.50787	2.52E-39
SLC27A6	45.09913	6.84E-13
CEACAM1	39.80012	0.000275
ADRA2C	37.08017	3.71E-19
LAMA4	33.77614	7.37E-07

Table C-2 contd..

Gene	Fold change CP70 v A2780	p-value
TLR6	29.39821	3.35E-52
DDAH1	29.00061	2.24E-08
PDGFA	26.28241	6.76E-48
PPP3CA	25.9321	1.86E-52
METRNL	23.79754	4.31E-22
VAMP8	22.47622	1.16E-76
PHLDA1	21.91634	7.25E-80
FBXL16	21.36917	6.07E-61
KIF5C	21.15128	1.1E-11
GSG1	20.89257	6.41E-14
CXADR	19.55366	5.48E-48
BMP7	16.89743	6.15E-57
ERRFI1	16.87938	6.82E-74
CACNA1H	15.30267	1.5E-99
SLC39A8	14.27447	1.75E-45
GATA5	14.08363	2.67E-08
C9orf150	13.94771	0.000205
MYOF	13.10654	6.7E-118
SLC45A1	11.89503	2.27E-33
TFEC	11.42253	4.19E-30
MT1X	11.12725	1.46E-16
ADAMTS20	9.88019	0.000319
RPS6KL1	9.801027	3.42E-47
OSBPL10	9.493201	3.3E-20
ART3	9.458352	1.27E-07
TNS3	8.628452	1.36E-80
TMEM130	8.565589	5.34E-10
GPR56	7.509891	1.24E-30
TRIM56	7.340083	8.77E-50

Appendix D

Original western Blots from the thesis

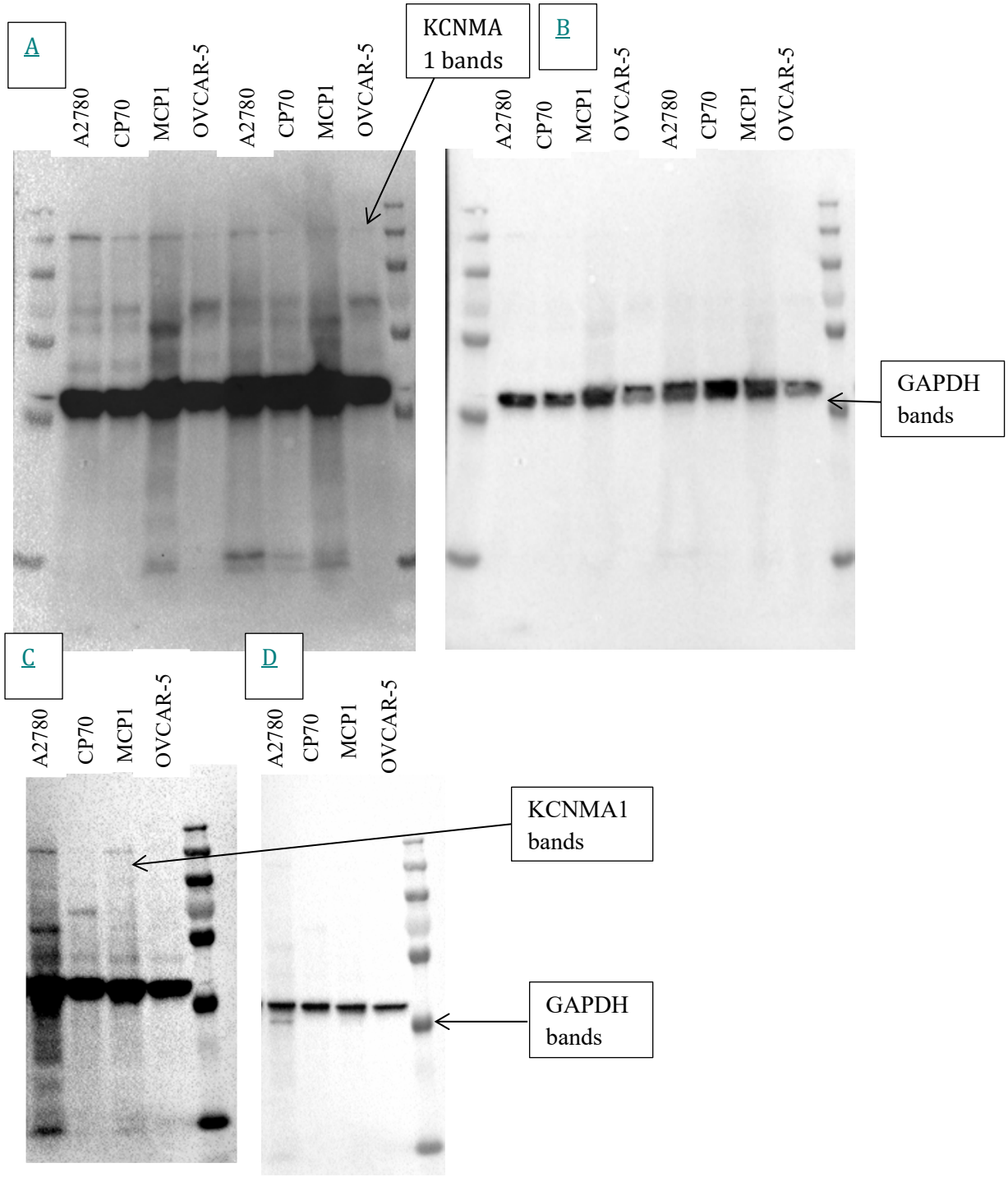

FIGURE D.1: ORIGINAL WESTERN BLOT SHOWING PROTEIN FROM A2780S, CP70S, MCP1 AND OVCAR-5 cell lines labelled for KCNMA1 (130 kD) and GAPDH (35 kD). Approximately 20µg of protein from each sample was denatured by heating to 70°C, electrophoresed on a polyacrylamide gel (Bio-Rad), transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked with 5% BSA in TBS with Tween, incubated with primary antibody (1 in 500 KCNMA1) at 4°C overnight and then with secondary antibody (1:10000) for 1 hour at room temperature. The Blots were immersed in ECL solution and digitally imaged on a Bio-Rad Chemidoc system and analysed using ImageLab software. The procedure was repeated with GAPDH primary antibody (1in 15000). Panel A shows the higher exposure used for KCNMA1 bands and panel B shows a lower exposure for GAPDH. KCNMA bands are not visible at this low exposure level.

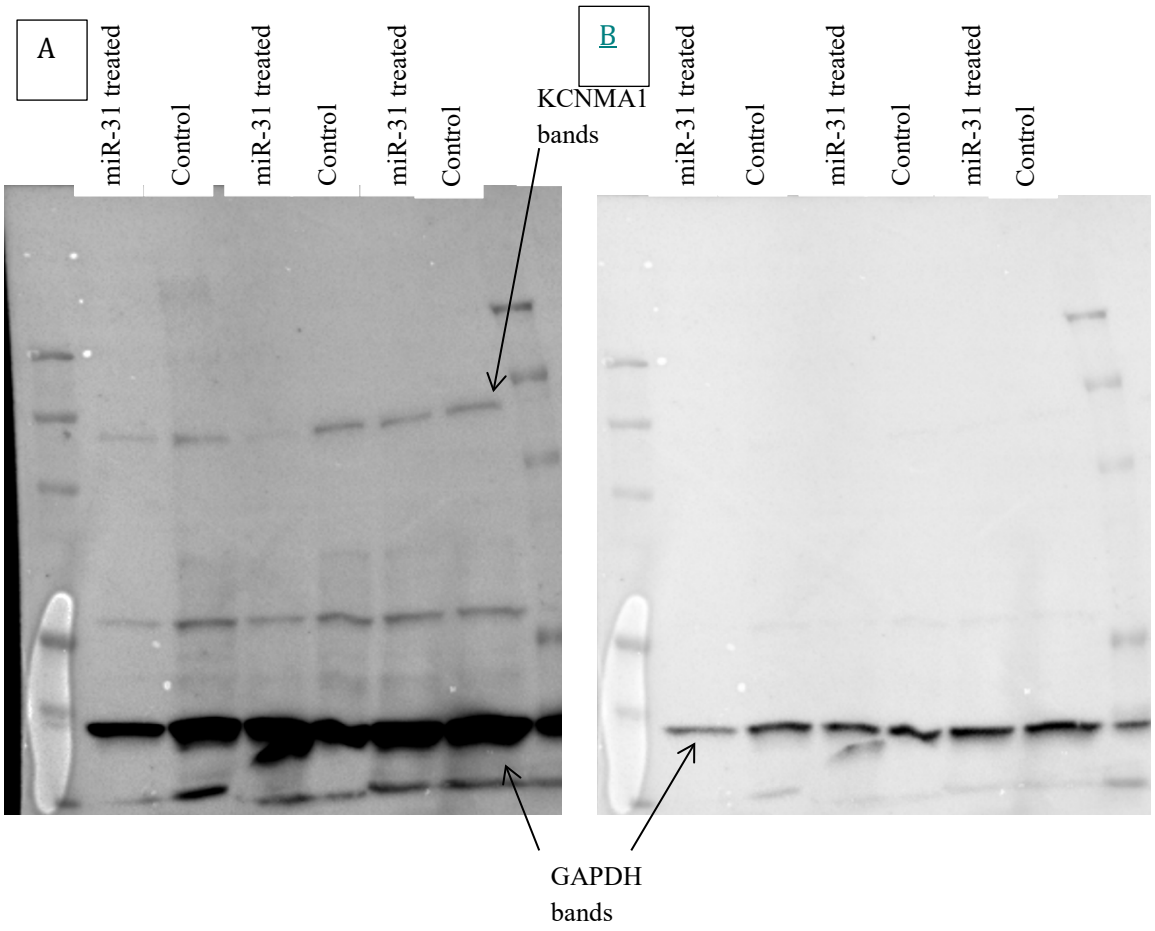

FIGURE D.2: ORIGINAL WESTERN BLOT SHOWING PROTEIN FROM A2780S TREATED WITH MIR-31 MIMIC OR CONTROL MICRORNA labelled for KCNMA1 (130 kD) and GAPDH (35 kD). Approximately 20µg of protein from each sample was denatured by heating to 70°C, electrophoresed on a polyacrylamide gel (Bio-Rad), transferred onto a PVDF membrane (Bio-Rad) using the Bio-Rad. The membrane was blocked with 5% BSA in TBS with Tween, incubated with primary antibody (1 in 500 KCNMA1) at 4°C overnight and then with secondary antibody (1:10000) for 1 hour at room temperature. The Blots were immersed in ECL solution and digitally imaged on a Bio-Rad Chemidoc system and analysed using ImageLab software.. The procedure was repeated with GAPDH primary antibody (1in 15000). Panel A shows the higher exposure used for KCNMA1 bands and panel B shows a lower exposure for GAPDH. KCNMA bands are not visible at this low exposure level.

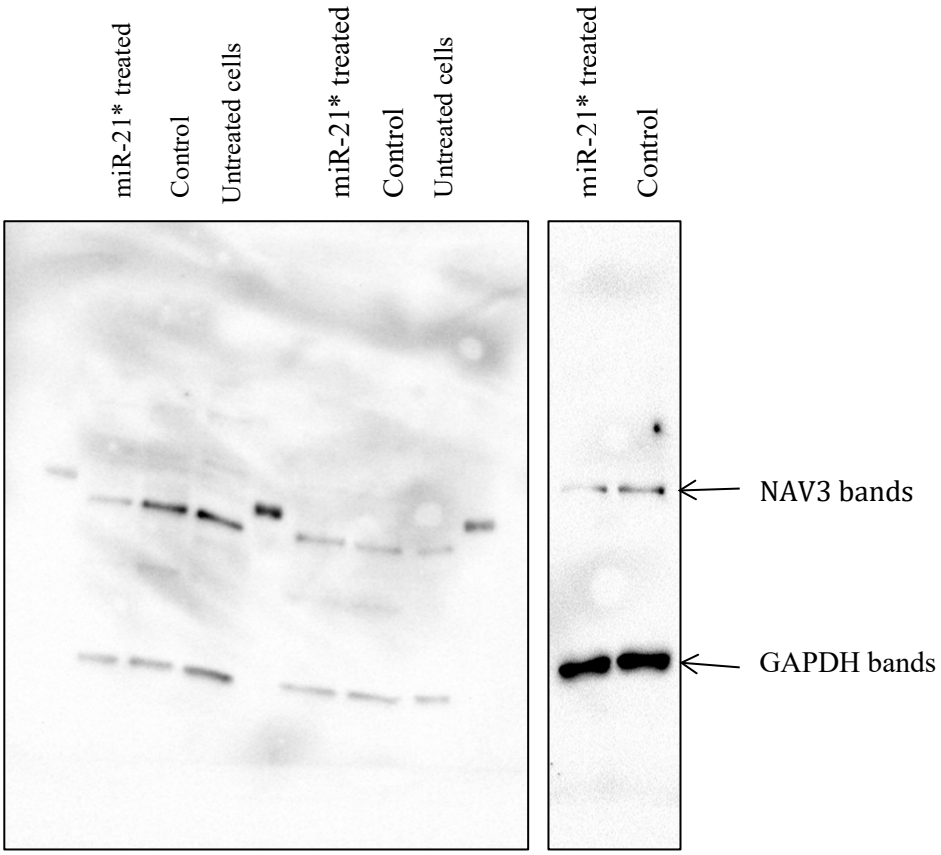

FIGURE D.3: ORIGINAL WESTERN BLOT SHOWING PROTEIN FROM A2780S TREATED WITH MIR-21* MIMIC OR CONTROL MICRORNA LABELLED FOR NAV3 AND GAPDH. Approximately 20µg of protein from each sample was denatured by heating to 100°C, electrophoresed on a polyacrylamide gel (Bio-Rad), transferred onto a PVDF membrane (Bio-Rad) using the Bio-Rad. The membrane was blocked with 5% BSA in TBS with Tween, incubated with NAV3 primary antibody (1in 1000) at 4°C overnight. The blot was then incubated with GAPDH primary antibody (1in 15000) overnight. The blot was incubated with secondary antibody (goat anti-rabbit HRP conjugate from Sigma, A9169) at 1:5000 concentration. The blot was imaged on a Bio-Rad Chemidoc system and analysed by ImageLab software from Bio-Rad.

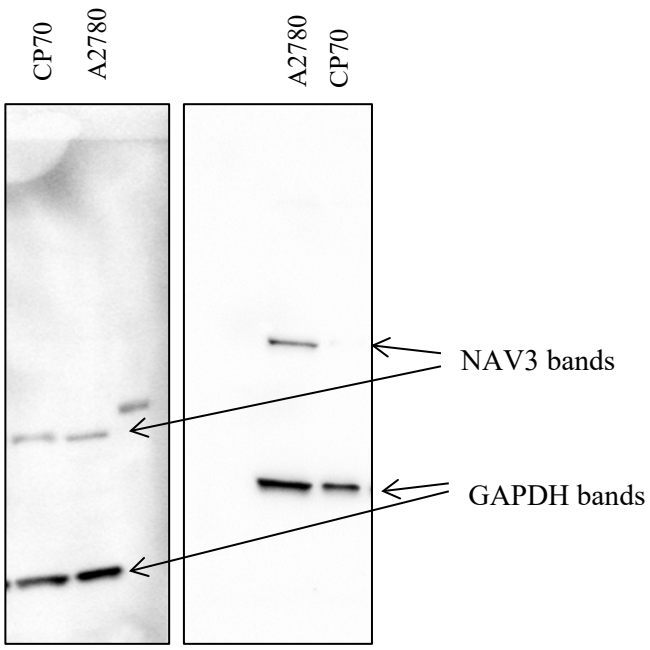

FIGURE D.4. ORIGINAL WESTERN BLOT SHOWING PROTEIN FROM A2780S AND CP70S LABELLED FOR NAV3 AND GAPDH. Approximately 20µg of protein from each sample was denatured by heating to 100°C, electrophoresed on a polyacrylamide gel (Bio-Rad), transferred onto a PVDF membrane (Bio-Rad) using the Bio-Rad. The membrane was blocked with 5% BSA in TBS with Tween, incubated with NAV3 primary antibody (1in 1000) at 4°C overnight. The blot was then incubated with GAPDH primary antibody (1in 15000) overnight. The blot was incubated with secondary antibody (goat anti-rabbit HRP conjugate from Sigma, A9169) at 1:5000 concentration. The blot was imaged on a Bio-Rad Chemidoc system and analysed by ImageLab software from Bio-Rad.

Appendix E

Publications from this thesis

1. Samuel, P., Pink, R. C., Brooks, S. A. and Carter, D. R. (2016) 'miRNAs and ovarian cancer: a miRiad of mechanisms to induce cisplatin drug resistance', *Expert Rev Anticancer Ther,* 16(1), pp. 57-70. DOI: 10.1586/14737140.2016.1121107

2. Samuel, P., Pink, R. C., Caley, D. P., Currie, J. M., Brooks, S. A. and Carter, D. R. (2015) 'Over-expression of miR-31 or loss of KCNMA1 leads to increased cisplatin resistance in ovarian cancer cells', *Tumour Biol,* 37(2), pp.2565-73. https://www.ncbi.nlm.nih.gov/pubmed/24244721Pink, R. C., Samuel, P., Massa, D., Caley, D. P., Brooks, S. A. and Carter, D. R. (2015) 'The passenger strand, miR-21-3p, plays a role in mediating cisplatin resistance in ovarian cancer cells', *Gynecol Oncol,* 137(1), pp. 143-51. DOI: https://doi.org/10.1016/j.ygyno.2014.12.042

3. Jacobs, L. A., Bewicke-Copley, F., Poolman, M. G., Pink, R. C., Mulcahy, L. A., Baker, I., Beaman, E. M., Brooks, T., Caley, D. P., Cowling, W., Currie, J. M., Horsburgh, J., Kenehan, L., Keyes, E., Leite, D., Massa, D., McDermott-Rouse, A., Samuel, P., Wood, H., Kadhim, M. and Carter, D. R. (2013) 'Meta-analysis using a novel database, miRStress, reveals miRNAs that are frequently associated with the radiation and hypoxia stress-responses', *PLoS One,* 8(11), pp. e80844. doi:10.1371/journal.pone.0080844. OPEN ACCESS

4. Bewicke-Copley, F., Samuel, P. and Carter, D. R. (2015) 'RNAi2015 - Ten years of RNAi Oxford', *J RNAi Gene Silencing,* 11, pp. 515-8. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4606920/. OPEN ACCESS