Can the Dual Inhibition of Extracellular Signal-Related Kinase (ERK) and Phosphoinositide-3 Kinase (PI3K) Increase Efficacy of Cisplatin in Endometrial and Ovarian Cancers?

A thesis submitted in partial fulfilment of the requirements for the degree of Masters in Science in Cellular and Molecular Biology

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ABSTRACT

Ovarian and endometrial cancers are two of the most common gynaecological malignancies in women. The chemotherapeutic drug cisplatin is a first-line therapy for both of these cancers, however despite the success of this treatment there are patients who suffer relapse. This is associated with acquired resistance of tumour cells to cisplatin and raises the need for an effective treatment that can re-sensitise the cancer to cisplatin. There is promise in targeting particular cellular growth and signalling pathways that contain over active proteins, as it is these that decide a cells fate (in terms of survival or death). This study investigates the inhibition of both the PI3K, Akt, mTOR pathway through phosphoinositide-3 kinase (PI3K) and the Ras, Raf, MEK, ERK pathway through extracellular signal-related kinase (ERK). The aim of the project is to re-sensitize cisplatin resistant cancerous cell lines Hec-1A (endometrial adenocarcinoma) and OVCAR-5 (ovarian adenocarcinoma) to cisplatin through PI3K and ERK inhibition. The dual inhibition therapy utilized inhibitors FR180204 and LY294002 who target ERK and PI3K respectively. This research utilized 3D cell culturing techniques, which provided an insight into the potential impact a tumour cells microenvironment may have on the treatment. The results of this research found no significant reduction in cellular metabolism or growth activity after treatment with FR180204, LY294002 and cisplatin (singly and in combination). Limited Western blotting of signalling proteins suggested the inhibitors had no significant effect upon the expression of ERK, Akt and cyclin B_2 proteins across all combination treatments in OVCAR-5 cells. The information provided in this research is capable of providing a platform for future studies investigating resistant cancers using targeted drug therapy and 3D cell culturing techniques.

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ABBREVIATIONS AND DEFINITIONS

AKT	protein kinase B
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BSA	bovine serum albumin
Cisplatin	cis-diamminedichloroplatinum (II)
CREB	CAMP responsive element binding protein
Crystal violet	(tris(4-(dimethylamino)phenyl)methylium chloride
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-related kinase
FBS	foetal bovine serum
FDA	Food and Drug Administration
FR180204	ERK inhibiting compound
FTI	farnesyltransferase inhibitor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanine triphosphate
Hec-1A	human endometrial adenocarcinoma cell line
HRP	horseradish peroxidise
JNK	c-Jun N-terminal kinase
LY294002	PI3K inhibiting compound
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase/ERK kinase
MPSC	micropapillary serous carcinoma
mTOR	mammalian

NER	nucleotide excision repair
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
OVCAR-5	human ovarian adenocarcinoma cell line
pAkt	phosphorylated Akt
PDES	prenyl-binding protein
PDK1	phosphoinositide-dependent kinase 1
pERK	phosphorylated ERK
PI3K	phosphoinositide-3-kinase
Pikk	Phosphatidylinositol 3-kinase-related kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3, 4, 5-triphosphate
PKC	protein kinase C
PNCA	proliferating cell nuclear antigen
poly-HEMA	poly-hydroxyethylmethacrylate
PTEN	phosphate and tensin homologue protein
PVDF	polyvinyl difluoride
rpm	revs per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	standard error of the mean
TBS-T	Tris-buffered saline and Tween20
TSC1	tuberous sclerosis 1
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

1.1 Overview

The prevalence of ovarian and endometrial cancer is increasing. It is estimated that worldwide more than 225,500 women are diagnosed with ovarian cancer each year and annually there are over 40,000 new cases of endometrial cancer (Mabuchi et al 2015, Tsoref et al 2014). The traditional treatment for these cancers includes a combination of cyto-reductive surgery and platinum-based chemotherapy. Cytotoxic drugs used in the chemotherapy of ovarian and endometrial cancers are relatively successful in eliminating cancerous cells. However, a number of patients who undergo this treatment relapse afterwards, indicating a resistance to this type of therapy and the need for more effective treatment options (Karaca et al 2013). Various ideas have been proposed as to what may be causing this phenomena but one holds particular promise. This is the over activity of particular proteins within cellular growth and signalling pathways that are potentially preventing cancers subsequent proliferation, growth and metastasis.

Cisplatin-induced DNA damage stimulates cellular growth and signalling pathways, which control the activation of apoptosis; or in the case of some tumour cells their survival. There is evidence that proteins such as extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase B (Akt) and protein kinase C (PKC) are thought to regulate signals influencing tumour survival or apoptosis. Studies have found that the occurrence of mutations within key oncogenes *KRAS*, *BRAF* and *PTEN* have resulted in the over-activity of proteins found within the Ras, Raf, MEK, ERK and PI3K, Akt, mTOR signalling cascades (Nakayama et al 2008, Wang et al 2000). The over expression of these proteins have been shown to increase the proliferation and metastasis of the tumour cells preventing cisplatin-induced apoptosis (Brozovic and Osmak 2007, Wang et al 2007).

In 2013, Schenk (2013) investigated a potential therapy combating acquired cisplatin resistance through ERK inhibition. This kinase was chosen, due to its

key role within the Ras, Raf, MEK, ERK pathway. MEK (mitogen-activated protein kinase), the kinase upstream from ERK, had shown promising results with respect to inhibition studies in the past. Thus, this success was thought to be applicable to ERK. Schenk (2013) used the ERK inhibitor FR180204 due to supporting evidence from a study by Ohori et al (2005). This study elucidated crystal structures of FR180204 and its target ERK indicating a strong bind between the two. Research by Schenk (2013) found both OVCAR-5 and Hec-1A cell lines to be cisplatin resistant. However, the targeted inhibition of ERK with FR180204 was neither effective alone, nor in combination with cisplatin. Limited western blot analysis indicated the OVCAR-5 cells had a low proportion of ERK proteins expressed, a potential reason for the inhibitors lack of response. It is thus plausible OVCAR-5 does not up-regulate ERK as a survival mechanism against cisplatin and its inhibition has no effect on diminishing resistance.

The proposed project will focus on developing an appropriate treatment strategy against cisplatin resistant gynaecological cancers using the same methodology and techniques as Schenk (2013). In addition, to the Ras, Raf, MEK, ERK pathway, the PI3K, Akt, mTOR pathway will be inhibited by LY294002 (a PI3K inhibitor). This was decided due to evidence of cross talk between both pathways as well as previous Western blot analysis by Schenk (2013) elucidating the over expression of Akt within OVCAR-5 cells (Engelman 2009). LY294002 (a PI3K inhibitor) was chosen as it has been widely used to elucidate the role of PI3K via inhibition. As well as this there are studies warranting its use, in particular with platinum based drugs, and within ovarian and endometrial cancer cultures (Liu et al 2015).

The following report is an assessment of FR180204 and LY294002 efficiency in preventing cell growth and proliferation within cisplatin-resistant ovarian and endometrial cancer cell lines.

1.2 Cancer

Cancer is defined as uncontrollable and rapid cell proliferation, and is a multitude of diseases that arise through a multi-step mutagenic process. Effected cells acquire a collection of properties induced by genetic alterations (Luo et al 2009, Xie et al 2014). Known as the "Hallmarks of Cancer" this collection includes self-sufficient growth signals, insensitivity to anti-growth signals, apoptosis evasion, sustained angiogenesis, tissue invasion, metastasis and limitless replicative potential (Hanahan and Weinberg 2000). Additionally, due to recent discoveries this hallmarks list now includes: evasion of immune destruction, tumour-promoting inflammation, genome instability and deregulation of cellular energetics (Berdasco and Esteller 2010, Hanahan and Weinberg 2011).

The limitless replicative potential of cancer cells defines the disease as a whole, making it one of the most fundamental cancer traits. The uncontrolled replication of cancer cells arises from specific mutations that allow the escape from numerous failsafe mechanisms including apoptosis (the bodies natural barrier to cancer) and DNA damage checkpoints. This enables the cells to continue proliferating, to propagate existing mutations and to acquire new ones (Hahn and Weinberg 2002, Luo et al 2003, Su et al 2015). This also encourages the transition of benign tumours to malignant tumours, capable of metastasis (the development of primary tumours spawning pioneer cells that are capable of invading adjacent tissues). These tumour cells travel to distant sites throughout the body and, if the environment is right, establish new metastatic colonies (Duan et al 2014, Hanahan and Weinberg 2000). It is said that metastasis causes approximately 90% of human cancer deaths (Hanahan and Weinberg 2000). What make cancers such intriguing, yet fatal, genetic diseases are the differences in molecular profile, histology, prognosis and chemo-sensitivity of each type. These factors can vary depending on the cancer's location, as well the subtype, proving the determination of cures to be difficult with numerous variables involved within a particular cancer's existence (Cheaib et al 2015).

The above highlights why cancer is one of the leading causes of death worldwide, with 8.2 million deaths and 14.1 million new cancer cases arising in 2012 alone (Organization 2014). The underlying cause behind cancer cell generation is an unstable genome and the occurrence of mutations within key cellular pathways (Rytelewski et al 2014). Hahn and Weinberg (2002) state that in humans at least 4-6 mutations are required in order for a cell to reach the state of cancer. With respect to the current worldwide situation, these requirements are being met and at a staggering pace, as it is approximated that 1 in 3 people die from cancer (Organization 2014).

Furthermore, the fight against cancer has been increasingly difficult due to the development of resistance to traditional chemotherapy. Cancer cells can acquire the ability to adapt and evade treatments and this has become one of the biggest medical research challenges to be overcome (Hanahan and Weinberg 2011).

1.3 Ovarian and Endometrial Cancers

Ovarian and endometrial cancers are two of the most common gynaecological malignancies, and these occur in the female reproductive system. The reproductive system is situated in the lower abdomen and consists of two ovaries connected by Fallopian tubes on either side of the uterus (or womb). The uterus is positioned above the cervix and vagina (Figure 1.1). Ovarian cancer originates in the ovaries; these are flat, nodular, oval structures that are similar in shape and size to almonds. Each ovary is suspended on either side of the uterus by peritoneal folds and ligaments, and is close to the end of the Fallopian tubes (Chen et al 2003). The ovaries role is to produce the female hormones; oestrogen and progesterone, as well as the ova (female reproductive cells) that travel through the Fallopian tubes into the uterus.

Endometrial cancer originates in the endometrial lining of the uterus. The uterus is located low in the pelvis and is joined to the vagina by the cervix. It is comparable in size and shape to an upside-down pear. The lining of the uterus is

called the endometrium; this is made up of several layers that include surface epithelium, blood vessels, tissue space and glands. The uterus is responsible for nurturing the fertilized ovum that develops into a foetus, via implantation into the endometrium. Ovarian cancer is thought to arise from the epithelial cells lining the surface of the ovaries while endometrial cancer arises from the cells lining the uterus (Network 2013).

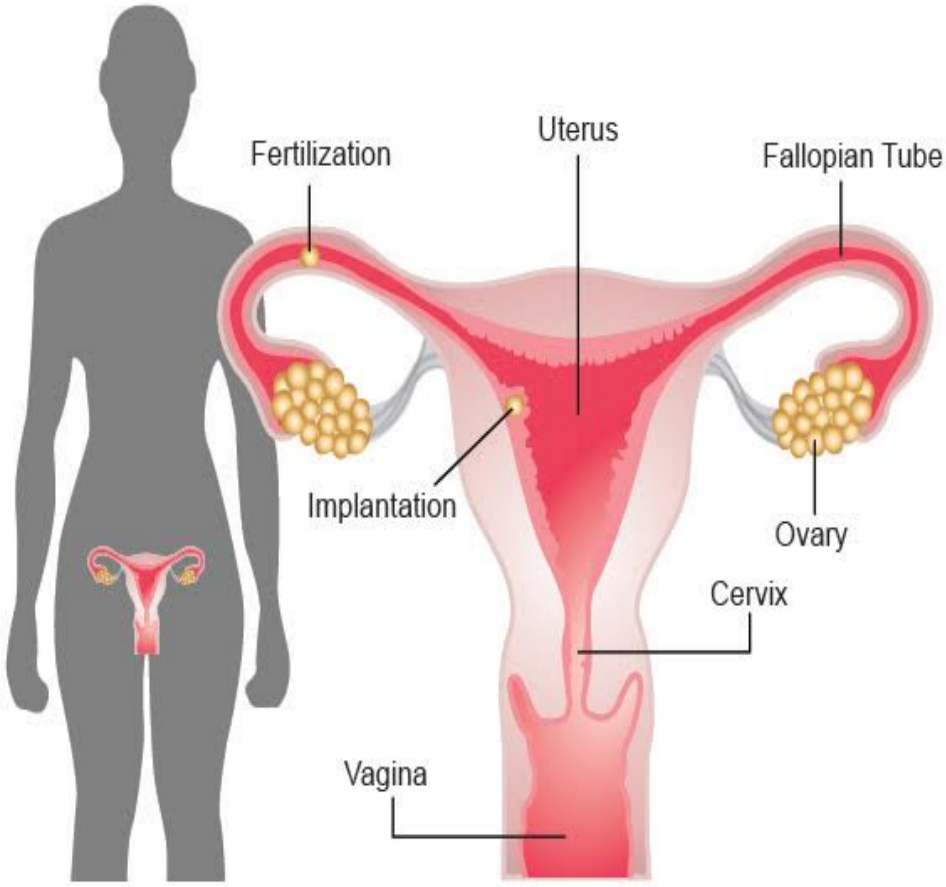


Figure 1.1 The female reproductive system. The majority of ovarian cancers arise within the ovaries, seen as the small almond shaped organs on either side of the uterus. The endometrium, or womb lining of the uterus is where endometrial cancers arise.

The pathology that underlies both ovarian and endometrial cancers lacks sufficient evidence, though there are several theories that have been proposed. The incessant ovulation theory proposed by Fathalla (1971) hypothesizes that the process of ovulation traumatizes the ovary's epithelium. The constant rupture and repair of this area results in increased cell division and entrapment of cells in the ovary stroma, leading to the development of tumours (Hawkridge 2014, Persson 2000). Several studies support this, showing that the shedding on the ovarian surface is a crucial stage in the sequence of events that progressively lead to the development of serous, endometrioid or clear cell cancers (Murdoch and McDonnel 2002, Vercellini et al 2011).

The gonadotrophin theory proposed by Persson (2000) hypothesizes high levels of gonadotrophin in some females' increases the risk of cancer. This increase could be attributed to the onset of menopause or premature ovarian failure and is detected by receptors for gonadotrophic hormones It is thought the receptors and gonadotrophic hormones stimulate growth of the epithelium and are linked to an increase in proliferation and a decrease in apoptotic rates within tumour cells (Konishi et al 1999, Murdoch and McDonnel 2002).

With regards to the pathology of endometrial cancer, it has been suggested that long standing proliferation of endometrial cells can lead to a gradual development of the endometrium into hyperplasia and then the development of atypical hyperplasia, thus potentially leading to cancer cell development (Persson 2000). Additionally, cancers like ovarian or endometrial originating in the female reproductive tract are then directly exposed to processes and factors such as ovulation and menstruation or high levels of estrogens, which inadvertently may be enhancing their development once the cancers have become established (Fortner et al 2015, Persson 2000).

Ninety percent of ovarian cancers are carcinomas, cancers that arise from the epithelium of the ovary (Bell 2005). However, ovarian cancer can also arise from sex-cord stromal cells or germ cells, albeit much less frequently (López et al 2013, Weiderpass and Labrèche 2012). Epithelial ovarian cancers can be broken

down into different sub-types on the basis of their morphological appearances. The most common is serous carcinomas that account for 50-70% of ovarian cancers followed by, endometrioid carcinomas (10-20%), clear-cell carcinomas (5-10%) and mucinous carcinomas (5-10%) (Kim et al 2012, Su et al 2015). Similarly epithelial endometrial cancers are also broken down into histological categories; beginning with the most common type - endometrioid (80%), followed by serous (10%) then clear-cell carcinomas (10%) (Amant et al 2005).

Ovarian and endometrial cancers can also be classified into two broad categories based primarily on their histology and tumour grade. The tumour grade of a cancer depends on the ratio of glandular or papillary structures versus solid tumour growth within an individual tumour. Grade 1 tumours have < 5% solid tumour growth, grade 2: 5-50% tumour growth and grade 3: > 50% solid tumour growth (Silverberg 2000). Both cancers are separated into Type 1 and Type 2.

Type 1 ovarian cancers are made up of clear-cell, endometrioid, mucinous and low-grade serous carcinomas. These tumours generally have a more positive prognosis, as their symptoms present sooner and thus are more likely to be diagnosed earlier (Cheaib et al 2015, Hsu et al 2004). They are slow growing tumours and tend to have *KRAS, BRAF, PTEN* and *PIK3CA* mutations (Thibault et al 2014). Type 2 ovarian cancers are primarily high-grade serous carcinomas. These are quick growing and tend to present their symptoms at a later stage. Thus, they are generally more aggressive and are advanced at the time of prognosis (Hsu et al 2004). These tend to have *BRCA1, BRCA2* and *p53* mutations (Thibault et al 2014).

Type 1-endometrial cancers are strongly linked to excessive estrogens, to obesity and to hormone receptor positivity. They account for 70-80% of endometrial cancers and are generally discovered at a lower grade thus have a more favourable prognosis (da Silva et al 2015, Network 2013). Type 2 endometrial cancers are primarily serous carcinomas and are more common in older, non-obese women. They account for 10-20% of endometrial cancers and are generally discovered at a higher grade. These cancers appear irrespective of

estrogens levels and have a tendency to recur (Dobrzycka et al 2009, Network 2013). Type 2 is thought to arise from atrophic endometrial tissues, frequently metastasise and thus has a poorer prognosis (Weiderpass and Labrèche 2012).

There are overlapping risk factors associated with ovarian and endometrial cancers. Primarily these are related to a westernized lifestyle and its associated factors, which include obesity, diabetes and lack of exercise (Weiderpass and Labrèche 2012). Olsen et al (2007) found that the risk of ovarian cancer increases as much as 30% in those who are obese compared to those with a healthy BMI (Body Mass Index). Similarly, it has been found that approximately 70-90% of Type I endometrial cancer patients are obese and incidence rates of this are 10 times higher in western cultures (Kaaks et al 2002, Pisani et al 1993). There is an increased risk of endometrial cancer associated with late menopause, possibly due to a greater lifetime exposure to estrogens (Fader et al 2009, Persson 2000). Whilst endometrial cancer does not have a strong familial influence, ovarian cancer does have a high risk of familial heredity, as having two or more first-degree relatives with the cancer significantly increases the risk of developing it. About 10% of ovarian cancer patients are likely to have had a genetic predisposition to the disease (Modan et al 2001).

Approximately 70% of ovarian cancer cases are diagnosed at an advanced stage, which causes the risk of recurrence after treatment to be as much as 60% (Mabuchi et al 2015, Spiliotis et al 2015). Due to this high rate of advanced tumours upon diagnosis, 50% of women do not survive more than 5 years (Coleman et al 2011). The key factor causing this is the difficulty to detect ovarian cancer at an early stage, due to its vague clinical symptoms (Su et al 2015). In comparison, around 80-85% of endometrial cancers are caught early, giving a 70-80% chance of survival after five years of diagnosis (Amant et al 2005, Eskander and Tewari 2014, Network 2013). Similarly to ovarian cancer, the majority of patients with aggressive, advanced endometrial tumours will have spread beyond the uterus within a year of diagnosis (Network 2013).

Although there is focus on finding therapies and treatments for this disease, the death rate of both cancers have not changed significantly over the last 50 years (Lau and Leung 2012, Vaughan et al 2011). An area of research that remains promising is the genomic instability of tumours and how mutations within the genomes may provide targets for cancer treatment.

1.4 Oncogenes

One of cancers fundamental characteristics is genomic instability and mutation; this distinguishes tumours cells from their normal counterparts. This lack of integrity in the cancer genome is often precipitated by mutations leading to compromised or incomplete DNA repair (Rytelewski et al 2014). This can lead to the development of oncogenes, which encode proteins controlling cell proliferation, apoptosis or both, causing the deregulation of growth and signalling pathways in resuming tumours (Hanahan and Weinberg 2000).

When an oncogene is affected by mutation, the encoded protein will change in a way that enhances or reduces its activity. For example, Ras oncogenes when mutated at codons 12, 13 or 61 remain in an active state, continuously transducing signals to downstream kinases, involved in continuous cell growth (Croce 2008, Mayr et al 2006, Nakayama et al 2008). Mutations of the *KRAS* gene have been involved in the development of numerous ovarian malignancies, with reports of *KRAS* mutations in 35% of ovarian invasive micropapillary serous carcinomas (MPSCs) (Dobrzycka et al 2009, Singer et al 2003). The mutation in codon 12 of *KRAS* has also been found in 10-30% of endometrial carcinomas (Dobrzycka et al 2009, Ryan et al 2005).

Another common mutation found in ovarian and endometrial carcinomas is *BRAF*. Mutations within the Raf oncogene are thought to be present in 40% of human melanomas, affecting the structures of the B-raf protein, resulting in constitutive signalling down the mitogen activated protein (MAP)-kinase pathway (Hanahan and Weinberg 2011, Nakayama et al 2008). Singer et al

(2003) found *BRAF* mutations in 33% of MPSCs, and 24% of ovarian endometrioid carcinomas. The point mutation V600E is thought to be the most common site of *BRAF* mutations in ovarian cancer (Nakayama et al 2008, Sieben et al 2004, Singer et al 2003). This mutation leads to changes within B-raf's kinase domain, constitutively activating the protein and uncontrollably stimulating MAPK cascades downstream, promoting G1/S transition of cell cycle regulation (Croce 2008, Davies et al 2002, Mayr et al 2006).

In a study by Hsu et al (2004) it was reported that either BRAF or *KRAS* mutations were present in approximately 60% of ovarian serous borderline and low-grade serous tumours. The Ras, Raf, MEK, ERK pathway was found to be active in 33% of ovarian carcinomas in and its activation depended on the mutational status of K-ras and B-raf. This highlights the influence these oncogenes have on the growth of tumour cells (Nakayama et al 2008). None of the tumours investigated by Singer et al (2003) contained both *KRAS* and *BRAF* mutations, which appears to be a common theme across a majority of the literature (Mayr et al 2006, Rajagopalan et al 2002).

PTEN (phosphatase and tensin homolog) is a tumour suppressor protein, which acts as a negative regulator for the PI3K, Akt, mTOR pathway (Eskander and Tewari 2014). When a mutation is present within the *PTEN* oncogene this results in the "silencing" of the protein and ultimately leads to an increase in phosphorylation and the activation of Akt (Eskander and Tewari 2014, Ying et al 2015). *PTEN* and other autophagy related genes are expressed in normal ovarian tissues, benign ovarian tumours and borderline tumours, but are down regulated in the cases of malignant ovarian cancers (Ying et al 2015). *PTEN* mutations have been found in 20% of ovarian endometrioid carcinomas (Cheaib et al 2015, Ryan et al 2005, Singer et al 2003). As well as this, 40-80% of Type 1 endometrial cancer cases have one or more somatic alterations affecting this pathway (Chitcholtan et al 2013, Eskander and Tewari 2014). The expression of *KRAS, BRAF* and *PTEN* oncogenes within these growth and signalling pathways of ovarian and endometrial cancer raises the potential for research to focus in this

area. There is an opportunity to inhibit the downstream actions of the pathways preventing the uncontrollable growth of tumours they cause.

1.5 Cisplatin and Acquired Resistance

Ovarian and endometrial cancers are typically treated with cytoreductive surgery and platinum-based chemotherapies (Mabuchi et al 2015, Spiliotis et al 2015). The major goal of chemotherapy is to commit tumour cells to apoptosis following exposure to the anti-tumour agents. One of these anti-tumours agents is the platinum based drug, cisplatin. Cisplatin or cis-diamminedichloroplatinum is a neutral, inorganic, square planar complex, which reacts with DNA via interaction with the nucleophilic N-7 sites of purine bases, forming DNA-protein and DNA-DNA intrastrand or interstrand crosslinks (Figure 1.2) (Siddik 2003).

An Italian chemist, Michele Peyrone in 1845, first described cisplatin and it was Rosenberg and Vancamp (1969) who first suggested cisplatin for clinical use in 1965, when examining the effect of electromagnetic fields on bacterial cell growth. It was reported that cisplatin inhibited bacterial cell division, which then led to the prediction that it would act similarly in cancer cells, and inhibit the proliferation of rapidly dividing cells (Rosenberg and Vancamp 1969). In 1968 this was shown to be the case, with platinum-based compounds shown to inhibiting sarcoma-180 growth in mice (Rosenberg and Vancamp 1969). The first patients were treated intravenously with cisplatin in 1971, via a short-term infusion in physiological saline and by 1978, the cytotoxic drug was FDA approved (Galluzzi et al 2012, Kelland 2007).

Cisplatin works as a chemotherapeutic agent through binding to DNA and causing damage via cross-linking. The DNA damage signals are transduced downstream resulting in G1 arrest in cell division and apoptosis (Siddik 2003). It has been suggested that the cisplatin adducts sequester transcription factors at the site of cross-linking preventing their participation in transcription and thus potentially serving as a trigger for transducing DNA damage signals (N'soukpoé-Kossi et al 2008, Rouette et al 2012, Siddik 2003).

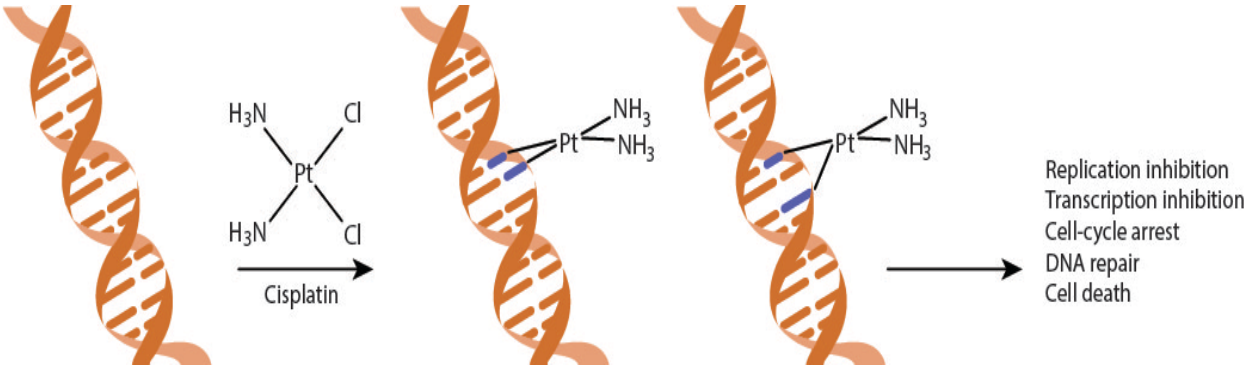


Figure 1.2 Cisplatin's mode of action. Cisplatin interacts with N-7 sites of purine bases (blue) in DNA (orange) to form DNA-protein and DNA-DNA interstrand and intrastrand cross-links. This leads to recognition of DNA damage by proteins within the cell and results in DNA damage signals being transduced.

Cisplatin has been shown to be one of the most efficacious agents against ovarian and endometrial cancers, with initial response rates in patients varying from 40-80%. However, as time has progressed other platinum-based drugs, including carboplatin and oxaliplatin have been favoured for use (Kelland 2007). This is due to cisplatin's toxicity, which can lead to side effects, including nephrotoxicity, neurotoxicity and to a lesser extent, cardiotoxicity (Basu and Krishnamurthy 2010, Yellepeddi et al 2011). Another reason cisplatin's popularity has declined is due to an increase in relapse frequency in cancer patients treated with the cytotoxic drug (Nakayama et al 2008). Thus second-generation platinum-based compounds were developed to reduce the side effects of cisplatin whilst retaining its anti-cancer properties (Galluzzi et al 2012, Parmar et al 2003). Fewer than 25% of women diagnosed with advanced ovarian cancer will show progression-free survival after 4 years, in spite of treatment. This has been attributed to the disease's aggressive nature given that diagnosis typically occurs at an advanced stage, but also due to the cancers reduction in sensitivity to cisplatin after treatment (Bamias et al 2012, Du and Ho 2001, Sherman-Baust et al 2003). Despite cisplatin's replacement with newer platinum-based drugs, these also have their limitations. An example of this is carboplatin who has

reduced toxic side effects in comparison to cisplatin, but does not cause a response in cisplatin resistant cancers (Parmar et al 2003). As such the 5-year survival rates of patients with ovarian and endometrial cancers can be less than 15%. This is attributed to a combination of the diseases' aggressiveness and the first line chemotherapies utilized lacking efficacy (Bamias et al 2012, Eskander and Tewari 2014, Nakayama et al 2008).

The efficacy of cisplatin depends on the cancer cells ability to detect and respond to DNA damage. Following cisplatin induced DNA damage; both pro-survival and pro-apoptotic signals are activated simultaneously. The relative intensity and/or duration of each are integrated downstream to determine the final fate of the cell. This is very important in terms of resistance. Cells will undergo apoptosis only when DNA repair is incomplete, as would be the case when damage (caused by the cisplatin) is extensive (Singer et al 2003). DNA damage via chemotherapeutic agents is used to exploit DNA fidelity in an effort to preferentially push tumour cells towards to apoptosis. However, tumour cells with intact DNA repair pathways are less sensitive to this strategy and are capable of acquiring resistance by outgrowth of clonal populations (Rytelewski et al 2014).

There are many theories behind what may allow cells to survive and acquire resistance. Mechanisms underlying tumour resistance to cisplatin are suggested to be multi-factorial, these include: decreased drug transport and accumulation, increased cellular detoxification due to increased glutathione levels, changes in DNA repair involving increased nucleotide excision repair (NER) and/or loss of mismatch repair, increased tolerance of DNA adducts and alterations in the apoptotic cell death pathway (Du and Ho 2001, Eskander and Tewari 2014, Holford et al 2000, Parker et al 1991, Siddik 2003).

In response to therapy, cancer cells have the potential to reduce their dependence on a particular hallmark capability, becoming more dependent on another (Hanahan and Weinberg 2000). Resistance mechanisms arise as a consequence of intracellular changes that either prevents cisplatin from

interacting with DNA, interfere with DNA damage signals from activating the apoptotic machinery or both (Siddik 2003). The cancer cells are able to survive DNA damage and this may occur via multiple overactive growth and signalling pathways, promoting proliferation, thus preventing apoptosis from occurring (Eastman and Schulte 1988, Siddik 2003). Several signalling pathways including PI3K, ERK, JNK and p38 MAPK can regulate cisplatin-induced apoptosis, thus these could be potential targets with regards to new therapies that combine chemotherapy and inhibitory agents (Basu and Krishnamurthy 2010, Galluzzi et al 2012, Nakayama et al 2008). Strategies to sensitise cancer cells to platinum agents are of high clinical importance (Rytelewski et al 2014). Combining cisplatin with molecular targeted therapy has the potential to lower the dosage of cisplatin currently employed, thus helping in alleviating its side effects as well as overcoming platinum resistance in ovarian and endometrial cancers (Basu and Krishnamurthy 2010, Karaca et al 2013).

1.6 The Ras, Raf, MEK, ERK signalling pathway

The transmission of extracellular signals into the nucleus is mediated by a network of proteins and pathways (Seger and Krebs 1995). One of these pathways is the evolutionarily conserved Ras, Raf, MEK, ERK signalling cascade (Samatar and Poulikakos 2014). Under physiological conditions, this pathway integrates extracellular signals with the transcription of genes involved in cell proliferation, differentiation and survival (Ohori et al 2005, Siddik 2003). In tumour cells, proteins within the pathway can be mutated causing deregulation or over-expression, leading to progression in tumour growth and signalling (Singer et al 2003).

The Ras, Raf, MEK, ERK signalling pathway is initiated when an extracellular signalling molecule such as epidermal growth factor (EGF) binds to the epidermal growth factor receptor (EGFR) on the cell surface (Figure 1.3). This coupling complex activates a guanine nucleotide exchange factor (GEF) to change

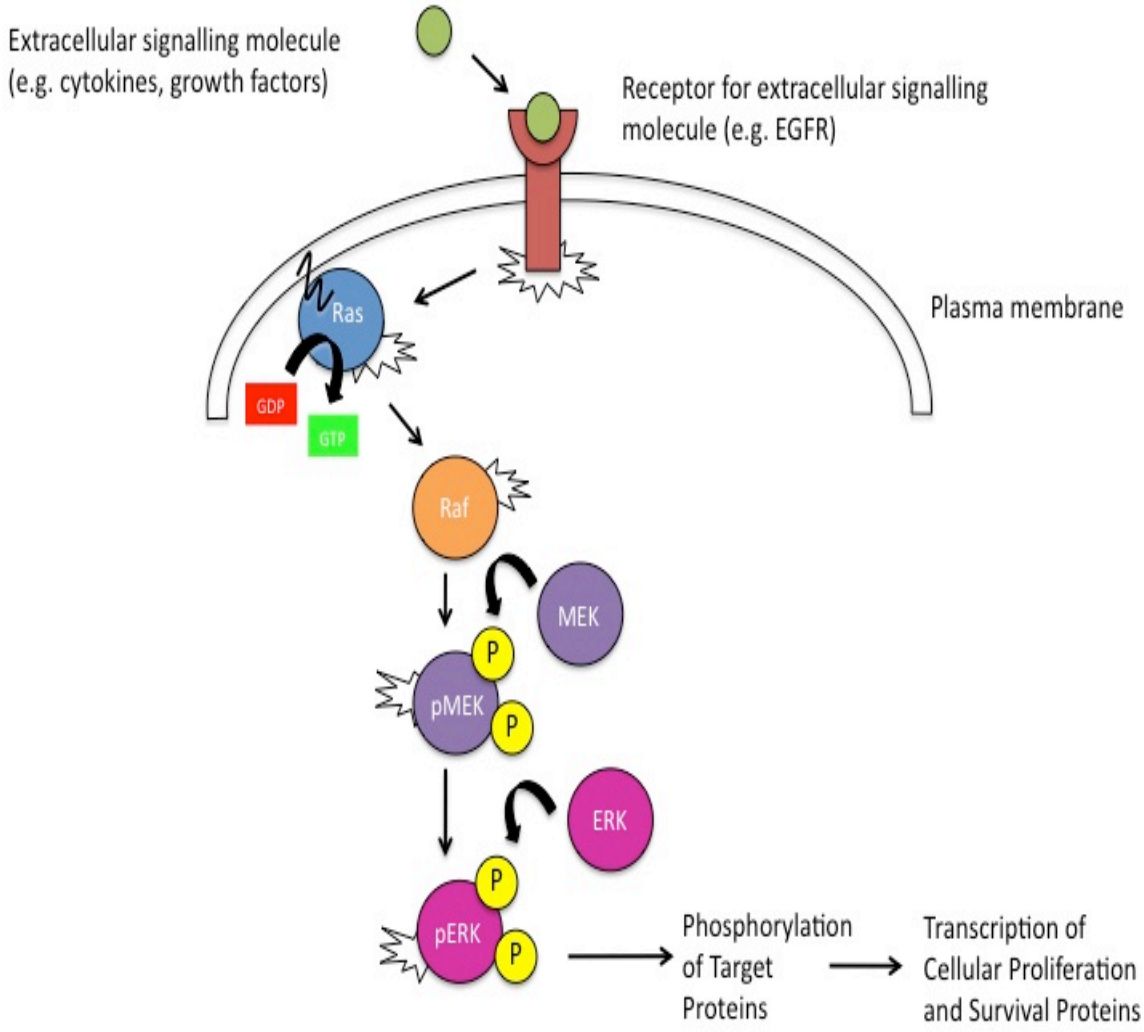


Figure 1.3 Overview of the Ras, Raf, MEK, ERK pathway. An extracellular molecule (green) binds to its respective receptor (red), which spans the plasma membrane. The active receptor stimulates Ras' (blue) exchange of GDP for GTP and once active, recruits Raf (orange) to the plasma membrane. Raf is activated by Ras and then phosphorylates MEK (purple) at a serine or threonine residue, which activates it. Phosphorylated MEK (pMEK) then goes on to activate the final protein in the part of the pathway that is shown, ERK (pink) again via serine/threonine residues. ERK has a variety of downstream targets, which are involved in cellular proliferation and survival. Image adapted from Schenk (2013).

the conformation of a GTPase such as Ras, enabling the exchange of GDP (guanosine diphosphate) for GTP (guanosine triphosphate). Ras (a small GTPase) has three isoforms; K-Ras, N-Ras and H-Ras (Baines et al 2011). The best understood GEF for Ras is the SOS (Son of Sevenless) protein. Fusing Ras' CAAX motif to the C-terminus of SOS renders Ras constitutively active (Burns et al 2014). Mammalian Ras proteins in active state bind and activate several other effectors in addition to Raf kinases, each shown to play a role in Ras-driven cancers. Once active, Ras recruits Raf to the plasma membrane for its dimerisation and activation. Raf is a serine/threonine kinase, meaning it can only phosphorylate another protein or substrate on either a serine or a threonine residue. There are three isoforms of the Raf protein A-Raf, B-raf and C-raf. Once Raf is activated, it seeks its main target, another serine/threonine kinase MEK (mitogen-activated protein kinase kinase). Active MEK goes on to phosphorylate the final protein in the pathway ERK (extracellular signal-related kinase). ERK is yet another serine/threonine kinase, and is a key regulatory protein within the cascade, having over 160 downstream targets. These targets include transcription factors and proteins involved in cell cycle regulation, cell growth and differentiation (McCubrey et al 2007, Nakayama et al 2008, Ohori et al 2005).

It is well known that components of this pathway are frequently mutated in tumour cells (Roberts and Der 2007). As mentioned prior, the proteins most commonly over-expressed are Ras and Raf, in particular the isoforms B-raf and K-ras. Ras signalling is frequently deregulated in a third of human cancers. This does not come as a surprise as *KRAS* mutations can prevent the essential switch of GTP to GDP in the Ras protein, evidently leading to the constitutive hyper-activation of Ras and consequently of those downstream (Turacli et al 2015). Whilst Raf signalling in particular signals involving mutant B-raf proteins, increase the activity of downstream proteins and are amongst the most common in human cancers (Davies et al 2002).

These mutations both have influences upon the expression of ERK who controls a wide range of cellular processes that remain under strict regulation. There is

evidence showing that the over expression of ERK occurs in cancers is due to upstream protein deregulation and this could influence cisplatin-acquired resistance (Zhang and Gu 2014). Losing the regulation of key proteins like ERK can give tumour cells the ability to evade cisplatin-induced apoptosis through constitutively active anti-apoptotic signals and up-regulated cellular proliferation (Hsu et al 2004, Karaca et al 2013, Ohori et al 2005, Turacli et al 2015).

This above raises the possibility of using the Raf, Ras, MEK, ERK signalling cascade as a target for cancer treatment, in particular inhibiting its activity to re-sensitise ovarian and endometrial cells to cisplatin (Karaca et al 2013, Ohori et al 2005).

1.7 Ras, Raf, MEK, ERK pathway inhibitors

There are a variety of inhibitors that target proteins within the Ras, Raf, MEK, ERK signalling cascade. Some of these have been around for a long time, whilst others are newly developed or are at various junctures of clinical trials.

As a first step in targeting this pathway, is inhibiting Ras. To date there have been few reports of an effective inhibitor. This task has been challenging due to Ras' substrate binding interface being structurally featureless. This limits targeting the protein, as it cannot be specifically inhibited, as there are few unique structural target sites due to similarities to other proteins. Additionally, Ras' affinity for GTP is much higher than that of other kinases for ATP, thus attempts to interfere with its nucleotide-binding pocket have proven difficult (Baines et al 2011, McCormick 2016, Young et al 2009). Nevertheless, there have been several strategies that have been developed to overcome these difficulties. The first option is to prevent Ras' attachment to the plasma membrane and its subsequent activation. Zimmermann et al (2013) achieved this via blocking the farnesylation of Ras through farnesyltransferase inhibitors (FTI). Ras undergoes several post-translational modifications that facilitate its attachment to the inner surface of the plasma membrane. The first modification is adding a farnesyl

isoprenoid moiety catalysed by farnesyltransferase. Inhibiting this enzyme prevents Ras from maturing into its active form thus inhibiting the entire pathway (Li et al 2012). Tipifarnib is an example of an FTI. It causes an accumulation of cells in G1/M phase of the cell cycle, and induces apoptosis in a variety of tumour cell lines irrespective of Ras mutation status (Andreopoulou et al 2013). Leshchiner et al (2014) found that SAH-SOS1 peptides can target the SOS-1 binding pocket on K-ras and block nucleotide association, impairing *KRAS* driven cancers cells. This strategy is still being explored, Burns et al (2014) and Evelyn et al (2014) found this method to be successful in the inhibition of this protein as well as the down regulation of the following pathway. All of the above provide an opportunity for the development of a specific Ras inhibitor to target cancer treatment but as of now development and trials continue.

Raf is the second protein within the signalling cascade. This protein can be inhibited by Sorafenib, which was the first Raf inhibitor to have gained regulatory approval, despite its weak specificity to B-raf (Eisen et al 2006). Alternatively another inhibitor that has been approved is Vemurafenib (Cox and Der 2012). This inhibitor is more specific to B-raf and has been used against melanomas, and colon and thyroid cancers as a main chemotherapeutic drug. It does however have side effects (Bollag et al 2010, Heakal et al 2011). Dabrafenib is a successful inhibitor of Raf. In 2013 it went through phase II clinical trials and now is the 2nd FDA approved B-raf inhibitor (Ascierto et al 2013, Fiskus and Mitsiades 2016). Similarly to Ras inhibitors there is limited knowledge and research surrounding their inhibition, as well as this cancer cells treated with B-Raf inhibitors are developing resistance. This remains an issue that is continuing to be investigated (Shi et al 2012, Solit and Rosen 2014).

MEK has been one of the more attractive proteins within the pathway to target drugs against as it exhibits a low prevalence of mutations in comparison to Ras and Raf (Marks et al 2008). One of the first MEK-targeted agents to enter clinical trials was the drug CI-1040. Though its' Phase I trials were halted due to low bioavailability and Phase II trials were stopped due to its failure to show anti-tumour activity (LoRusso et al 2005). However, this drug was then modified to

create PDO325901, which has improved solubility but also it had adverse side effects, which caused its trials to stop (Brown et al 2007). A 2nd generation MEK inhibitor is Selumetinib. This agent has been shown to be highly selective, and patients have had good toleration in Phase I studies, although, there are issues with dosage and it can have severe side effects (Archibald et al 2016, Temraz et al 2015). Alternatively in 2013 the MEK inhibitor, Trametinib became the first to be approved by the United States FDA for treatment on metastatic melanoma patients (Yao et al 2015). This drug was utilised by Greger et al (2012) in combination with a B-raf inhibitor and together they effectively suppressed gene expression and cell proliferation in melanoma cells. This drug binds unphosphorylated MEK, thus preventing its activation and this remains a promising drug for the treatment of cancers (Gilmartin et al 2011). In addition to this there are other drugs including PDO98059 and UD126 within preclinical trials (Chappell et al 2011). Thus far in cancer treatment MEK inhibitors have been popular, what makes them not as desirable for the inhibition of the Ras, Raf, MEK, ERK pathway is their dose-limiting properties that lead to severe side effects within patients (Marks et al 2008).

The final protein in the pathway to target is ERK. This protein, like MEK, is another good target due to its effects on numerous proteins downstream that are important with respect to cancer. There has however been limited progress in development of selective inhibitors for ERK. That said, interest has recently intensified, because of the rise of Raf and MEK inhibitor resistance.

Ohori et al (2005) discovered the first selective inhibitor for ERK 1/2. FR180204 acts as an ATP-competitive inhibitor blocking access to the ATP binding site, which is required for phosphate transfer, when ERK phosphorylates a target protein (Figure 1.4.). The inhibitor binds in a novel way to the unique residues of ERK1/2 but does not show selectivity between the two, which is desirable. It is cell permeable and has been shown to inhibit the ERK signalling cascade (Dirican et al 2015, Ohori et al 2005).

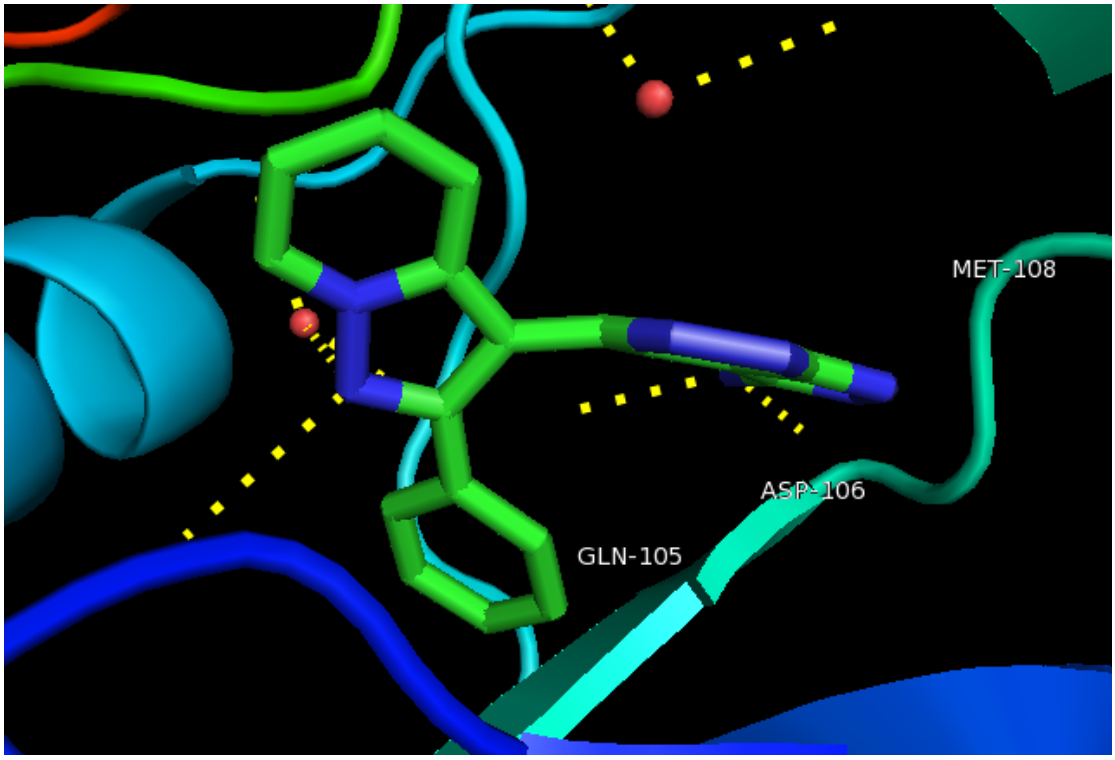


Figure 1.4 ERK inhibiting compound FR180204 and its' interactions with ERK's ATP binding pocket. Hydrogen bonds are observed between FR180204 and ERK, at residues Met 108, Gln 105 and Asp 106. Crystallographic image from Ohori et al (2005), adapted in PyMOL.

Ohori et al (2005) suggest that therapeutic applications such as the anti-cancer activity of MEK inhibitors may also be applicable to FR180204. There is evidence suggesting that it could inhibit the Ras, Raf, MEK, ERK pathway and aid in cancer treatment. FR180204 was used in studies by Doghman and Lalli (2012) who showed that the drug could act synergistically with a PI3K/mTOR inhibitor, to achieve efficient inhibition of adrenocortical cancer cell proliferation. This is a common suggestion in studies, which state that dual inhibition of signalling pathways (particularly those that are overactive) within tumour cells may be a more effective form of treatment against cancer growth (Chappell et al 2011, Dirican et al 2015). This is applicable in cases of acquired-cisplatin resistance where by the inhibition of Ras, Raf, MEK, ERK signalling cascade as well as the likes of the PI3K, Akt, mTOR pathway is desirable. This is because cross talk

between the two pathways could limit efficacy of single drug treatment thus eliminating both could be a more effective treatment strategy (Hanahan and Weinberg 2000, Mendoza et al 2011).

In addition to FR180204, there are several ERK inhibitors in development; these include SCH772984 (which is an ATP competitive inhibitor) that is derived from small molecules that bind specifically to unphosphorylated ERK2 (Morris et al 2013). As well as this there is BVD-523 and RG7842, which have recently entered clinical trials but are not yet available (Samatar and Poulikakos 2014).

1.8 The PI3K, Akt, mTOR signalling pathway

Alongside the Ras, Raf, MEK, ERK pathway there are alternate signalling cascades, thought to be involved in tumour progression and acquired cisplatin resistance. One of these pathways is the PI3K, Akt, mTOR signalling cascade. This pathway is promiscuous and works in parallel with the Ras, Raf, MEK, ERK cascade contributing to the regulation of cell cycle progression and cellular growth (Cheaib et al 2015, Luo et al 2003). It is also one the most frequently deregulated signalling cascades in human malignancies (Dienstmann et al 2014).

The PI3K, Akt, mTOR signalling pathway, is mediated by a wide range of cellular signal communicating molecules including hormones (such as insulin), growth factors, nutrients (such as amino acids and glucose) and cellular stress (Kang et al 2012). It controls many intracellular processes including: proliferation, differentiation and anti-apoptotic signalling and the pathway comprises three key proteins PI3K (phosphatidylinositol kinase), Akt (protein kinase B) and mTOR (mammalian target of rapamycin) (Figure 1.5).

The PI3K pathway is initiated through a growth factor or ligand binding to a receptor tyrosine kinase (RTK)(Eskander and Tewari 2014). This receptor in turn stimulates a GTPase i.e. Ras, which stimulates the activation of PI3K. PI3K can be grouped into three different classes on the basis of properties such as primary structure, regulation and function (Chang et al 2015). Within PI3K class

I there are 4 different isoforms (Wu and Hu 2012). Each protein within class I consists of regulatory (p85) and catalytic (p110α) subunits, producing a heterodimer (Cantley 2002, Cheaib et al 2015).

Direct binding of the p110α subunit to Ras initiates the activation of PI3K. p85 binds directly to phosphotyrosine residues on a RTK, which relieves intermolecular inhibition of the p110α subunit by p85. PI3K is then localised to the plasma membrane where its substrate phosphatidylinositol-4,5-biphosphate resides (Duan et al 2014). Here the conversion of phosphatidylinosital-4,5-bisphosphate (PIP2) to phosphatidyinositol (3, 4, 5)-triphosphate (PIP3) begins (Cantley 2002).

Once PIP3 accumulates at the plasma membrane, PI3K recruits a subset of signalling proteins with pleckstrin homology (PH) domains to the membrane, these include PDK1 (phosphoinositide-dependent kinase 1) and Akt (Chang et al 2015). Akt then sub-localises from the plasma membrane to sub-cellular compartments, where it phosphorylates substrates to exert distinct functions (Duan et al 2014).

Akt belongs to the serine/threonine kinase family and is composed of three structurally similar isoforms; Akt1, Akt2 and Akt3, each of which has a different role (Arboleda et al 2003, Eskander and Tewari 2014). Each isoform consists of three domains, an N-terminal PH domain, a central catalytic domain and a C-terminal extension with a hydrophobic motif (Chang et al 2015). Akt's plethora of downstream effectors are involved in a variety of cellular processes that include glucose metabolism, cell migration, transcription, proliferation and apoptosis (Altomare et al 2004). In addition, Akt is a well-established survival factor and works by exerting anti-apoptotic effects through the prevention of cytochrome c release and additionally inactivating pro-apoptotic factors such as BAD, pro-caspase-9, GSK3, p27 and p70S6K (Altomare et al 2004, Hu et al 2002). Akt promotes the transcription of anti-apoptotic and proliferative genes through the regulation of p53, NF- κB and CREB (Cheaib et al 2015, Siddik 2003).

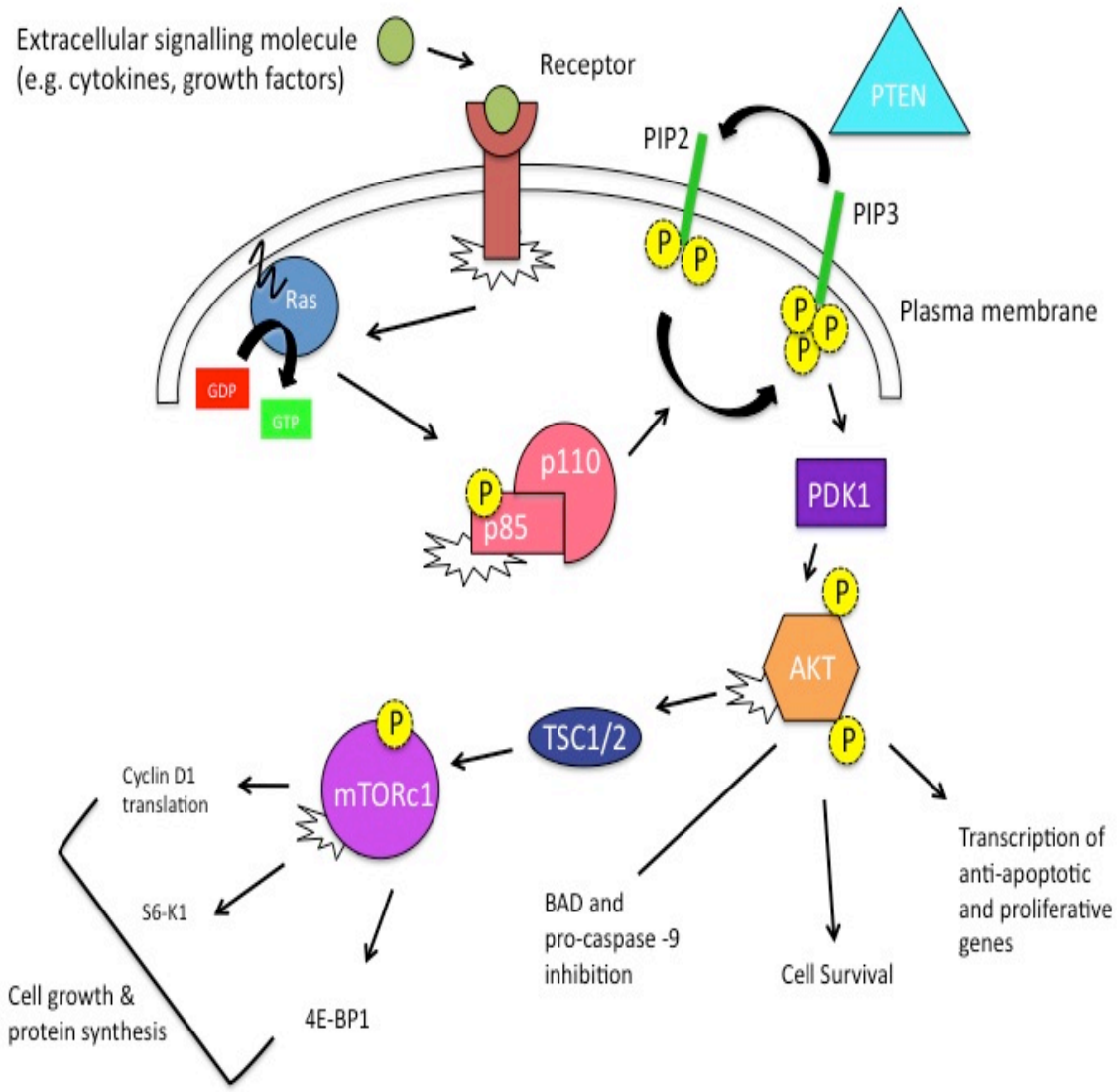


Figure 1.5 Overview of the PI3K, Akt, mTOR signalling cascade. An extracellular molecule (green) binds to its respective receptor (red) that spans the length of the plasma membrane. The active receptor stimulates Ras' (blue) exchange of GDP for GTP and once active Ras binds the p110 subunit of PI3K (pink) to initiate PI3K activation. PI3K controls the conversion of PIP2 to PIP3. PTEN (aqua) can revert the activity of PI3K, in turn inhibiting the pathways downstream actions. PI3K facilitates the activation of PDK1 (violet), which then activates Akt via phosphorylation (orange). Akt has a number of downstream effectors itself including the activation of TSC1 (navy). TSC1 activates mTORC1 (magenta), which goes on to initiate a plethora of downstream targets involved in cell growth, migration and survival.

It's involvement in various cellular processes means its regulation is of key importance. This regulation is controlled by the phospholipid phosphatase PTEN. PTEN serves at the molecular level to counteract the function of PI3K thus deactivating Akt. This means it is a mediator of agonist-induced apoptosis, achieving this via up-regulation of apoptotic machinery and down-regulation of anti-apoptotic proteins (Chitcholtan et al 2013, Hanahan and Weinberg 2000).

One of Akt's downstream effects, cell growth is for the most part controlled by its effector mTOR (Duan et al 2014). mTOR is a member of the phosphatidylinositol 3-kinase-related kinase (Pikk) family of proteins and is involved in an array of cellular processes. It exists as two functional complexes called mTORC1 (complex 1) and mTORC2 (complex 2), which localise to different sub-cellular compartments (Chang et al 2015, Wu and Hu 2012). mTOR is activated indirectly by Akt through the inhibition of TSC2 (tuberous sclerosis complex 2), although it is unknown whether this process alone is critical or sufficient for the full activation of mTOR. It functions by integrating signals from upstream pathways to up-regulate the translation of genes involved in angiogenesis and cell-cycle progression, including VEGF, cyclin D1, and c-myc as well as regulating cell growth, proliferation, motility, survival, protein synthesis, autophagy and transcription. In addition to motor's downstream effects and potentials, it also exerts positive feedback activity upon Akt (Cheaib et al 2015).

There is accumulating evidence showing that this pathway is involved in tumour growth, proliferation, metastasis and cisplatin resistance (Duan et al 2014, Mabuchi et al 2015). Arboleda et al (2003) found an over-expression of Akt2 with *in vitro* experiments on human ovarian cancer cells. This demonstrated that increased cancer cell invasion and metastasis was associated with over-expression of Akt2 and the PI3K pathway. The cause behind Akt over expression can be linked to *PI3KCA* mutations commonly found in ovarian and endometrial tumour cells (Bjornsti and Houghton 2004, Hanrahan et al 2012). This gene encodes the p100α subunit of PI3K and is found in more than 30% of solid tumours (Wu and Hu 2012). It is thought this mutation causes PI3K to be constitutively active thus influencing the activation of its downstream effectors

including Akt. In addition, *PTEN* mutations are commonly found in ovarian and endometrial cancers and these mutations can also lead to uncontrollable Akt expression (Hanahan and Weinberg 2000). This is due to the mutant PTEN protein lacking the ability to bind PI3K and regulate its activity thus Akt becomes hyper-activated (Bjornsti and Houghton 2004, Chitcholtan et al 2013).

Akt2 over expression promotes survival through BAD (Bcl-2 associated death promoter), which inactivates apoptosis (Hu et al 2002). Ovarian cancer cell lines with constitutively active Akt1 or Akt2 have been found to be highly resistant to paclitaxel (a platinum based drug) (Altomare et al 2004). Other studies have demonstrated that Akt directly phosphorylates XIAP (X-linked inhibitor of apoptosis protein), leading to inhibition of cisplatin-induced ubiquitation and degradation. This protects tumour cells from drug-induced apoptosis. Furthermore, the presence of Akt has been shown in tumour cells throughout endometrial cancer progression, this suggests Akt may have a role in chemo-protection making both this pathway and protein attractive targets (Basu and Krishnamurthy 2010, Gagnon et al 2004, Yang et al 2004).

Girouard et al (2013) showed that simultaneous down regulation of Akt1 and Akt2 restored cisplatin-induced apoptosis in resistant endometrial cells. This demonstrates that the inhibition of this pathway could be used in combination with chemotherapeutic drugs like cisplatin to restore the platinum-sensitivity to tumour cells (Altomare et al 2004, Cheaib et al 2015, Kang et al 2012, Luo et al 2003).

1.9 PI3K, Akt, mTOR pathway inhibitors

It has been suggested that in order to inhibit a particular protein within a signalling pathway, and at the same time illicit a strong tumour response or initiate tumour regression, combinatory therapy is advantageous (Hanrahan et al 2012, Mabuchi et al 2015). Such dual inhibition therapy is becoming an increasingly popular approach when investigating growth and signalling pathways. This is because single inhibitors have limited efficacy due to potential

feedback loops that can reduce any inhibitory effects, therefore targeting several pathways is an effective option (Chang et al 2015, Engelman 2009). Combination therapies are needed as they both enhance initial response and reduce subsequent onset of drug resistance (Cox and Der 2012). This suggests that inhibiting the Ras, Raf, MEK, ERK pathway and the PI3K, Akt, mTOR pathway to reduce their potential over-activity within ovarian and endometrial cancers may be an effective approach. As previously discussed ERK is an attractive target for Ras pathway inhibition leaving the question of what protein is the most appropriate to target in the PI3K pathway.

The first target is PI3K, and this has been suggested to be the most direct approach for PI3K pathway inhibition (Hu et al 2002). Two of the first PI3K prototype inhibitors developed were LY294002 and wortmannin. Both of these target the catalytic site of the p110 α subunit and have become invaluable tools in elucidating the roles of the PI3K protein (Walker et al 2000, Wu and Hu 2012). Wortmannin is a microbial product and a potent irreversible inhibitor of PI3K but is difficult to work with due to problems with its stability, solubility and toxic side effects (Arcaro and Wymann 1993, Chang et al 2015, Dan et al 2009, Powis et al 1994, Yaguchi et al 2006). The first study involving wortmannin was shown to inhibit phagocytosis induced respiratory bursts, and it was elucidated this was due to specific PI3K inhibition (Baggiolini et al 1987). Recently a newer compound has been developed based on the structure of wortmannin, this is called ZSTK474. It has a 20 fold greater activity and has been showing efficacy within clinical trials (Barollo et al 2014, Kong and Yamori 2007, Yaguchi et al 2006).

LY294002 was the first known synthetic inhibitor of PI3K, with a structure that was based on quercetin (a naturally occurring bioflavanoid). LY294002 has a much lower potency than wortmannin but its stability is far superior as well as reversible, allowing it to become more widely used in studies on PI3K (Guo et al 2014, Wu and Hu 2012). LY294002 acts as a competitive and reversible inhibitor at PI3K's ATP binding site and selectively inhibits the PI3K-ATP nexus (Badinloo and Esmaeili-Mahani 2014, Guo et al 2014). Another specific-PI3K inhibitor is

NVP-BKM120, within preclinical studies; this agent has been shown to suppress proliferation and induces apoptosis.

With respect to its effects on cancer cells, LY294002 has featured in many studies. Hu et al (2002) demonstrate that LY294002 has the ability to halt tumour growth *in vivo* in a mouse ovarian cancer model. Altomare et al (2004) used LY294002 alongside paclitaxel within *in vitro* and *in vivo* ovarian cancer. They found reduction in tumour growth and dissemination in combination treatments compared to single treatments of the individual drugs. Similarly, Luo et al (2013) found LY294002 to reduce protein expression of Akt1 and pAkt1 in spheroid cells and in combination with paclitaxel the cells were re-sensitised to the chemotherapeutic agent.

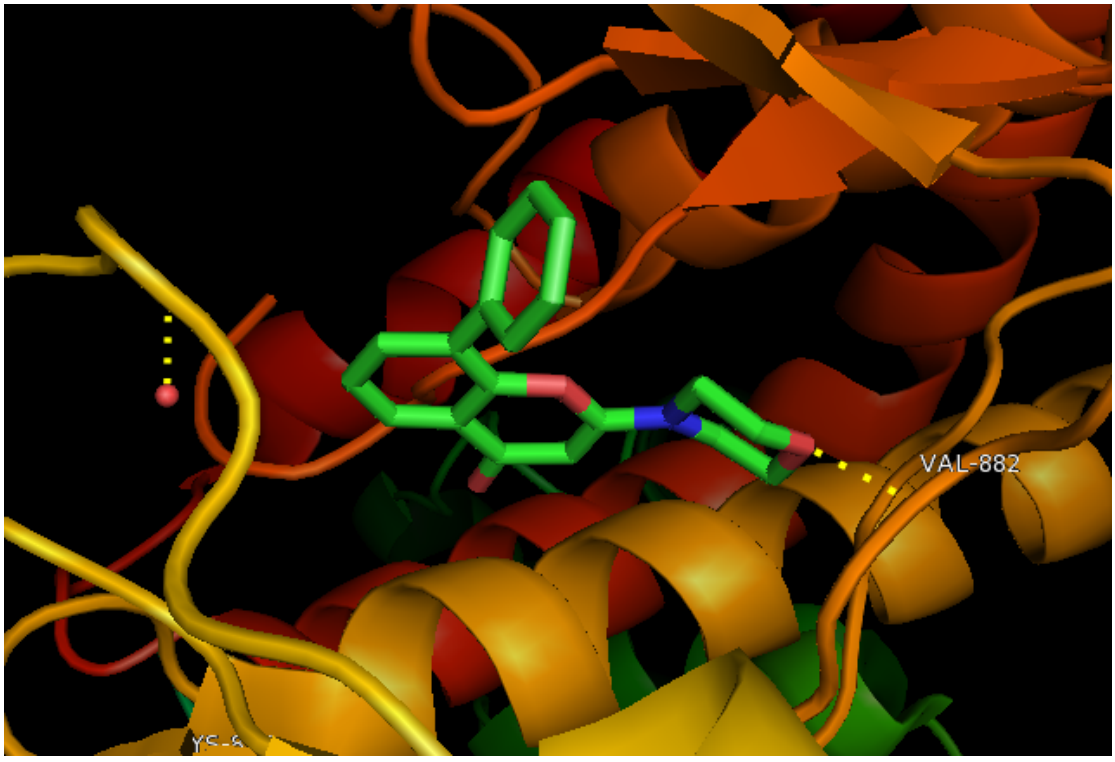


Figure 1.6 PI3K inhibiting compound LY294002 and its' interactions with PI3K's ATP binding pocket. A hydrogen bond with Val 882 is shown as the primary interaction between LY294002 and PI3K. Crystallographic image from Walker et al (2000), adapted in PyMOL.

With respect to the use of LY294002, in cisplatin-based studies, Liu et al (2015) showed after cisplatin treatment, the PI3K pathway was activated and the expression of XIAP was enhanced, which restrained cell apoptosis and lead to further cisplatin resistance. However, when treated with LY294002 and cisplatin, the expression of pAkt decreased and the elevated expression of XIAP induced by cisplatin was inhibited. This encourages the use of LY294002 in combination with cisplatin and shows promise for its application in cancer treatment.

There is still uncertainty in whether specific or broad PI3K inhibitors are clinically more effective and further research is required to clarify this. There is likely to be additional toxicity caused by the complete inhibition of all PI3K isoforms (Engelman 2009). Unfortunately, PI3K inhibition does come with some adverse effects resulting from the inhibition of PI3K dependant cellular processes. One of which is glucose homeostasis, and PI3K inhibition disrupts insulin's effects on metabolism, which are mediated through this pathway (Hu et al 2002).

Another inhibition option that has been was suggested in several studies is the use of a dual PI3K/mTOR inhibitor. Both PI3K and mTOR are apart of the PI3K-related kinase super family and there are inhibitory compounds that can target both proteins. These inhibitors target the proteins both upstream and downstream of Akt. Thus they avoid activation of Akt by the mTOR-S6K-IRS1 negative feedback loop (Chappell et al 2011, Mazzoletti et al 2011). An example of this type of inhibitor is NVP-BEZ235 (Schnell et al 2008). This inhibitor exhibited reduced tumour growth in mouse models (Serra et al 2008). The most recent studies of this inhibitor show it within preclinical trials slowing tumour progression as well as working in combination with cisplatin and effectively inducing apoptosis (Gobin et al 2014, Yang et al 2013). These inhibitors may represent an option as they provide maximal therapeutic efficacy, especially as mTOR is a positive regulator of Akt (Roper et al 2011). Although Dienstmann et al (2014) suggest that broad-PI3K inhibitors are better suited to combination therapy rather than PI3K-mTOR ones.

The next option would be to inhibit Akt and advances in drug design have seen the development of more potent Akt inhibitors. Currently the most promising inhibitor is perifosine, which is in phase III trials and has promising use in combination with drugs such as sorafenib and effectively inhibits Akt (Carlo-Stella et al 2010, Gills and Dennis 2009, Guidetti et al 2014, Hennessy et al 2007). Another highly studied Akt inhibitor is GSK690693, is a selective broad-Akt inhibitor. It is in clinical development and has shown promising results inducing apoptosis in combination treatments on colon cancer cells (Heerding et al 2008, Pal et al 2010). Unfortunately so far these second generation inhibitors have shown more anti-proliferative effects rather than anti-tumour effects but further development may improve this for future studies.

Investigations of mTOR inhibition began with the discovery of rapamycin or serolimus, the product of a bacterium. First generation mTOR inhibitors were derivatives of rapamycin and called rapalogs (Zaytseva et al 2012). These have been proven to be effective in some trials and examples of these are temserolimus and everolimus (Hudes et al 2007). Unfortunately, the positive effects of these inhibitors were limited to rare cancers and they did not exhibit robust, broad anti-cancer effects. This is due to the inhibitors failing to repress mTOR's negative feedback loop. Second generation mTOR inhibitors are commonly called mTORC1/mTORC2 dual inhibitors. These compete with ATP at the catalytic site of mTOR and unlike first generation inhibitors, block the negative feedback loop preventing the activation of PI3K and Akt. These inhibitors shown limited success with KRAS driven tumours, thus it is advised to use these in combination with other inhibitors (Falcon et al 2011, Gupta et al 2012).

From the studies described above the potential efficacy of LY294002 when combined with cisplatin, towards ovarian and endometrial cancers warrants the further investigation of this drug, In combination with an ERK inhibitor such as FR180204 these agents have the potential to create a novel treatment for cancers that show resistance to chemotherapeutic agents such as cisplatin.

1.10 Hypotheses and Aims

The overall aim for this project is to investigate the potential of inhibitors FR180204 and LY294002 in re-sensitising OVCAR-5 and Hec-1A cell lines to cisplatin treatment as well as the opportunity of developing a novel combination therapy that is effective against cisplatin resistant cancers.

Objectives:

1. To investigate FR180204 and LY294002's efficacy individually in preventing cellular growth and proliferation.

2. To investigative FR180204 and LY294002's efficacy in preventing cellular growth and proliferation in combination with cisplatin to re-sensitise resistant cell lines.

3. To investigate the expression of cell cycle, signalling and growth pathway proteins in both OVCAR-5 and Hec-1A cell lines. Justifying the rationale in targeting specific signalling proteins, ERK and Akt.

4. To investigate the expression of cell cycle, signalling and growth pathway proteins in both OVCAR-5 and Hec-1A after treatment with FR180204, LY294002 and cisplatin. This will justify the use of these inhibitors and what effects they may have on the cells. As well as the efficacy of dual inhibition in combination with cisplatin as a novel therapy.

It is hypothesized that upon addition of FR180204, LY294002 and cisplatin to OVCAR-5 and Hec-A1 cell lines, a reduction of cell growth, proliferation and protein expression within both cultures will occur. Ultimately the addition of inhibitors FR180204 and LY294002 will re-sensitise the cancer cells to cisplatin, thus producing a novel targeted combination therapy. This research has the potential to be incorporated into current cancer treatments in order to provide additional treatment strategies.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Cell Lines

Unless stated otherwise, all chemicals and solutions were purchased from Sigma-Aldrich (Auckland, New Zealand), and all buffers were prepared with MilliQ water. The human cancer cell lines used (ovarian adenocarcinoma, OVCAR-5 and endometrial adenocarcinoma, Hec-1A) were provided by Dr Kenny Chitcholtan (Department of Obstetrics and Gynaecology, University of Otago, Christchurch). Both cell lines were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12 catalogue number 12500-062) base media (GIBCO®, Invitrogen, New Zealand), PenStrep antibiotics (GIBCO, Invitrogen, New Zealand) at a concentration of 100 units/mL penicillin and 100 µg/mL streptomycin, 2 mM glutaMAX™ (GIBCO®, Invitrogen, New Zealand), and 2µg/mL Fungizone® (Invitrogen, New Zealand). Henceforth, this supplemented DMEM/F-12 media is referred to as the working media.

2.2 2D Cell Culture

The following procedures were completed under sterile conditions in a cell culture laminar flow cabinet. Both cell lines were continuously maintained at 37°C in a humidified 5% CO_2 incubator in culturing flasks containing working media. The culture medium was replaced every two days with fresh working media until the cells reached near confluence, as assessed using a light microscope. At this stage the cells were sub-cultured. The sub-culturing protocol involved discarding the media, rinsing the flask with 1x sterile phosphate buffered saline (PBS) pH 7.4, and adding 1x trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA). Flasks were incubated with trypsin-EDTA for 20-30 minutes to remove the adhered cells from the flask bottom. Cells were then pelleted by centrifugation at 1500 rpm for five minutes and the supernatant discarded. The remaining pellet was re-suspended in working media. Resuspended cells were diluted two times with 1x PBS, and a cell count carried out using a haemocytometer. The average cell count was used to calculate the

volume of cells required for $2x10^5$ cells per well. This volume was then added to each well in a 12-well cell culture plate, and working media added to make a total volume of 1000 µl per well. Approximately 500 µl of the remaining cell suspension was added to a new culture flask that contained fresh working media for maintenance of the cell line.

2.3 Drug Treatment

After two days of cell growth in 12-well plates drug treatments were added. Cells were treated singly with the cytotoxic drug cisplatin (*cis*-diamminedichloroplatinum (II)), the ERK inhibiting compound FR180204 or the PI3K inhibiting compound LY294002. Stock solutions of 16.6 mM cisplatin, 1.6 mM FR180204 and 16.6 mM LY294002 were prepared in 100% dimethyl sulphoxide (DMSO). Three concentrations of LY294002 (2 µM, 4 µM and 8 µM) and three concentrations of cisplatin (5 µM, 10 µM and 15 µM) were tested. For the control, DMSO was added at a volume equal to that which the cells are exposed to with the highest concentration of drug treatment (0.01%(v/v) in the cisplatin treatment and 0.005%(v/v) in the LY294002 treatment). In the combination experiments, four different combinations of drugs were used 8 µM FR180204 and 8 µM LY294002 combination, 8 µM FR180204 and 10 µM cisplatin combination, 8 µM LY294002 and 10 µM cisplatin combination and 8 µM FR180204, 8 µM LY294002 and 10 µM cisplatin combination. The combination treatment control contained 0.07 % (v/v) DMSO. In each experiment there were three replicate wells for each treatment. Treated cells were returned to the incubator for either 24 or 48 hours before analysing cell metabolism and growth activity.

2.5 Alamar Blue Assay

Cellular metabolism after drug treatment was assessed by addition of Alamar Blue (Invitrogen, New Zealand) after either 24 or 48 hours of exposure to the drugs. Alamar Blue (resazurin) is a blue dye that is reduced to a pink compound (resorufin) in the presence of respiring cells due to mitochondrial activity. Half of the media was removed from each well before adding Alamar Blue, followed by an incubation period of four hours. After this duration, solution (not cells) from

each well was loaded onto a 96-well plate. A change of colour from blue to pink was measured by absorbance at 570 nm and 600 nm using a microplate reader (SpectraMax® M5, Molecular Devices). The difference between the absorbance at 570 nm and 600 nm was used as a measure of the cells metabolic activity.

2.6 Crystal Violet Assay

Following the Alamar Blue assay, cell numbers were indirectly determined by measuring cellular DNA content using crystal violet staining. Crystal violet (tris(4-(dimethylamino)phenyl)methylium chloride) is a purple dye that binds to DNA. Its absorbance at 570 nm enables a quantification of the amount of DNA within a sample, which is proportional to the total cell number. The Alamar Blue solution was discarded and 1x PBS pH 7.4 added to each well to rinse out the Alamar Blue. Cells were then detached from the bottom of the wells by adding 10x trypsin-EDTA and incubating at 37°C for 10-20 minutes. Cells were pelleted by centrifugation at 2500 rpm for five minutes and the PBS supernatant discarded. 2mg/mL crystal violet in 2% (v/v) ethanol in MilliQ water was added and incubated at room temperature for 30 minutes. The cells were washed with MilliQ water to remove non DNA-bound crystal violet dye. Washed cells were pelleted by centrifugation at 2000 rpm for five minutes and the supernatant discarded. This washing step was repeated four times before lysing cells with 2% (w/v) sodium dodecyl sulphate (SDS). The lysed cell solutions from each well were then loaded onto a 96-well plate and the optical density at 560 nm (OD_{560}) was measured using the microplate reader.

2.7 3D Cell Culture

Alternatively to 2D cell culture, the majority of experiments were done in 3D cell culture conditions. This method uses culture plates coated with a polymer that prevents cells from adhering to the plates causes the cells to cluster together and form 3D spheroids. This 3D model is likely to be a more appropriate representation of *in vivo* solid tumours. 12-well plates were pre-coated with poly-hydroxyethylmethacrylate (poly-HEMA). Poly-HEMA was added to 95% ethanol at a concentration of 12mg/mL and dissolved by heating at 70°C. Once

completely dissolved, 500 µL per well of poly-HEMA solution was added per well to the 12-well plates, which were then incubated overnight on an orbital shaker at 37°C. Before adding cells, the coated wells were washed with 1x PBS pH 7.4. The remaining procedures were similar to that of the 2D cell cultures, except with minor modifications. Whenever drawing up media from the wells, the plates were left to rest at an angle for approximately five minutes to allow the spheroids to settle. For the crystal violet assay, the remaining Alamar Blue solution and cells were transferred to centrifuge tubes with 3 mL 1x PBS pH 7.4 as a wash for the wells. The tubes were spun at 2000 rpm for five minutes and the Alamar Blue-containing supernatant discarded. Cells were then digested with 10x trypsin-EDTA and incubated at 37°C for 10-20 minutes. Tubes were spun again for five minutes at 2000 rpm and the supernatant discarded before adding crystal violet to each tube. The remainder of the protocol was as described before for the 2D cell cultures.

2.8 Western Blot Analysis

Cells were lysed by adding 200 µL of 0.1% SDS to12-well plates and incubating on ice for 20 minutes. The cell lysates were then collected.

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) was used to separate the cell lysates. The protein concentration of each cell lysate was determined using Micro-BCA™ Protein Assay Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and this concentration was used to determine the volume of each cell lysate that was required to load 10 µg of protein per cell lysate onto the gel (see Appendix). To prepare them for loading onto the gel, the cell lysates were mixed with sample buffer (20% (v/v) bromophenol blue, 50% (v/v) glycerol, in Tris-HCl pH 6.8) and heated at 90°C for 10 minutes, followed by centrifugation for seven minutes at 12,000 rpm. Cell lysates were loaded onto Bolt™ 4-12% Bis,-Tris Plus gels (Invitrogen, New Zealand) and the SDS-PAGE was run for approximately 120 minutes at 120 Volts using Bolt™ MES SDS Running Buffer (Invitrogen, New Zealand). MagicMark™ Western Protein Standard (Invitrogen, New Zealand) and Precision Plus Protein™ standard (BIO-

RAD, Hercules, USA) were used as SDS-PAGE markers. Following SDS-PAGE, the separated proteins were electro-blotted onto polyvinyl difluoride (PVDF) membranes (BIO-RAD, Hercules, USA), running at 15 Volts for 20 minutes in cold Tris-glycine running buffer. After electro-blotting, membranes were blocked for 60 minutes using either 5% (w/v) non-fat skim milk (PAMS brand, New World , New Zealand) in Tris-buffered saline and Tween20 (TBS-T) buffer or 1% (w/v) bovine serum albumin (BSA) in TBS-T buffer. The blocking solution was decanted off and diluted primary antibody solution was added prior to incubation at 4^{o}C overnight. All primary antibodies were diluted 50% (v/v) in TBS-T using the appropriate blocking solution. All antibodies were purchased from Thermofisher Scientific, Rockford, IL, and their working concentrations are described in Table 2.1.

The primary antibody solution was decanted off and the membranes were washed by the addition of TBS-T buffer, shaken on an orbital shaker for 10 minutes, and decanting off of the TBS-T buffer. This wash step was repeated four times before the addition of the secondary antibody solution. As before, the antibody solution was diluted 50% (v/v) with TBS-T buffer and the appropriate blocking solution. Secondary antibodies were either anti-rabbit-goat or anti-mouse-goat (see Table 2.1 for working concentrations). Membranes were incubated with secondary antibody for 90 minutes at room temperature on an orbital shaker. Following this incubation the secondary antibody solution was discarded and the TBS-T buffer wash step repeated (as before after the removal of the primary antibody solution). A chemiluminescence detection kit (Amersham™ ECL™ Prime Western Blotting Detection Reagent Kit, GE Healthcare) was used to determine antibody localization. Protein bands were visualized and densiometry analysis performed using Alliance 4.7, Unitec software (Cambridge, UK). Cell lysates were collected for each 24-hour combination experiment (FR180204, LY294002, cisplatin and the combinations) and each treatment was carried out in triplicate.

2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism® software (La Jolla, CA, USA). Experimental results were assessed for significance using either one-way or two-way ANOVA analysis (using $P < 0.05$ as a measure of statistical significance).

Table 2.1 Working concentrations of antibodies used for Western Blots.

Primary antibodies	(clone): catalogue number, lot number	Species antibody raised	1/(working concentration of primary antibody)	Blocking solution and concentration	1/(working concentration of secondary antibody)
Anti-ERK	44-654G	Rabbit	1/2000	5% (w/v) non-fat skim milk	1/5000
Anti-pERK	PA5-15305	Mouse	1/500	1% (w/v) BSA	1/5000
Anti-Akt	MA5-14916	Rabbit	1/500	1% (w/v) BSA	1/1000
Anti-pAkt	44-621G	Rabbit	1/500	1% (w/v) BSA	1/10,000
Anti-GAPDH	MA5-15738	Rabbit	1/2000	5% (w/v) non-fat skim milk	1/5000
Anti-PCNA	PA5-27214	Mouse	1/500	5% (w/v) non-fat skim milk	1/10,000
Anti-cyclin D_2	AHF0112	Mouse	1/500	1% (w/v) BSA	1/10,000
Anti-cyclin B_2	PA529233	Rabbit	1/2000	1% (w/v) BSA	1/5000

CHAPTER 3: RESULTS

3.1 Single Treatment Effects on Cellular Metabolism and Growth

To assess cellular metabolism and cell growth of Hec-1A and OVCAR-5 cell lines following treatment with cisplatin, FR180204 and LY294002, Alamar Blue and crystal violet assays were used respectively. FR180204 experiments were not completed due to issues with contamination and time constraints - see section 4.4 for elaboration.

3.1.1 Cisplatin

Overall, treatment with cisplatin did not significantly affect cellular metabolism or growth activity in either cell line after 24 hours (Fig 3.1.1 a, b, c, d). There was a trend for Hec-1A cells to increase metabolism after 24 hours with cisplatin treatment, although the increase was not statistically significant ($P > 0.05$, ANOVA, n = 9) (see Fig. 3.1.1 a). Increased metabolism could indicate resistance, as the anticipated metabolic reduction characteristic of cisplatin treatment is absent. This is unexpected, although may require more replicates to confirm this result. Cisplatin also affected cell growth of the Hec-1A cells (see Fig 3.1.1 b), as there was an increase after 24 hours with the 5 μM treatment. However, again this was not a statistically significant result ($P > 0.05$, ANOVA, n = 9). Cisplatin had little effect on the metabolic activity of the OVCAR-5 cells ($P > 0.05$, ANOVA, n = 9) (Fig 3.1.1 c). However there was a trend toward reduction in cell growth activity (see Fig 3.1.1 d) with both 5 μM and 10 μM cisplatin treatments having an effect after 24 hours although again this was not statistically significant ($P > 0.05$, ANOVA, n = 9).

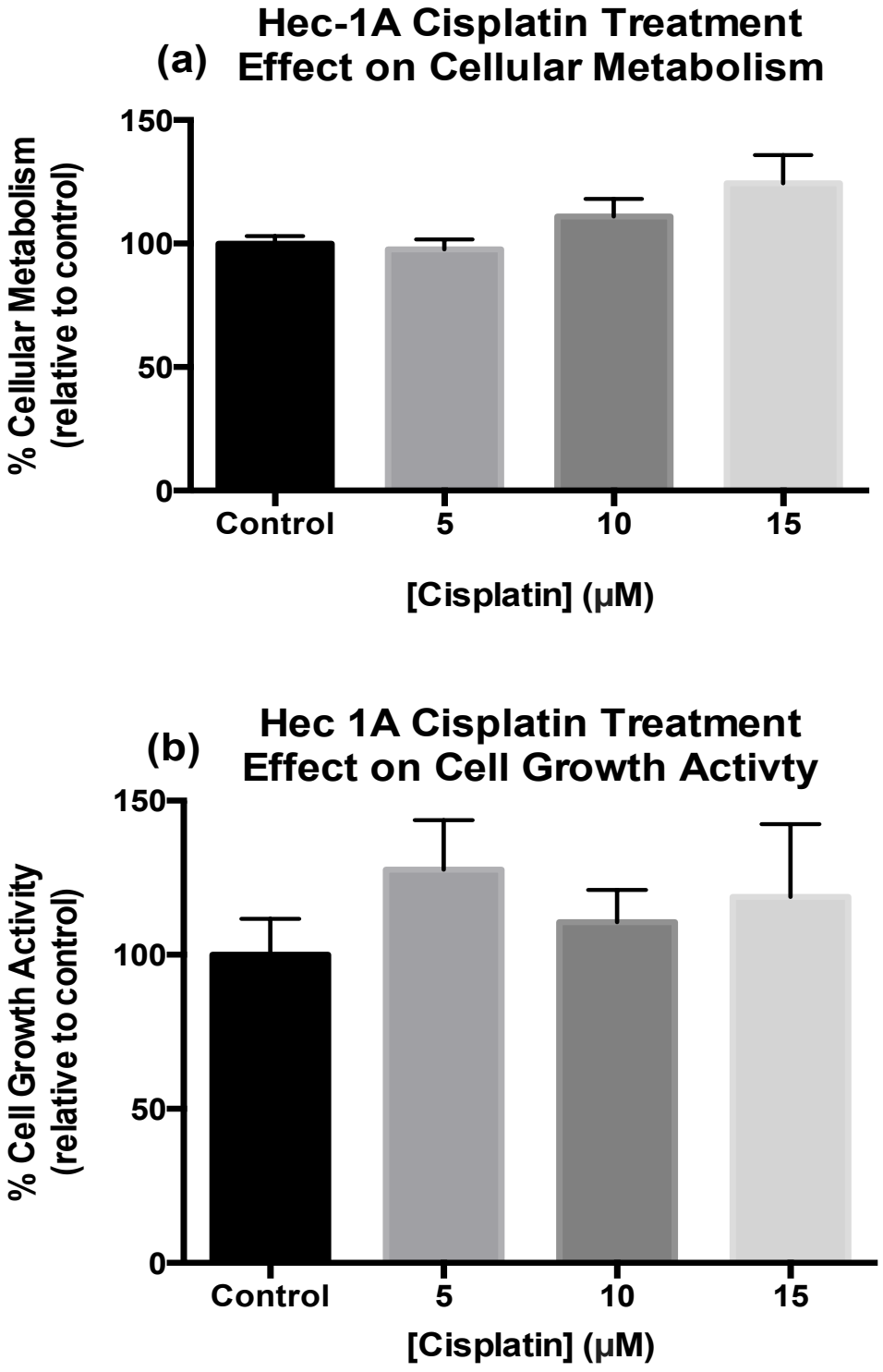
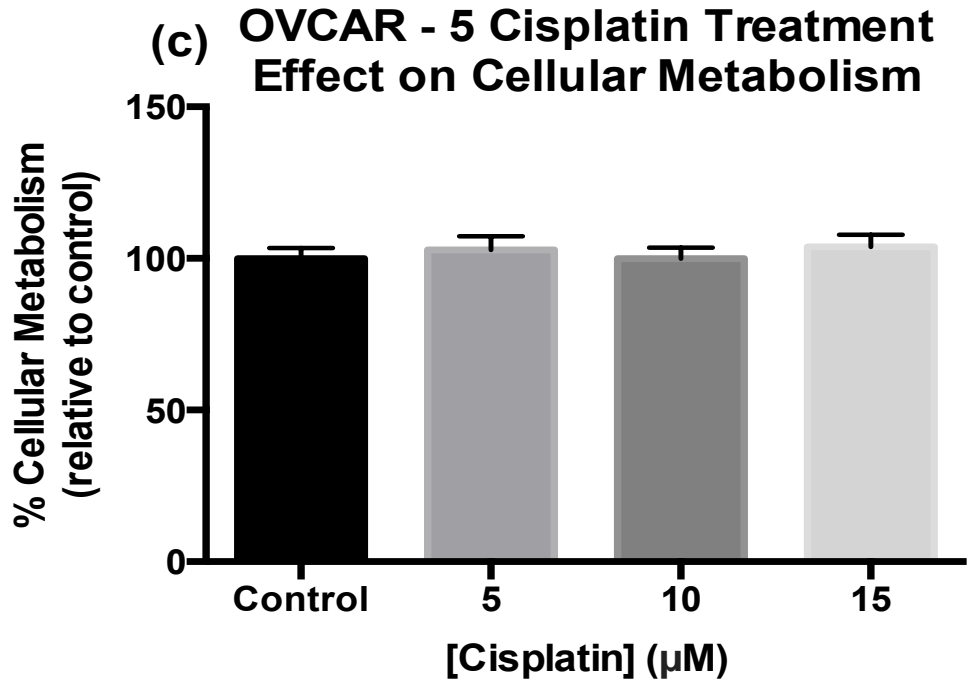


Figure 3.1.1 Single Treatment Effect of Cisplatin on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay) (a) and growth activity (determined by a crystal violet assay) (b) of Hec-1A cells treated with various concentrations of cisplatin for 24 hours. Data are from three independent experiments carried out in triplicate ± SEM.



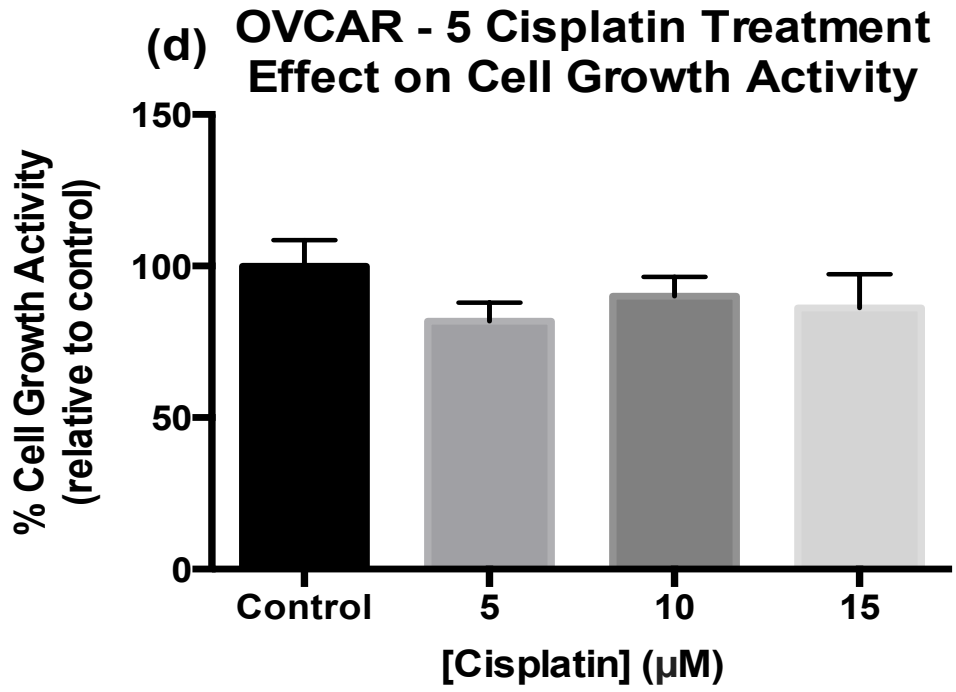


Figure 3.1.1 Single Treatment Effect of Cisplatin on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay) (c) and growth activity (determined by a crystal violet assay) (d) of OVCAR-5 cells treated with various concentrations of cisplatin for 24 hours. Data are from three independent experiments carried out in triplicate ± SEM.

3.1.2 LY294002; PI3K inhibitor

Overall, LY294002 treatment had little effect on cellular metabolism or growth activity after 24 or 48 hours in either cell line (Fig 3.1.2 a, b, c, d). Figure 3.1.2 a shows that there was no significant effect on cellular metabolism in the Hec-1A cells. In comparison, cell growth was largely unaffected (Fig 3.1.2 b), although it was significantly decreased after 24 hours with the 8 μM treatment ($P = 0.0012$, ANOVA, n = 9) and after 48 hours with the 4 μM treatment ($P = 0.0225$, ANOVA, n = 9). Although both 24 and 48 hours results show there is a reduction in cellular growth, because these are not consistent with the other data points (of either longer duration at the same concentration or a higher concentration at the same concentration), so these are difficult to explain. However there is a trend towards a decrease in cell growth with all treatments. Further research and an increase in replicates could clarify these results. OVCAR-5 cells showed a trend towards a small decrease in cellular metabolism after treatment with LY294002 compared to the control (Fig 3.1.2 c) although of these only the 24-hour exposure to 4 μM was statistically significant ($P = 0.0387$, t-test, n = 3). As there was no significant difference in metabolism at the higher concentrations (8 μM) this suggests that further experiments/replicates are required to clarify these results (Fig 3.1.2 d). There was a trend for cell growth of the OVCAR-5 cells to increase as the concentration of LY294002 increases after 24 hours, although again there was no statistical significance ($P > 0.05$, ANOVA, n=9).

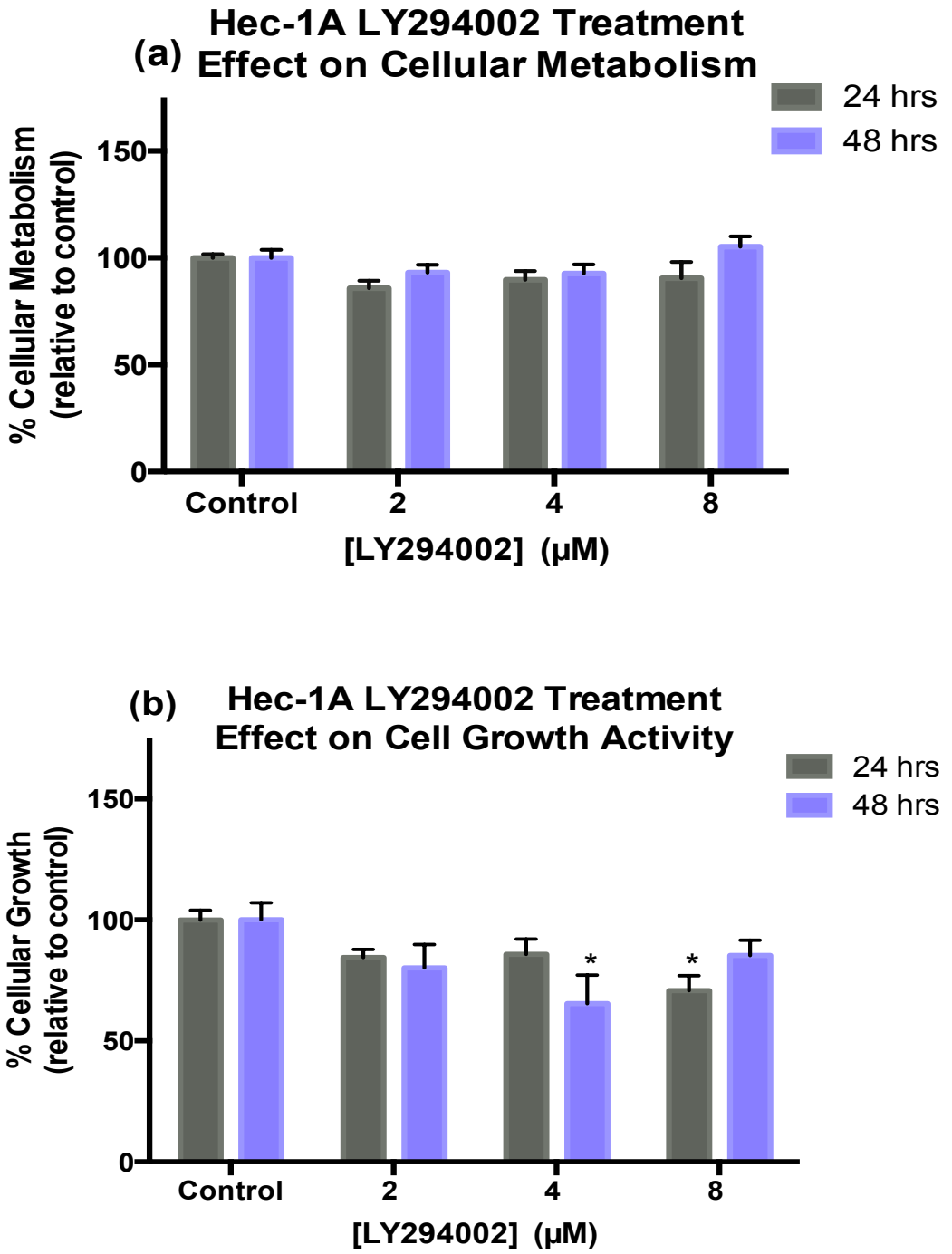


Figure 3.1.2 Single Treatment Effect of LY294002 on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay)(a) and growth activity (determined by a crystal violet assay)(b) of Hec-1A cells treated with various concentrations of LY294002 for 24 hours (grey columns) or 48 hours (blue columns). Data are from three independent experiments carried out in triplicate ± SEM. * denotes a statistical significance (t-test, $P< 0.05$) relative to the control.

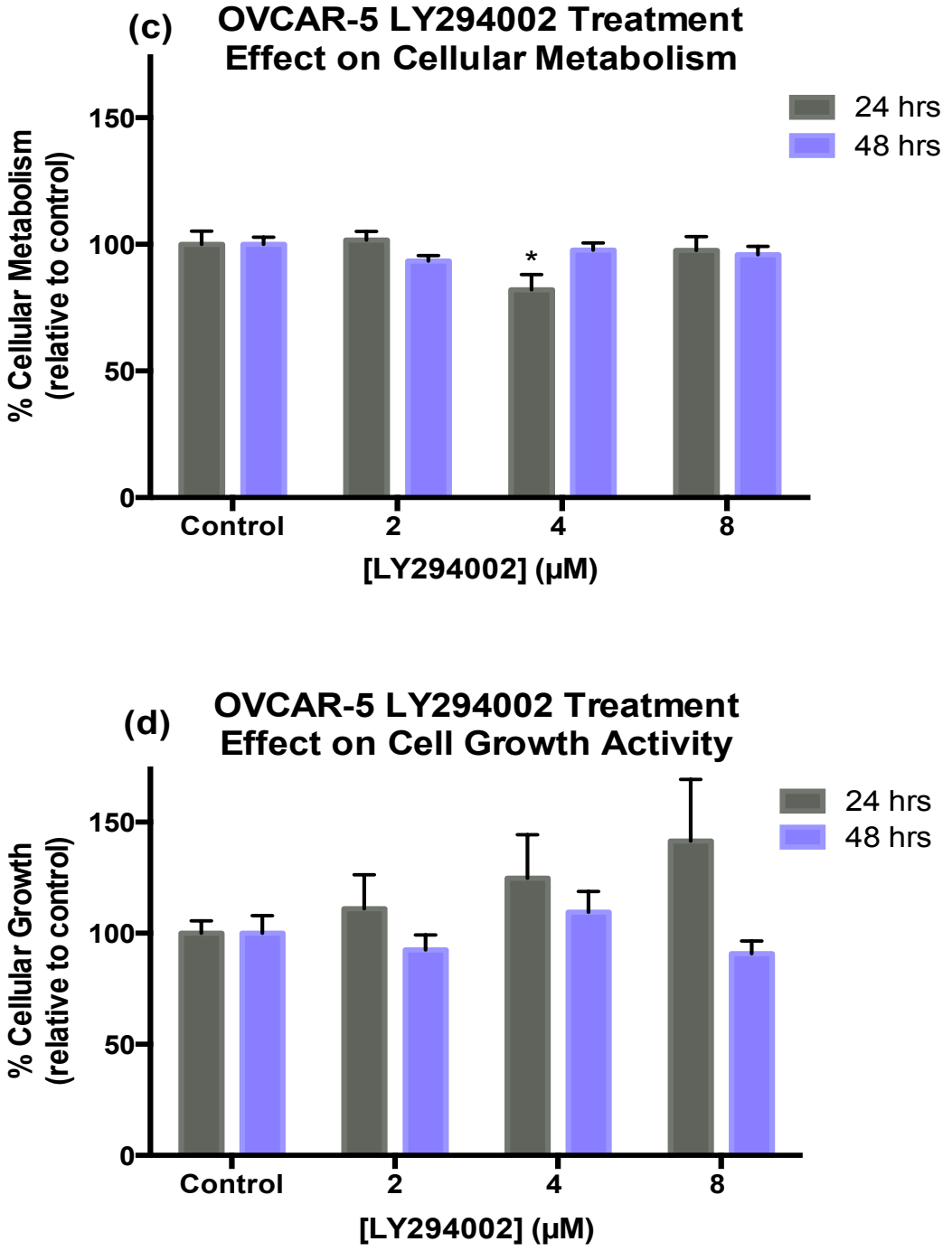


Figure 3.1.2 Single Treatment Effect of LY294002 on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay)(c) and growth activity (determined by a crystal violet assay)(d) of OVCAR-5 cells treated with various concentrations of LY294002 for 24 hours (grey columns) or 48 hours (blue columns). Data are from three independent experiments carried out in triplicate ± SEM. * denotes a statistical significance (t-test, $P< 0.05$) relative to the control.

3.1.3 20 μM LY294002

Due to the lack of any consistent significant effect of LY294002 treatment at the lower concentrations, a higher concentration range of 8 μM to 20 μM was tested to investigate whether this would be more influential in affecting the cell lines.

After treatment with 20 μM LY294002, both cell lines showed no significant decrease in cellular metabolism (Fig 3.1.3 a). However, there was a decrease in cellular metabolism in the OVCAR-5 cells after treatment of 20 μM LY294002, this was not statistically significant (see Fig 3.1.3 b)($P > 0.05$, ANOVA, n = 3). Crystal violet assays could unfortunately not be completed due to time constraints, but in order to have accurate results on the growth activity of LY294002, both Alamar Blue and crystal violet assays are suggested to be repeated increasing the sample size and producing more conclusive results.

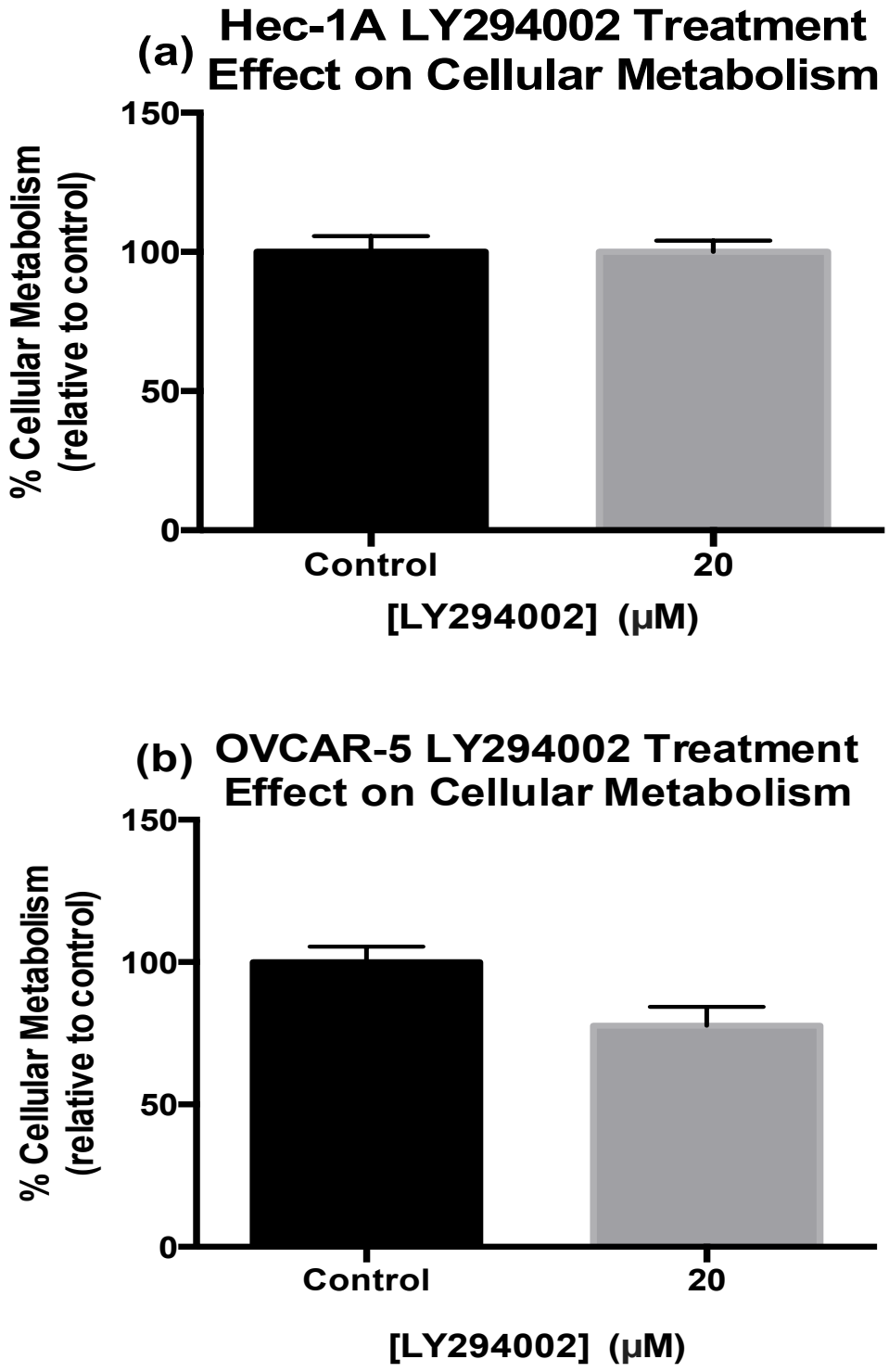


Figure 3.1.3 Single Treatment Effect of LY294002 (20μM) on Cellular Metabolism. Relative cellular metabolism (determined by an Alamar Blue assay) of Hec-1A (a) and OVCAR-5 (b) cells treated with 20 μM of LY294002 for 24 hours. Data are from one independent experiment carried out in triplicate ± SEM.

3.2 Comparison of 2D and 3D Cell Culture

To investigate if the method of culturing the cell lines influences the way they react to the inhibitors, 2D and 3D cultures treated with LY294002 were compared.

Overall regardless of culturing technique, there was no significant difference in cellular metabolism or growth activity following treatment with LY294002. The Hec-1A cell lines metabolism results, comparing 2D and 3D showed no significant difference (Fig 3.2 a). However, the 3D 2 μM treatment shows a trend towards reduction, although this is not significant ($P > 0.05$, ANOVA, n = 3). Similarly the cell growth activity results have one significantly reduced result of 2D 4 μM treatment (Fig 3.2 b) ($P > 0.05$, ANOVA, n = 3), again however this was not significant. This is not consistent with the treatment causing a significant effect on metabolism results either, which suggests this may be an anomaly. Otherwise all other data points are very similar in results between 2D and 3D cultures.

The metabolism of the OVCAR-5 was reduced with the 3D 4 μM treatment (Fig 3.2 c) although statistically it was not significant ($P > 0.05$, ANOVA n = 3). This is not consistent with the cell growth activity results, which showed no significant reduction with any treatments or culturing method (Fig 3.2 d). This result may be an anomaly and further experiments may elaborate this finding.

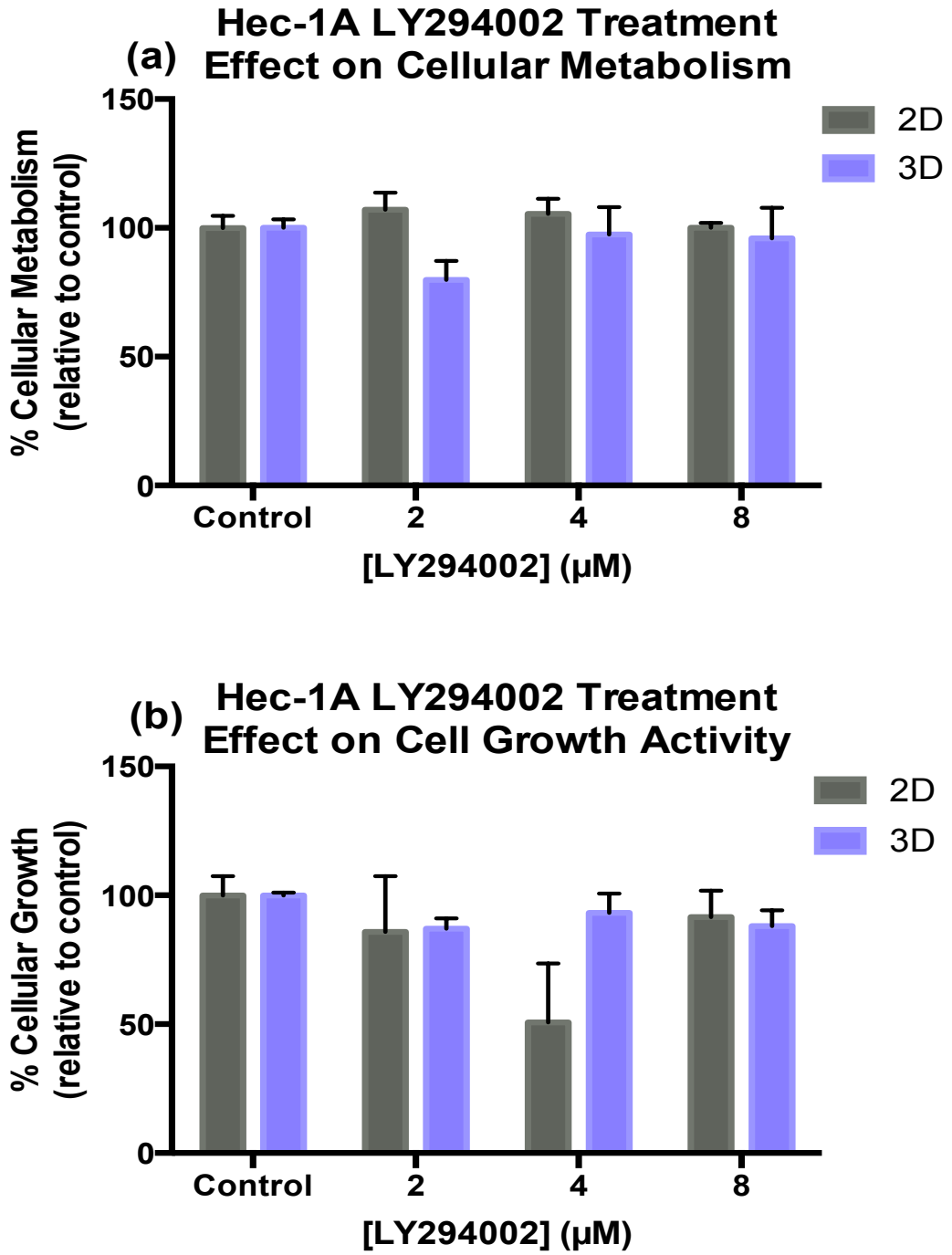


Figure 3.2 Comparison of 2D and 3D Cell Culture Effects on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay)(a) and growth activity (determined by a crystal violet assay)(b) of Hec-1A cells treated with various concentrations of LY294002 and grown in 2D plates (grey columns) or 3D plates (blue columns). Data are from one independent experiment carried out in triplicate ± SEM.

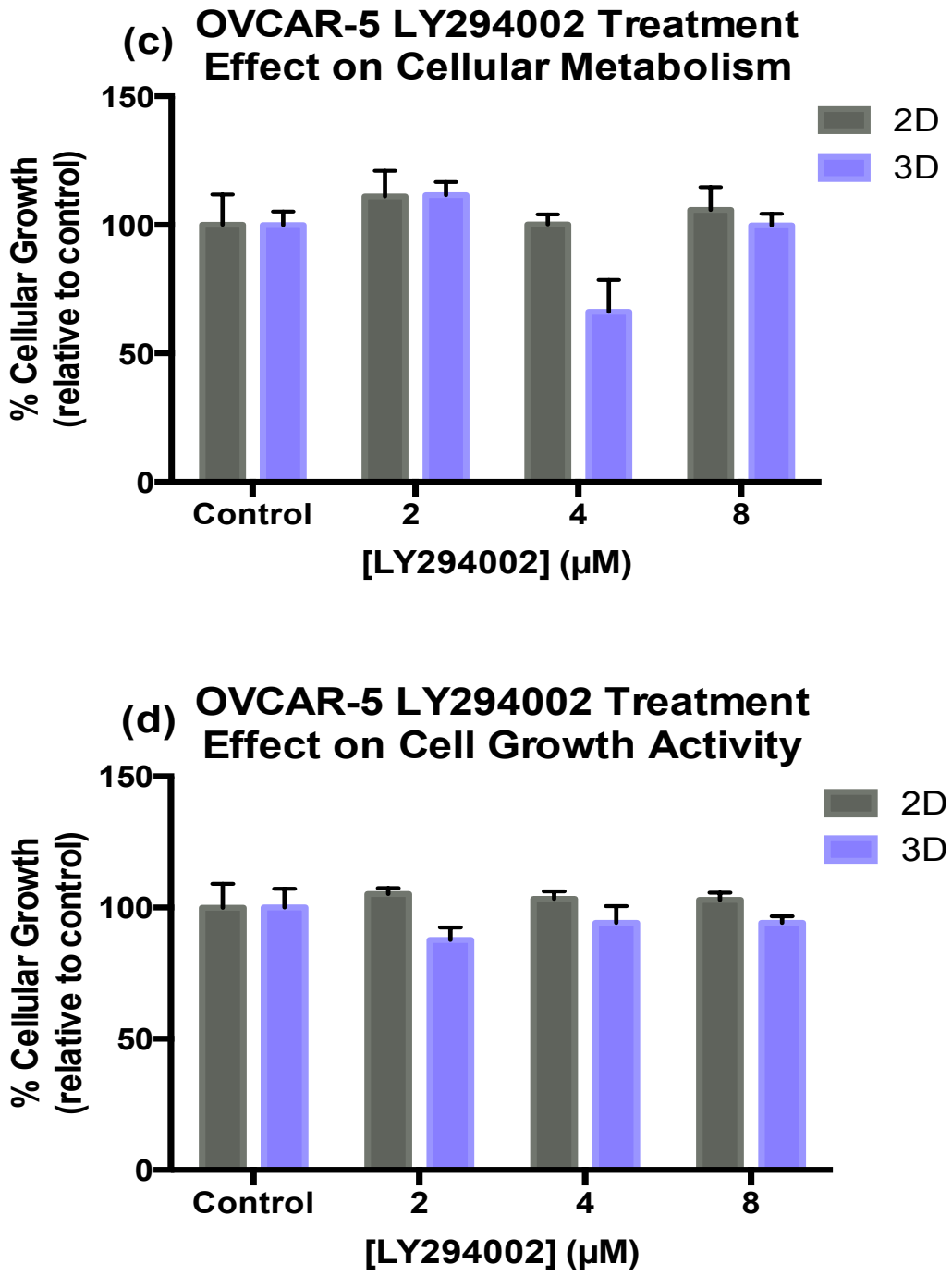


Figure 3.2 Comparison of 2D and 3D Cell Culture Effects on Cellular Metabolism and Growth. Relative cellular metabolism (determined by an Alamar Blue assay)(c) and growth activity (determined by a crystal violet assay)(d) of OVCAR-5 cells treated with various concentrations of LY294002 and grown in 2D plates (grey columns) or 3D plates (blue columns). Data are from one independent experiment carried out in triplicate ± SEM.

3.3 Combination Treatment Effects on Cellular Metabolism and Growth

Hec-1A and OVCAR-5 cells were treated with four different combinations of FR180204 (8 μM), LY294002 (8 μM) and cisplatin (10 μM) to determine whether or not the cells could be re-sensitised to cisplatin by the inhibition of PI3K, ERK and their downstream effectors by LY294002 and FR180204 respectively. Both cellular metabolism and cell growth activity were measured as before using the Alamar Blue and the crystal violet assays, respectively.

Hec-A1 cells exhibited no increase or decrease in cellular metabolism after 24 hours exposure to all treatments relative to the control (Fig 3.3 a). Cell growth activity was decreased after 24 hours in Hec-1A cells (Fig 3.2 b) with the FR180204, LY294002 and cisplatin combination treatment, which showed a slight decrease in growth activity ($P > 0.05$, ANOVA n = 6). This is a promising finding as this could be a result of dual inhibition re-sensitising the cells to cisplatin. But the Alamar Blue results showed no reduction in metabolism with this treatment.

The cellular metabolism of OVCAR-5 cells decreased in all treatments after 24 hours (Fig 3.3 c). This was most marked with the treatment with FR180204 and cisplatin ($P = 0.0035$, ANOVA n = 6). Similarly to Hec-A1 cells, the OVCAR-5 cells showed no decrease in cell growth activity with the combinatory treatments after 24 hours (Fig 3.3 d). In fact all treatments resulted in increased cell growth activity suggesting there needs to be alterations in drug treatment to achieve inhibitory results.

48-hour combination experiments were planned but due to contamination and time restrictions (see section 4.6) they were unable to be completed.

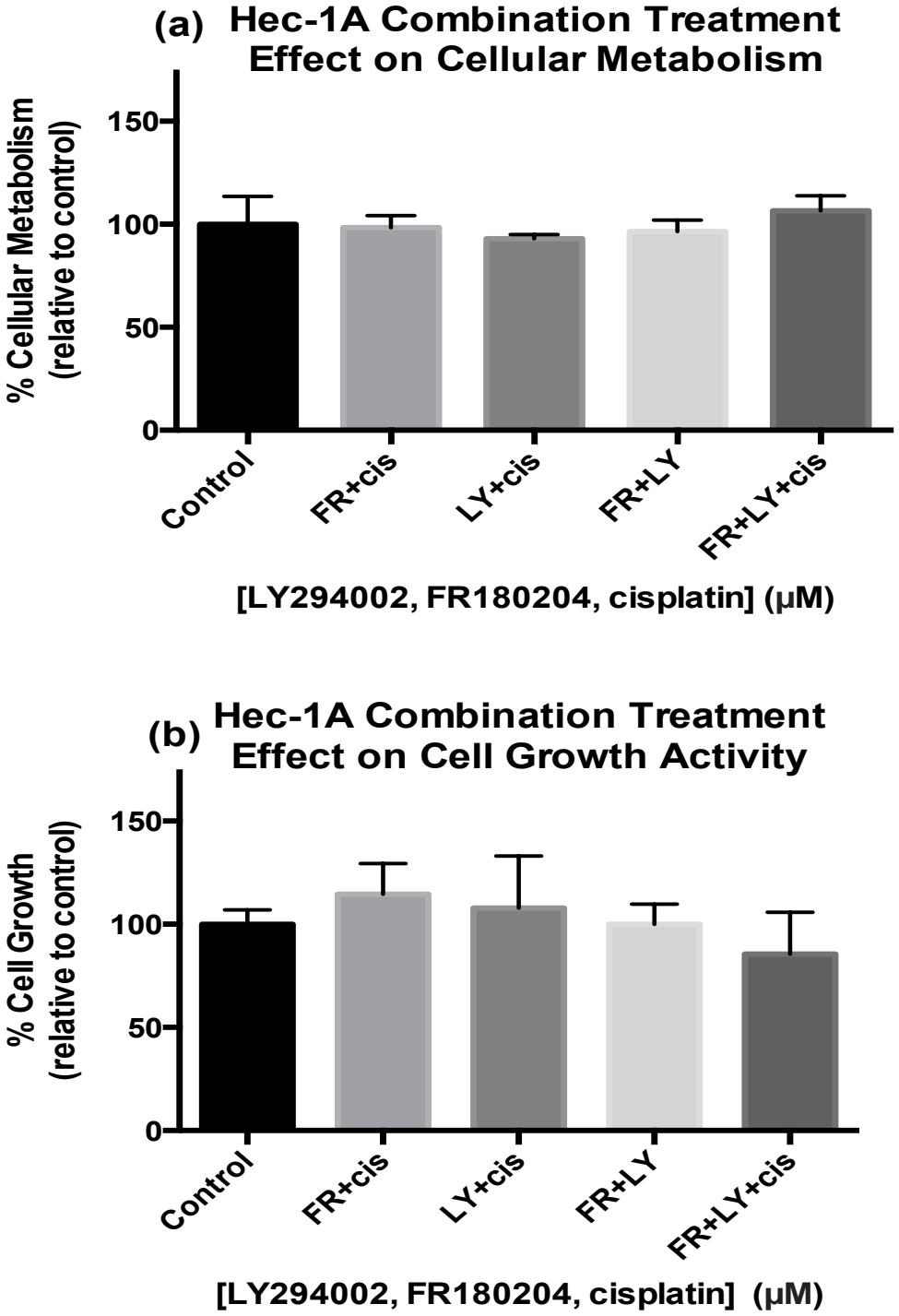


Figure 3.3 Combination Treatment Effect on Cellular Metabolism and Growth. Hec-1A cells were subject to combination treatment of 10 μM cisplatin, 8 μM FR180204 and 8 μM LY294002. After 24 hours (grey columns) or 48 hours (blue columns) of treatment, cellular metabolism and growth activity were analysed by Alamar Blue (a) and crystal violet assays (b), respectively. Data from two independent experiments carried out in the triplicate ± SEM.

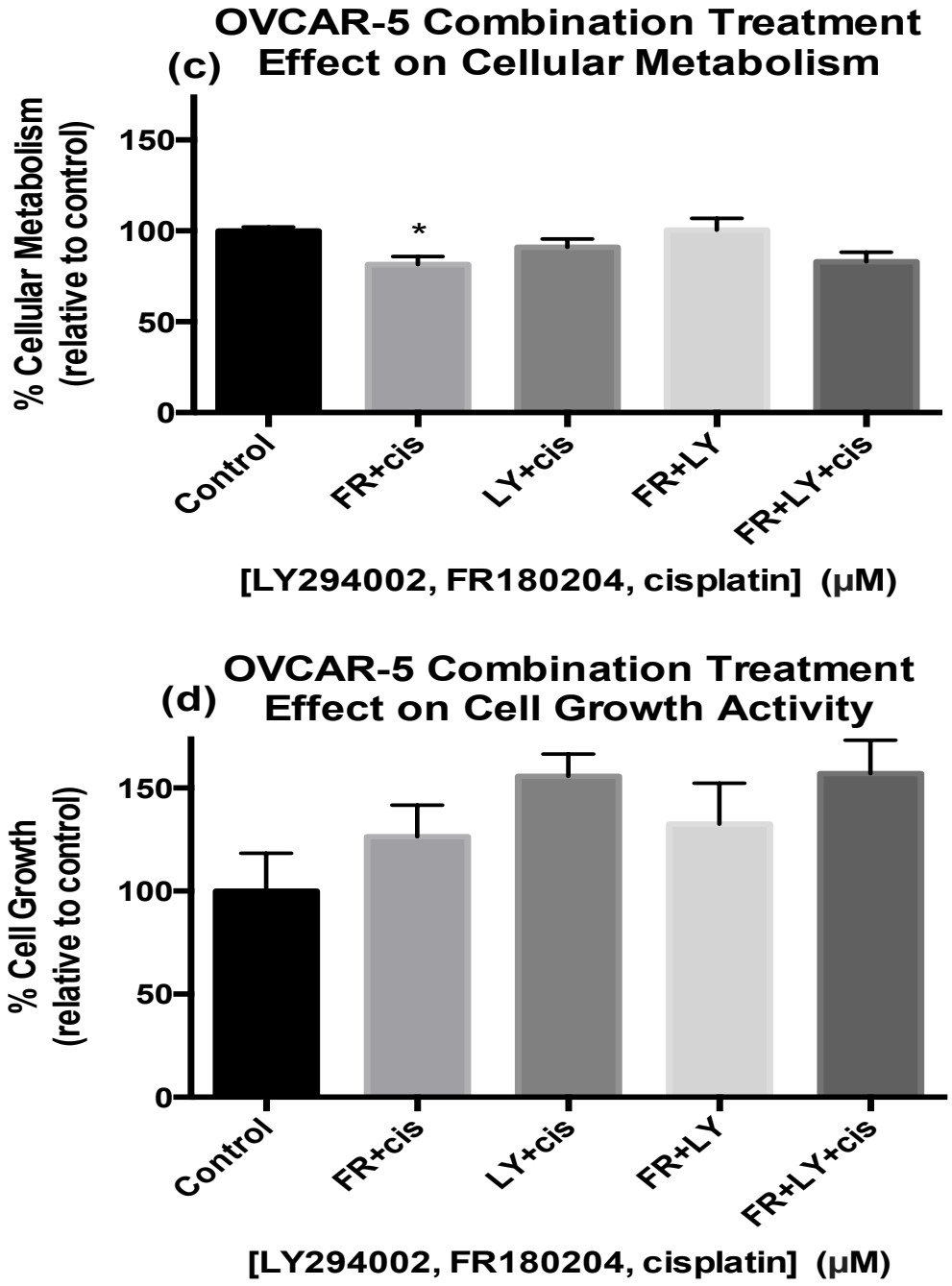


Figure 3.3 Combination Treatment Effect on Cellular Metabolism and Growth. Hec-1A cells were subject to combination treatment of 10 μM cisplatin, 8 μM FR180204 and 8 μM LY294002. After 24 hours (grey columns) or 48 hours (blue columns) of treatment, cellular metabolism and growth activity were analysed by Alamar Blue (c) and crystal violet assays (d), respectively. Data from two independent experiments carried out in the triplicate ± SEM. * denotes a statistical significance (t-test, $P< 0.05$) relative to the control.

3.4 Protein Expression

Western blotting was carried out to determine the expression of a number of cell cycle and signalling proteins in OVCAR-5 and Hec-1A cells after their exposure to FR180204, cisplatin and LY294002 over 24 hours. Cell cycle proteins included cyclin D_2 (G1/S phase), cyclin B_2 (G2/M phase), and PCNA (proliferating cell nuclear antigen, S and G2/M phase). The expression of these proteins was used to analyse the impact of the drug treatments upon cell cycle progression. ERK and its more active phosphorylated form, pERK, were examined to determine whether or not ERK signalling was active in these cells. Additionally the expression of PI3K pathway proteins; phosphoinositide-3 kinase (PI3K), Akt and its active form, pAkt were investigated to determine their active within the cell lines. Due to time and contamination constraints only two experiments (n=2) using OVCAR-5 cells were assessed for the combination treatments after 24 hours. Of the two Western blots, only one produced usable data, and within these blots only three proteins produced readable bands. Therefore these results are not statistically analysable. However, it does give a qualitative indication of protein expression in cells treated with these drugs over a 24 hour period. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference housekeeping protein, due its relatively constant expression despite changing cellular conditions. Ideally this would have been quantitatively affirmed by the density of the GAPDH bands, unfortunately on both occasions of experiments the GAPDH blots did not work. In further research, it would be useful to utilise a second housekeeping protein such as actin. Each well was loaded with 10 μg of protein, as determined using the protein concentration of each cell lysate (see Appendix).

Figure 3.4 shows the Western blot results for the OVCAR-5 cell line, with combinatory drug treatments. Caution is advised, bearing in mind that there is no statistical analysis due to only one replicate being completed.

In the OVCAR-5 cell line, expression of the cell cycle protein cyclin B2 remains constant between the different concentrations of drugs. There is potentially a slightly lower expression in the last treatment (8 μM FR18204 + 8 μM LY294002

+ 10 μM cisplatin). Akt expression is constant across all drug treatments also, the weakest expression would be the 8 μM LY + 10 μM cisplatin, although this still indicates Akt expression within the cells. Finally ERK protein expression shows expression across all treatments apart from 8 μM FR + 8 μM LY + 10 μM cisplatin. This treatment has no ERK band/ expression.

Across the board all three proteins blotted show relatively consistent expression across varying drug treatments. The only treatment that has shown promising results is the combination of LY, FR and cisplatin. Although if this combination is effective in reducing protein expression considerably, it would be expected to see some reduction in expressions in combinations with only two of the drugs. Nevertheless, further replicates would be required to confirm this as well as investigate the other cell cycle and signalling proteins in order to make stronger assumptions.

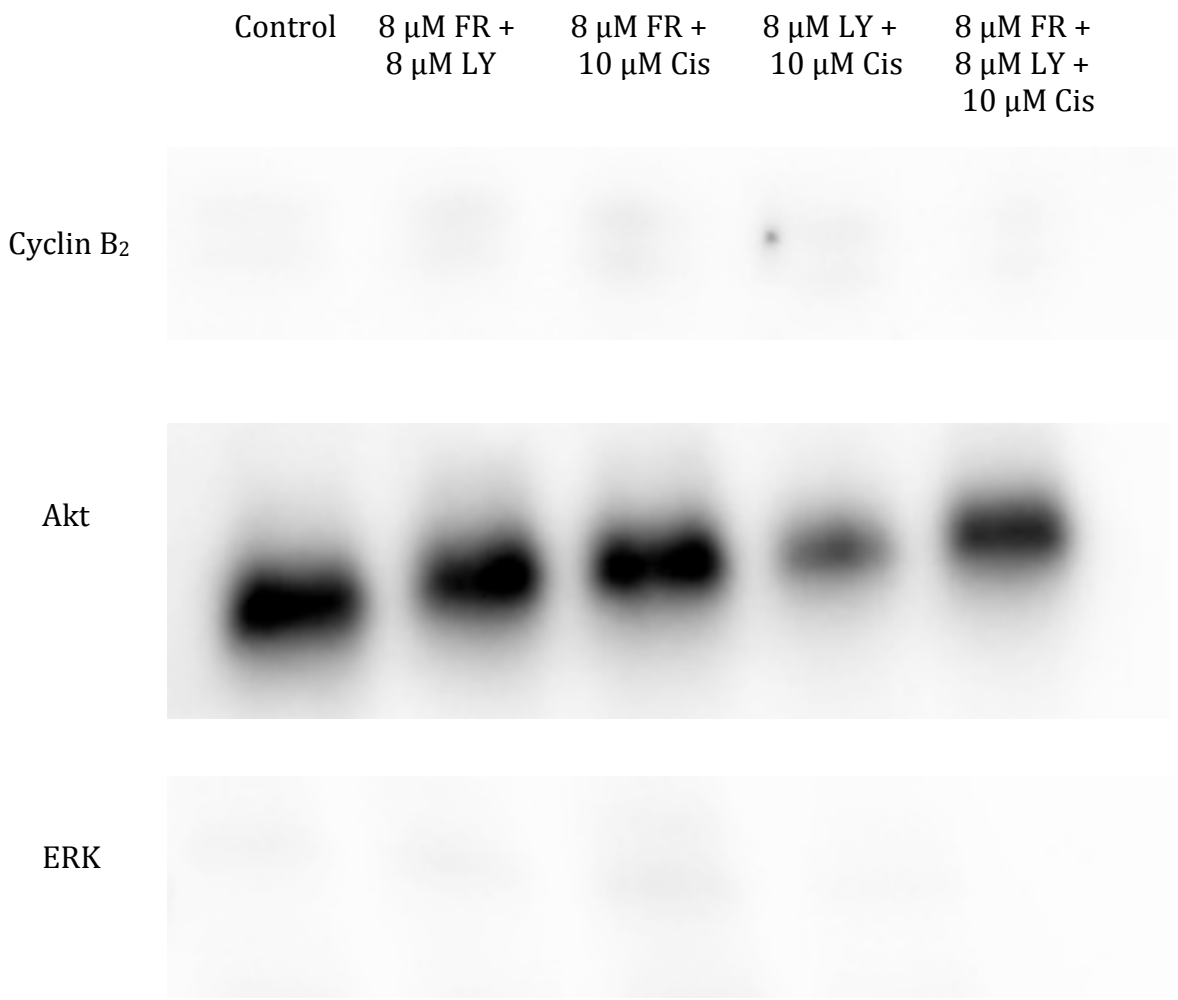


Figure 3.4 Western blots of cell cycle protein cyclin B_2, and signalling proteins Akt and ERK in OVCAR-5 cells. Cells were treated with: 8 μM FR180204 (FR), 8 μM LY294002 (LY) and 10 μM cisplatin (Cis) in varying combinations for 24 hours. Images are representative of one experiment, 10 μg of protein per well was loaded for each sample.

CHAPTER 4: DISCUSSION

The aim of this study was to investigate FR180204 and LY294002 (ERK and PI3K inhibitors respectively) and their efficacy in re-sensitising resistant ovarian and endometrial cells to cisplatin. Overall the study showed no successful inhibition of the Ras, Raf, MEK, ERK pathway or the PI3K, Akt, mTOR pathway, singly or in combination. Thus successful re-sensitisation to cisplatin treatment was not achieved. However, the information found has provided insights into alternative approaches of pathway inhibition, and has built a platform for future studies aimed at developing a novel combination therapy for cisplatin resistant cancer cells.

4.1 Cisplatin Treatment Demonstrates Resistance in Hec-1A and OVCAR-5 cells

Cisplatin treatment confirmed OVCAR-5 and Hec-1A resistance to the first-line therapeutic drug. In response to the cisplatin treatment, no significant reduction of cellular metabolism or growth activity after 24 hours of exposure was observed. Previously Rosenberg and Vancamp (1969) found 5 μM cisplatin to be effective in their studies of the platinum-based agent. This concentration is also relevant as it is the highest concentration that would be seen *in vivo* after standard cisplatin treatment (Lerza et al 1997, Rajan et al 2009). Thus it is likely that the OVCAR-5 and Hec-1A cells are resistant given the higher concentrations that they were exposed to in the current study.

4.2 PI3K Inhibition Did Not Prevent Cell Proliferation

The PI3K pathway and its proteins are the subjects of numerous therapeutic approaches, and the majority of success in clinical trials comes from targeting either PI3K or mTOR proteins. This project used a broad-PI3K inhibitor; LY294002 on the basis of its success in previous studies and evidence showing its efficacy at varying concentrations; in particular with cancer cells (Gupta et al 2012, Yu et al 2015). This inhibitor has featured in several studies using the OVCAR-5 and Hec-1A but these are limited (Altomare et al 2004, Kang et al

2012). However, it has been used against similar cell lines (i.e. OVCAR-3, OVCAR-8), as well as cisplatin-resistant cell lines, Thus it was hypothesized that it might have similar effects against OVCAR-5 and Hec-1A (Eckstein et al 2009, Lee et al 2005, Mazzoletti et al 2011). Previously preliminary observations from Schenk (2013), suggested low levels of ERK expression, but conversely high levels of Akt in the OVCAR-5 cell line when treated with an ERK inhibitor; FR180204. This prompted the investigation of an alternate pathway (PI3K), and dual inhibition of the Ras, Raf, MEK, ERK pathway and the PI3K, Akt, mTOR pathway in order to re-sensitise tumour cells to cisplatin.

Single treatment with LY294002 (2 μM, 4 μM and 8 μM) showed no significant decrease in cell metabolism or growth activity of cell lines after 24 or 48 hours of exposure. It is difficult to explain this result given the abundance of literature reporting its efficacy. Hu et al (2002) treated ovarian cancer cells (OVCAR-3) with 5 μM LY294002 and found cell growth to decrease as well as inhibition of PI3K. This would suggest that the concentration chosen in this study should have been sufficient to elicit an effect. For this study it was decide to treat the cells at low concentrations as there is evidence suggesting that the lower the drug concentrations, the less likely there will be side effects *in vivo* if the therapy makes it to clinical trials. It is of interest that Altomare et al (2004) treated OVCAR-4 and OVCAR-5 cell lines with 20 μM LY294002 and found it to have little effect on the cells. They suggested that the cells constitutive activation of Akt increased their resistance to cisplatin through protection from cisplatin-induced apoptosis. This could explain why OVCAR-5 cells did not respond to LY294002 treatment in this study. As for studies treating Hec-1A cells with LY294002, Gagnon et al (2004) found 50 μm treatment to reduce the expression of the Akt protein and increase of apoptosis within these cells. This does not explain the results found in this study. As there is the potential that the low concentrations of LY294002 were insufficient for the inhibition of the cell lines used in this study a single experiment was completed testing the effect of 20 μM LY294002 on the OVCAR-5 and Hec-1A cell lines. This experiment likewise showed no significant decrease in cellular metabolism, though this experiment was only carried out once due to time constraints and as such should be treated with

caution. Previous research has shown that 20 μM is sufficient to inhibit PI3K and to induce apoptosis in ovarian and endometrial cancer cells (Eckstein et al 2009, Yuan et al 2000). In addition, Lee et al (2005) used 25 μM of LY294002 on ovarian cancer cells and this re-sensitised them to cisplatin. With respect to Hec-1A cells, Guo et al (2006) around 50 μM of LY294002 to inhibit Akt activation and activity of the PI3K pathway. These studies suggest that it is possible to not only use LY294002 on ovarian and endometrial cancers at 20 μM and get successful inhibition, but also that it can work in combination with cisplatin and thus could work effectively as a therapy that reduces cisplatin resistance. Without existing data and animal models, it is difficult to say what concentration would be relevant *in vivo*, with respect to LY294002. There are clinical trials involving LY294002 but unfortunately it has a history of adverse side effects (as do many PI3K inhibitors) and thus results using this drug *in vivo* are not available as of yet (Turacli et al 2015, Yu et al 2015).

Another variable in this experiment that may have influenced how the cell lines reacted to the PI3K inhibitor is the time of exposure to the inhibitory agent. Preferable treatments would be expected to begin working after 24 to 48 hours and in some experiments there can be reduction in signalling pathways after as little as 6-12 hours exposure (Kang et al 2012). However, it would still be of interest to investigate the effect of increasing the exposure time to LY294002 to 72 or 96 hours, instead of 24 and 48 hours.

Selective targeting of one pathway may result in compensatory up-regulation in another and vice versa (Cheaib et al 2015). As Schenk (2013) suggested with single FR180204 exposures and as these results further support, there is more than one factor influencing resistant cancers, thus dual inhibition was of interest.

4.3 Dual Pathway Inhibition Did Not Prevent Cellular Proliferation

In the present study the combination experiments used 8 μM FR180204, 8 μM LY294002 and 10 μM cisplatin in various combinations, to investigate the viability of dual inhibition in re-sensitising cell lines to platinum-based

chemotherapy. Despite previous studies implicating re-sensitisation with dual inhibition or combination treatment the results from this experiment did not show any significant reduction of cellular metabolism or cell growth activity after 24 hours exposure. It had been hypothesised that the PI3K inhibitor, in combination with the ERK inhibitor, would reduce any active pathway crosstalk or protein over-expression and reduce cell growth and metabolism to render cells sensitive to the cisplatin treatment. As this was not the case, this may suggest factors that are additional to signalling pathway proteins are influencing cisplatin resistance in the cell lines that were used. Doghman and Lalli (2012) used NVP-BEZ235 (a PI3K/mTOR inhibitor) in combination with FR180204 and found this duo to be synergistic and achieve efficient inhibition of adrenocortical carcinomas proliferation. Yang et al (2013) also used this inhibitor in combination with cisplatin upon human tumour cells and found it inhibited proliferation and induced apoptosis. Dirican et al (2015) similarly to this study used both FR180204 and LY294002 (concentrations not specified) to investigate the possible synergistic apoptotic effects of combination treatment with docetaxel and thymoquinone. They stated that a major benefit of combination therapies is the ability to reduce the development of drug resistance, since tumour cells are less likely to have resistance to multiple drugs simultaneously. Interestingly, Dirican et al (2015) found that LY294002 had no effect upon the combination treated cells while FR180204 did have an effect on the prostate cancer cells, significantly decreasing their viability. This is somewhat relatable to this study, as the cancer cell lines used also did not respond to LY294002. however it differs from the effect of FR180204 treatment in this study and also from that of Schenk (2013).

A theory discussed frequently in literature surrounding cisplatin and the Ras, Raf, MEK, ERK pathway is ERK's role in cisplatin-induced apoptosis and cisplatin resistance. There are studies supporting that cisplatin treatment activates ERK and causes cisplatin-induced apoptosis. On the other hand, there is literature that suggests upstream proteins that are over-expressed can increase ERK activity, thus causing cisplatin resistance and the prevention of apoptosis. Wang et al (2000) demonstrated that ERK2 is activated by cisplatin and that this

activation is necessary for cisplatin-induced cell death. In comparison, Wang et al (2007) found that knockdown of ERK2, decreases MPK-1 (mitogen-activated protein kinase 1) phosphorylation and increases cisplatin sensitivity and thus suggested that ERK protects ovarian cancer cells from cisplatin-induced death. It is possible that the pro-death or the pro-survival roles of MAPKs, like ERK may depend on the type of activation signals it receives in order to commit to one of these roles. Thus further investigation into the way cisplatin interacts with ERK may elaborate how this protein is involved in both pro-apoptotic and anti-apoptotic signalling (Wang et al 2007).

There is the possibility that the Ras, Raf, MEK, ERK pathway may not be critical to, or involved in, cisplatin resistance in Hec-1A and OVCAR-5 cells and that oncogenes within the cell lines may have a role in FR180204's lack of efficacy. Both OVCAR-5 and Hec-1A possess *KRAS* mutations but they do not carry *BRAF* mutations and because of this it raises the question to whether or not *KRAS* mutation is influencing pathways other than the Ras, Raf, MEK, ERK pathway (Schenk 2013). Certainly Ras has been shown to integrate between different pathways and proteins within cells. There is the potential for another pathway, such as JNK or p38 MAPKs, to act in a similar fashion to ERK and to induce cancer resistance, but at the same time to be unaffected by the inhibitors used in this study.

Signalling pathways are integrated into a complex network, and the final response of a signal depends on the balance of activity of the pathways, the cell type, proliferation and differentiation status of tumour cells (Brozovic and Osmak 2007). Thus, investigating signalling pathways can prove difficult, as for the most part we still do not fully understand them and exactly how cellular networks act in response to events such as chemotherapy induction. As such the search for an effective and appropriate target for inhibition and re-sensitisation of cisplatin in cancer cells is complex. In addition, there is at present limited research surrounding endometrial cancers and cisplatin, and combination drug treatments, thus it is difficult to pin-point what may be influencing resistance in these cells.

4.4 Analysis of PI3K and ERK Signalling

Western blots demonstrated that the proteins ERK, Akt and cyclin B_2 were expressed in OVCAR-5 cells. Cyclin B_2 expression in the ovarian cancer cells was lowered after the 8 μM FR + 8 μM LY + 10 μM Cis treatment. This suggests the cell cycle has been affected and that the dual inhibitors may have worked in re-sensitising the cells to cisplatin. However, these results are not consistent with the growth data although it may be that a threshold decrease in protein levels required for an effect on cell growth may not have been reached. Deng et al (2003) found LY294002 treatment to inhibit ovarian cancer cell proliferation, and to induce G1 cell cycle arrest. Although Deng et al (2003) did not investigate cyclin B_2 expression; it suggests that the PI3K inhibitor has the ability to prevent cell cycle activity in ovarian cancer cells. Further Western blots should be completed involving cyclin D_2, as these proteins are present across the entire cell division process thus are a stronger indicator of cell cycle inhibition than cyclin B_2 which is only expressed through G2/M phases (Deng et al 2003).

Akt protein expression was lowered by the 8 μM LY294002 + 10 μM cisplatin treatment. Altomare et al (2004) treated OVCAR-5 cells with LY294002 and observed diminished Akt phosphorylation. Although, as per above, the cellular metabolism and growth activity assays showed no noticeable changes in the cells after this particular treatment. Again there may be a required threshold decrease that was not reached.

ERK expression in OVCAR-5 cells appeared to be lower in comparison to Akt. This supports findings from Schenk (2013) who found there to be high expression of Akt in OVCAR-5 cells, with lower levels of ERK. The blot for ERK shows a faint band with the 8 μM LY + 10 μM Cis treatment, and no band with the 8 μM FR + 8 μM LY + 10 μM Cis treatment. This is unexpected as LY294002 is a PI3K protein inhibitor and has no specificity for any of the proteins within the Ras, Raf, MEK, ERK pathway. However, there is a possibility that due to convergence and cross talk between the two pathways PI3K inhibition may indirectly affect ERK (Cheaib et al 2015). This is supported by Jacob et al (2002)

who suggested that ERK activation was in some way dependent upon the PI3K protein within B cells.

Further Western blot experiments are required to fully elucidate the effects FR180204, LY294002 and cisplatin have upon the OVCAR-5 and Hec-1A cell lines, although the data gathered in this experiment has given an idea towards what may be happening within the cells growth and signalling pathways. However it should be noted that all of these conclusions are based on a single experiment and that the housekeeping proteins did not produce any bands. Thus we are restricted to qualitative assessments of only single replicates and therefore due caution is advised. Had there been no contamination issues and thus sufficient time available to optimise the conditions of the Westerns and to vary out replicates we may have been able to come to more solid conclusions.

4.5 2D and 3D Cell Culture

The comparative data from this study on 2D and 3D cell cultures and how these may affect the response of cell metabolism and activity to inhibitors suggested that there was no significant difference between the two cell cultures. That said, it would have been preferable to have greater numbers of replicates to strengthen any conclusions. However due to time constraints and significant and continuing contamination issues this was not possible (see section 4.6). 2D and 3D cell culture was included in the present study as an aspect of the investigating the effect that a cancer cells microenvironment has on its growth and metabolism in the presence of inhibitors. 3D cell culture is seen as a more appropriate representation of the *in vivo* environment of a tumour cell compared to a 2D culture, thus making it an increasing popular technique in cancer research (Adissu et al 2007, Thoma et al 2014).

Traditionally 2D cell culturing causes cells to lose their tissue-specific functions and morphological organization (Chitcholtan et al 2013). 3D cell cultures aim to restore the 3D architecture that characterises normal tissues and solid tumours. It has been reported that 3D multi-cellular aggregates contain hypoxic and

necrotic areas found in primary tumours and these are thought to be associated with chemo-resistance (Lee et al 2013). Whether the cells are grown in a 2D or 3D microenvironment will also impact on cell shape and the architecture of cell-cell adhesions. Although the results from this study did not show any differences between the two types of culture, this is an area that warrants further investigation.

Lee et al (2013) investigated chemo-sensitivity to cisplatin and paclitaxel in 11 ovarian cancer cell lines and 63% (7/11) of cell lines had significantly increased survival rates against cisplatin in 3D cultures. This suggests that cells in 3D cultures may more accurately reflect characteristics of primary human epithelial ovarian cancer cells *in vivo*, but also be more resistant to chemotherapies as well. These 3D cultures are thus likely to be better for studying the underlying biology of the disease (Lee et al 2013). The importance in utilising alternative cell culturing techniques is invaluable to cancer research. The more precise *in vitro* microenvironments are developed to culture and grow cancer cells, the closer we get to developing ways to treat these tumours. 3D cell culture encourages cell-cell interactions, which are important for growth and metastasis as well as drug resistance. This is highly relevant and important with respect to ovarian and endometrial cancers which are have often metastasized to different locations within the body when the patient is initially diagnosed with the diseases (Zietarska et al 2007).

4.6 Obstructions in Research

As mentioned in previous sections, there were factors that arose that prevented the completion of all the planned experiments in this project. The main reason behind this was cell culture contamination, and as a consequence of this, time constraints prevented critical experiments from being completed. There was the intention of completing 48-hour combination experiments, in addition to further replicate the 24-hour combination experiment, but because of contamination continuously arising this could not be achieved. FR180204 3D treatment experiments were also planned along with extensive western blot analysis.

New cultures of cells were obtained on numerous occasions in order to collect data for these intended combination experiments but the media would become contaminated with bacteria and thus the cells were un-usable. As a result when contamination appeared the experiment was discontinued immediately. New cells were obtained from Christchurch Hospital at the University of Otago School of Medicine and the process of culturing was repeated. After the first round of contamination, a complete clean of the culture room was completed, additionally the CO_2 incubator was cleaned with ethanol (routinely), both flow laminar cell culture hoods were cleaned with ethanol and detergent (TriGene) as well as fumigated, the use of TriGene on top of 70% ethanol was employed to wipe down all apparatus before entering the hood. New base media, trypsin-EDTA, 1x PBS and working media were freshly made on multiple occasions when they were suspected to be the cause of contamination. New pipette tips with filters were purchased to prevent any contamination from this apparatus. This action appeared to resolve the problem for a few weeks but contamination would eventually reappear. To ensure that poor aseptic technique was not a contributing factor to the contamination, several researchers from the laboratory observed aseptic techniques. Nothing obvious was seen that might be the cause of the contamination. Another colleague within the laboratory encountered similar contamination problems around the same time period and additionally there have been previous contamination issues within in the lab (A.Tino personal communication). It is suggested that future research should be completed in a dedicated mammalian lab to reduce the cross over of research between lab workers and allow for the precise elimination of causes of contamination if it arises. As it currently stands research in this multi-user lab is currently carried out on mammalian cells, bacterial cells, plant cells, algal cells and fungal cells.

4.7 Targeting Alternative Signalling Pathways

As mentioned previously there are potential alternative growth and signalling pathways that may influence a cancer cells resistance to cisplatin. Pleiotropic interactions with other parallel pathways, or interacting proteins may allow tumour cells to escape from PI3K inhibition. The published literature describing Akt's interactions with Ras as well as Ras' interaction with the likes of JNK, P38-MAPK and PKC proteins and pathways, may be pertinent to the responses of OVCAR-5 and Hec-1A cells that are reported in this study.

SAPK/JNK (or stress activated protein kinase/Jun N-terminal kinase) is a serine-threonine kinase, which is activated in response to cellular stress (i.e. UV radiation, cytotoxic drugs and reactive oxygen species (ROS)). Mammalian cells have 3 JNK genes; JNK1, JNK2 and JNK3 and have at least 10 isoforms (Brozovic and Osmak 2007). JNK1/2 phosphorylates the N-terminus of its substrate c-Jun. Phosphorylation of c-Jun increases the activation of AP-1 (a transcription factor that enhances gene expression), which can influence processes such as proliferation, differentiation and apoptosis. Both JNK and ERK are activated by cisplatin-induced DNA damage and are required for cell survival after cisplatin treatment (Hayakawa et al 2000). It is suggested that future studies could investigate the JNK pathway, as the literature suggests its activity to be similar to that of ERK and its associations with cancer. Another MAPK pathway that may be pertinent is the p38/MAPK pathway. Rho family GTPases activate the p38 protein. Following activation, several protein kinases are activated through phosphorylation. $P90^{MAPKAPK2}$ and MSK1/2 phosphorylation lead to activation of transcription factors (i.e. CREB) that regulate cell survival. The duration of activated JNK and p38 signal is a critical factor in determining cell survival or apoptosis. Resistance is due, in part, to lack of prolonged activation of these stress kinases (Mansouri et al 2003). This could also be the case with ERK and may be a factor in why this study did not observe inhibition.

Ras, Raf, MEK, ERK and PI3K, Akt, mTOR pathways can negatively regulate each other's activity. Evidence of this includes IGF-1 (insulin growth like factor 1)

inducing cross-inhibition between Akt and Raf and additionally Akt can negatively regulate ERK activity through phosphorylating inhibition sites on Raf's N-terminus (Mendoza et al 2011). In addition, once ERK, RSK, Akt and S6K proteins are active, any of these can act upon the same substrate, sometimes in concert, to promote cell survival, proliferation, metabolism and motility. Ras proteins have strong interactions with both the PI3K and ERK pathways, influencing, and indirectly interacting with the majority of proteins involved within these pathways. For example Ras-ERK interaction can lead to mTORC1 activity by ERK, whilst Akt can be phosphorylated via crosstalk with Ras also (Cheaib et al 2015).

Similarly to Ras, it is thought that signalling molecules and receptors that activate the pathways discussed may be potential targets for inhibition. This idea is especially pertinent to endometrial cancers, where EGFR is commonly up regulated, and thus could be influencing the tumour cells activity or cellular signalling pathways (Jayson et al 2014). One inhibitor that has already been developed and is in clinical trials is Bevacizumab. This is a humanised monoclonal antibody used against VEGF. It did not have great results in phase II trials, but has recently shown big improvements in survival in lung cancer patients and has been treated on platinum resistant cancers with docetaxel (Kelland 2007, Wenham et al 2013). Another factor commonly associated with endometrial cancer is high levels of insulin. Insulin binding to an insulin receptor triggers the activation of IRS-2 and activates PI3K/Akt pathways (Slomovitz and Coleman 2012).

The key point to take away from all of the above is that resistance is likely to be highly multi-factorial. This is well put by Siddik (2003) who state that several mechanisms are encountered simultaneously within the same tumour cell, and that a high level of resistance is a net effect, of several unrelated mechanisms (Parker et al 1991).

4.8 Considerations for Future Research Endeavours

As described above a number of experiments could not be done due to the significant contamination issues. These included a comprehensive investigation of protein expression using Western blot analysis. This is highly recommended to continue investigating, as it would not only ultimately show whether the inhibitors were effective or not, but also give an insight into how the signalling pathways were reacting to the treatments.

As mentioned prior experiments treating the cell line with higher concentrations of inhibitors would be beneficial, as well as exposing the cells to the inhibitors for an increased time period of up to 96 hours.

Another idea to come out of the single treatment experiments was the potential of using alternative inhibitors, instead of LY294002 or FR180204. An example of this, which is mentioned frequently in the literature, is PI3K/mTOR inhibitors. Serra et al (2008) recommend the use of NVP-BEZ275 (a PI3K/mTOR inhibitor) that successfully blocks cellular proliferation. A PI3K/mTOR inhibitor works by inhibiting both PI3K, and mTOR. This is important as mTOR is not only under the control of PI3K, but integrates many other inputs from outside factors, thus highlighting the downside to using a PI3K specific inhibitor (Dienstmann et al 2014). Sheppard et al (2013) used the PI3K/mTOR inhibitor, PF502 alongside PD901 (a MEK inhibitor) as a dual inhibition system and found a reduction in proliferation of OVCAR-5 cells (Engelman 2009). This provides encouraging results as this inhibitor is shown to work effectively with other inhibitors in a dual system as well as working to effectively inhibit proliferation of one of the cell line used in this study.

An undesirable outcome of inhibition and development of cancer treatment, are *in vivo* side effects to the drugs. An example of this is the pharmaceutical inhibition of Akt, which has the potential to impact glucose metabolism, due to Akt's critical role in insulin signalling and maintenance of glucose homeostasis (Altomare et al 2004, Whiteman et al 2002). One potential approach to

minimising toxicity when targeting pathways is to selectively inhibit, for example only those Akt isoforms within a specific tumour that have proven to promote tumour progression (Hanrahan et al 2012). This idea proposed is something to investigate once signalling pathways or individual proteins within these specific cell lines are identified as causes of resistance, then more specific treatments can be developed to target particular overactive proteins or even specific isoforms for each cell line.

With future development of drugs hopefully comes a reduction in side effects or that the use of effective combination therapies will possibly reduce this. When drugs with different effects are combined, each drug can be used at its optimal dose, without intolerable side effects. This was the desired idea behind this study, using lower concentration to reduce the likelihood of adverse side effects *in vivo*. This is why this research used lower levels of inhibitors to begin with, as the idea of having the lowest level of inhibitor needed to sensitise cells to cisplatin is ideal for reducing the amount of toxic side effects, and may even allow the reduction in cisplatin treatment also.

There is the possibility for future research following this study to use alternative cell viability assays (Kepp et al 2011). Although crystal violet is a method that is relatively accurate, over time it has been largely superseded by more modern options, which provide faster and more accurate results of cell numbers and cellular death. The first and most commonly used option is propidium iodide staining (Sheppard et al 2013). This dye is membrane impermeable thus is generally excluded from viable cells and only stains dead cells (Martinelli et al 2013). This test also can differentiate between necrotic, apoptotic and normal cells. Another option, which utilises a dye, is trypan blue. Similarly to propidium iodide, is not absorbed through the membrane of live cells. It only traverses the membrane of dead cells, and these can then be observed and counted through microscopy (Dirican et al 2015). Again another dye used to stain dead cells is 7-aminoactinomycin D, this chemical compound has a strong affinity for DNA and is used as a fluorescent marker for DNA in fluorescence microscopy and flow cytometry as it cannot pass through intact cell membranes like many others

(Zembruski et al 2012). Another way of measuring cell death is through lactate dehydrogenase (LDH) expression. This enzyme is found in cells membranes catalysing the reaction between lactate and pyruvic acid. It is readily detected when cell membranes are no longer intact (Doherty and Cleveland 2013). The final method is the Annexin A5 affinity assay (Martinelli et al 2013). This is used to quantify the number of cells undergoing apoptosis. Annexin A5 is used to tag apoptotic and dead cells, and the numbers are counted either by flow cytometry or fluorescence microscopy. Having the ability to use a more precise and accurate tests would aid the results of dual inhibition, in particular providing a good representation of live or dead cells would aid in quantifying to efficacy of inhibitors.

4.9 Conclusions

In conclusion this study has demonstrated that both the single treatments of LY294002 and combination treatments using FR180204 and LY294002 are not effective in rendering Hec-1A or OVCAR-5 cell lines sensitive to cisplatin. The most plausible explanations for this are that the concentrations of, and/or duration of exposure to, LY294002 were too low/short to elicit an effect in these cells, or there could be the involvement of alternate pathway proteins. The results of this study add to research surrounding cisplatin resistance and the treatments being developed to combat it. This research may not have fully tested the hypotheses set out and provided the basis for a novel combination therapy, though none the less it has resulted in the opportunity to investigate various approaches in the future that have the potential to provide information towards strategies that may aid those who suffer from platinum-based drug resistant cancers.

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APPENDIX

Protein Concentration of Cell Lysates

Prior to Western blotting the concentration of each cell lysate was used to determine the volume required to load 10 µg of protein for each treatment. This was achieved by generating a standard curve using known concentrations of BSA (Figure A1). Absorbances at 562 nm of protein samples with working reagent added were measured and used to determine the protein concentration via the standard curve. Protein concentration was then used to determine the volume for 10 µg of protein for each sample (Table A1).

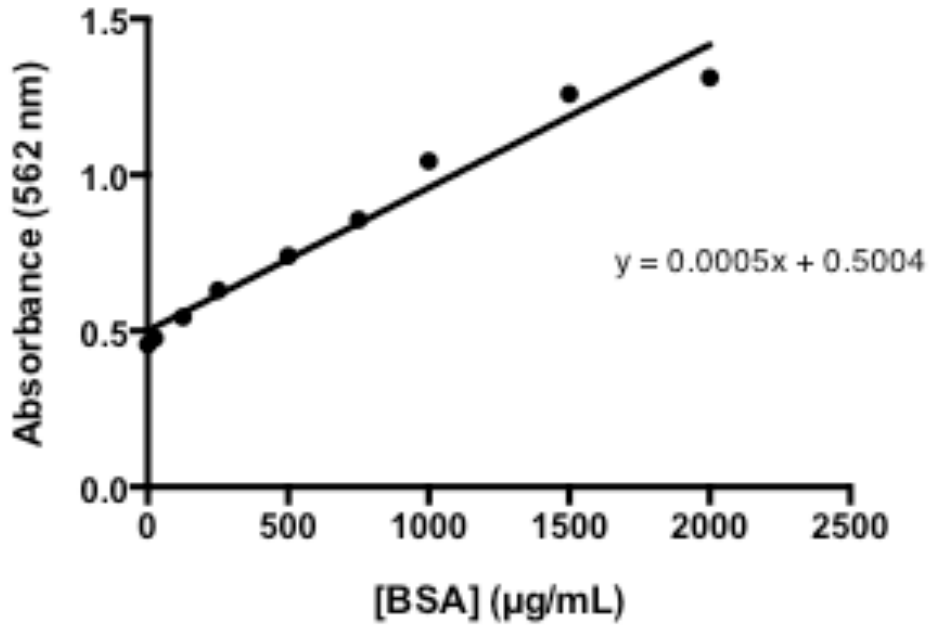

Figure A1: Standard curve generated for determining protein concentrations of the OVCAR-5 cell lysates treated with varying combinations of FR180204, LY294002 and cisplatin for 24 hours. Curve was generated using BSA standards of known concentration, measuring at 562 nm of standards mixed with working reagent. Standards were measured in duplicate.

Table A1: Absorbance of cell lysates mixed with working reagent at 562 nm, subsequent protein concentration, and volume required for 10 µg of protein per sample for OVCAR-5 cells treated with various concentrations of FR180204, LY294002 and cisplatin for 24 hours.

Hec-1A 24hr Combination Treatment		Control	8 µM FR180204 + 8 µM LY294002	8 µM FR180204 + 10 µM cisplatin	8 µM LY294002 + 10 µM cisplatin	8 µM FR180204 + 8 µM FR180204 + 10 µM cisplatin
A_{563nm} (blank adjusted)	Replicate #1	287	139.2	176.8	154.6	505
	Replicate #2	254.6	129	320.8	337	361.2
	Replicate #3	180	50.6	175.2	337	209.4
[Protein] (µg/mL)	Replicate #1	0.287	0.1392	0.1768	0.1546	0.505
	Replicate #2	0.2546	0.129	0.3208	0.337	0.3612
	Replicate #3	0.180	0.0506	0.1752	0.337	0.2094
Volume for 10 µg protein	Replicate #1	34.84	71.83	56.56	64.68	19.80
	Replicate #2	39.27	77.51	31.17	29.67	27.68
	Replicate #3	55.55	197.62	57.07	29.67	47.75