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The effect of AKT pathway inhibition on reversal of platinum resistance in ovarian cancer: a translational approach

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Declaration of Originality

I declare that the content of this thesis is the product of my own work and that all the sources used for the preparation of this thesis and all the assistants received to complete this work have been acknowledged.

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To my parents

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Abstract

Dysregulation of the phosphotidylinositol-3-kinase (PI3-kinase)/AKT signalling pathway is one of the most frequent mutational events in various human malignancies, including ovarian cancer. Increased PI3K/AKT activity in malignant cells is associated with resistance to chemotherapeutic agents.

GSK2141795 is a potent pan-AKT kinase inhibitor, being developed for ovarian and other cancers. The aim of the present study was to evaluate the ability of GSK2141795 to restore platinum sensitivity to platinum-resistant ovarian cancer cell lines. Effective pharmacodynamic (PD) biomarkers are critical for the implementation and assessment of targeted therapeutics. The second aim of this study was to explore the utility of using fluoro-deoxy-glucose positron emission tomography (FDG-PET) imaging as a PD marker by correlating changes in glucose metabolism (as measured by changes in FDG uptake) with changes in AKT pathway biomarkers, as an alternative to invasive tumour biopsies. The third aim of this study was to assess the role of DNA-PK mediated activation of AKT, compared to PI3K, in mediating drug resistance.

The results demonstrate that GSK2141795 synergistically enhances cisplatin induced apoptosis in platinum-resistant ovarian cancer cells, grown as either 2-dimensional monolayers or 3-dimensional multi-cellular tumour spheroids (MTS). Similar results were achieved when cisplatin was combined with the DNA-PK inhibitor NU7441, but not in combination with the selective PI3K inhibitor GSK2126458 *in vitro*. *In vivo*, combination of GSK2141795 or NU7441 with cisplatin led to superior tumour growth inhibition in murine xenografts, compared to either agent alone. In all three model systems (2D monolayers 3D MTS and *in vivo* xenografts), GSK2141795 decreased levels of phospho-PRAS40 and FDG uptake. FDG uptake and pPRAS40 expression were strongly correlated. In addition, reverse

phase protein arrays (RPPA) was used to study the effects of GSK2141795 on cell signalling pathways *in vitro*, *in vivo* and using tumour biopsies from a phase I clinical trial of GSK2141795 in ovarian cancer. This identified a signature of AKT-pathway inhibition that includes consistent changes in phosphorylation of S6, 4E-BP1 and AKT itself.

Taken together, this report shows that GSK2141795 inhibits AKT signalling in platinum-resistant ovarian cancer cells, and can reverse platinum resistance in pre-clinical systems, and merits clinical exploration. In addition, the results demonstrate significant differences in the role of DNA-PK and PI3K mediated AKT activation in modulating the response to platinum chemotherapy, and support further clinical development of AKT or DNA-PK inhibitors in combination with cisplatin to overcome platinum resistance in ovarian cancer. FDG uptake was also found to be potential non-invasive PD biomarker, subject to clinical validation, for guiding dose selection in ovarian cancer patients. RPPA also identified a proteomic PD signature that could be used for pre-clinical development and applied to clinical tumour biopsies.

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1 Introduction

1.1 Ovarian Cancer

Ovarian cancer is the most lethal gynaecological malignancy and the fifth most common cancer in women in developed countries. In 2010, 7,011 new cases of ovarian cancer were diagnosed in the UK. The cause of ovarian cancer is unknown; however, risk factors for developing ovarian cancer include older age, low parity, and a family history of breast and ovarian cancer. Ovarian cancer is a heterogeneous disease, with a 5-year overall survival of only 35-40%. This is due to the presence of advanced disease in most patients at initial presentation, and the development of drug resistance. Novel standards for reversal of drug resistance are urgently required to improve the outlook for this disease.

1.2 Classification of Ovarian Cancer

Ovarian cancer is classified according to the histology of the tumour into three main groups: epithelial ovarian cancer (EOC); sex cord-stromal tumours and germ cell tumours (Cho and Shih Ie 2009). More than 90% of ovarian cancers are epithelial, and historically were thought to be derived from the mesothelial cells that cover the ovarian surface or that line sub-surface inclusion cysts (Dubeau 2008). Recent histopathological studies, however, suggest that the majority of high-grade, serous carcinomas arise from high-grade intraepithelial serous carcinomas in the fallopian tube, which then spread to the ovary (Lee, Miron et al. 2007).

Ovarian tumours of epithelial origin are classified as benign, borderline / low malignant potential (LMP) or malignant, depending on the extent of cellular atypia and invasion of the basement membrane. Tumours are further sub-classified into four main histotypes: serous (60-70%), endometrioid (10-20%), mucinous (5%) and clear cell (5%). These histotypes differ with regard to the frequency of different genetic alterations, gene expression profiles,

immuno-histochemical markers, clinical response to chemotherapy and prognosis (Kaku, Ogawa et al. 2003, Carlson, Roh et al. 2008)

More recently, these ovarian tumours have been divided into type I and type II tumours. Type I tumours are composed of low grade serous, low grade endometrioid, clear cell, mucinous and transitional carcinomas. Type II tumours include conventional high grade serous carcinoma, high grade endometrioid, undifferentiated carcinoma and malignant mixed mesodermal tumours. Type I and type II tumours have distinct molecular and clinical features. Type I tumours are generally slow growing, lack TP53 and BRCA1/2 mutations, have a high frequency of RAS pathway activation, and are chromosomally stable. In contrast Type II tumours have a high frequency of TP53 mutation, rarely have RAS pathway mutations, usually have dysfunctional BRCA1/2 and are genomically unstable with widespread DNA copy number changes (Shih Ie and Kurman 2004, Kobel, Kalloger et al. 2008, Vang, Shih Ie et al. 2009, Bowtell 2010).

1.3 Diagnosis

Most patients with EOC present, initially, with non-specific symptoms of bloating, early satiety and alteration of bowel or bladder function. Clinical examination, complemented by transvaginal ultrasound (US), usually shows evidence of ovarian masses with increased vascularity (van Nagell, Higgins et al. 1990). The tumour marker CA125 is often raised and is used, in combination with imaging, to make a provisional clinical diagnosis of ovarian cancer, using pre-operative algorithms such as the RMI (Risk of Malignancy Index). Subsequent pre-operative tumour staging is performed using computed tomography (CT) of the chest abdomen and pelvis. In addition, magnetic resonance imaging (MRI) and positron

emission tomographic imaging (PET) are sometimes used (Das and Bast 2008, Su, Graybill et al. 2013).

1.4 Staging

In EOC, the tumour stage is one of the most important prognostic factors and definitive staging is performed at debulking surgery. EOC is classified into four stages. Stage 1 EOC is defined as a tumour confined to the ovaries, and is further sub-divided into: stage 1a, 1b and 1c disease. In Stage 1a disease, only one ovary is involved. In stage 1b, both ovaries are involved and in stage 1c, there is evidence of cancer cells in peritoneal washing and/or presence of cancer cells on the surface of the ovary or of the tumour cyst rupture. Stage 2 EOC refers to spread of the cancer to other pelvis organs, such as the uterus or bowel surface. Stage 3 disease is defined as spread of the cancer beyond the pelvis with involvement of the omentum and/or upper abdominal cavity, usually. Finally, stage 4 disease refers to spread beyond the peritoneal cavity, most commonly due to the presence of pleural effusions, and/or with intra-parenchymal visceral liver/spleen metastases such as liver and spleen (Cannistra 2004, Cho and Shih Ie 2009).

1.5 Treatment

The standard management of EOC comprises a combination of debulking surgery and combination chemotherapy (Coleman, Monk et al. 2013)

1.5.1 Surgery

EOC is unique in oncology in that debulking surgery is performed even in the context of metastatic peritoneal disease (Ie Stage 1-3 disease). The three goals of initial debulking surgery for patients with suspected ovarian cancer are: establishing a histological diagnosis,

staging and achieving complete macroscopic cytoreduction (Coleman, Monk et al. 2013) as the extent of residual disease after debulking surgery is the most significant prognostic factor in EOC.

One of the first reports on debulking surgery in EOC indicated that patients with post-surgery residual tumours greater than 1.5cm in size, compared to patients with residual tumours of 1.5cm or less, had a median overall survival of 12 months versus 26 months, respectively (Griffiths 1975). Since then, the definition of optimal debulking has varied between less than 2cm of residual disease to no gross residual disease (Fader and Rose 2007). Currently, cytoreductive surgery, resulting in less than 1cm maximum diameter of residual disease, is classified as 'optimal debulking', whereas those resulting in any larger residual tumours are defined as 'suboptimal' (Hoskins, McGuire et al. 1994, Bristow, Tomacruz et al. 2002). However, the best outcomes are achieved in patients who have a complete macroscopic debulk and should be the primary goal of surgery (du Bois, Reuss et al. 2009, Coleman, Monk et al. 2013). Debulking is thought to impact prognosis by removing hypoxic chemo-resistant tumour cells, as well as increasing drug penetration into residual disease by reducing tumour bulk (Harter, Hilpert et al. 2009).

Historically, patients have undergone primary debulking surgery followed by adjuvant chemotherapy. An alternative strategy to primary cytoreduction is neo-adjuvant chemotherapy with interval debulking. The rationale of neo-adjuvant therapy is to achieve 'chemical' cytoreduction, to increase the likelihood of optimal debulking at interval surgery. However, there have been concerns that if the benefit from cytoreductive surgery is related to increased drug penetration, then this would be compromised in the pre-surgical cycles of chemotherapy with a neo-adjuvant approach. Other concerns have included the difficulty of surgery post-chemotherapy and potential increase in the risk of infective complications

(Vergote, De Wever et al. 2000, Schwartz 2002, Ivanov, Ivanov et al. 2004, Le, Faught et al. 2005, Inciura, Simavicius et al. 2006, Lee, Kim et al. 2006).

The largest study to date, to address this issue, was performed by Vergote et al through the EORTC. In this study stage, IIIC-IV EOC patients were randomised between primary surgery followed by adjuvant combination platinum-taxane chemotherapy versus 3 cycles of neo-adjuvant chemotherapy using the same drugs, followed by interval debulking surgery (IDS), and at least a further 3 cycles of completion chemotherapy. The study showed that 41% of patients achieved optimal debulking after primary surgery, compared to 80% at IDS. Duration of surgery was also shorter during IDS, required fewer blood transfusions and was associated with less surgical mortality. Patients who have been treated with neoadjuvant chemotherapy also had non-inferior disease-free and overall survival, compared to primary surgery (Vergote, Trope et al. 2010). Extent of residual disease, whether performed after neoadjuvant chemotherapy or as primary treatment, was the strongest independence variable in predicting overall survival, and complete cytoreduction remains the main goal of surgery. The results of this study have led to a greater use of neoadjuvant chemotherapy and IDS, compared to primary surgery in EOC.

1.5.2 Chemotherapy

First-line chemotherapy for ovarian cancer has evolved in the past three decades. The platinum compounds, cisplatin and carboplatin, are the most active single agents in ovarian cancer and form the backbone of current therapy. Since the publication of GOG 111, which compared cisplatin in combination with cyclophosphamide versus paclitaxel, with an apparent survival advantage of the platinum-taxane arm, this has become standard first-line chemotherapy (McGuire, Hoskins et al. 1996). Carboplatin was introduced into the clinic in the 1980s and has been shown, in three randomised trials in combination with paclitaxel, to

be as effective as cisplatin-paclitaxel, but with significantly less toxicity, and since then it replaced cisplatin in most centres (Neijt, Engelholm et al. 2000, du Bois, Luck et al. 2003, Ozols, Bundy et al. 2003). First line chemotherapy with paclitaxel (175 mg/m^2) and carboplatin (AUC 5-6) is usually given every three weeks, for 6 cycles. Although the initial response to this treatment is excellent, with a response rate of more than 80% with 40-60% complete responses, the majority of patients relapse with a median progression free survival of only 18 months (Greenlee, Hill-Harmon et al. 2001, Agarwal and Kaye 2003).

In an interesting clinical trial in the early 1990s, patients with disease progression, after initial platinum-based chemotherapy, were re-challenged with carboplatin. This study uniquely showed that, in EOC, the response rate to carboplatin re-challenge was inversely proportional to the platinum free interval (PFI; time from last dose of carboplatin) (Gore, Fryatt et al. 1990). Tumours which relapse with a PFI greater than 12 months are classified as platinum sensitive and have response rates to platinum re-challenge of greater than 40%. Tumours which relapse between 6 and 12 months are classified as partially sensitive (response rates of 20-30%) and those which relapse with a PFI less than 6 months are classified as platinum-resistant (response rate 10%) (Harries and Kaye 2001, Agarwal and Kaye 2003). In the case of patients with first platinum sensitive relapse (PFI >6months), the current standard of care is to use a platinum-based doublet chemotherapy regimen. This is based on the results of several trials that have shown a survival benefit of combination over single agent carboplatin chemotherapy, in this setting, using paclitaxel, gemcitabine, and liposomal doxorubicin (Pfisterer, Plante et al. 2006, Sufliarsky, Chovanec et al. 2009, Wagner, Marth et al. 2012, Lawrie, Bryant et al. 2013). Subsequent relapse after second line therapy is similarly managed, based on PFI. With subsequent relapse, response rates to platinum chemotherapy falls with, ultimately, all patients developing platinum resistance (Agarwal and Kaye 2003).

In patients with platinum-resistant disease, liposomal doxorubicin and weekly paclitaxel are most commonly used. Other agents used include topoisomerase inhibitors such as topotecan. However, only small numbers of platinum-resistant tumours respond to these agents, with relatively short response durations (ten Bokkel Huinink, Gore et al. 1997, Rose, Blessing et al. 2003, Gordon, Tonda et al. 2004 , Naumann and Coleman 2011).

Two recent strategies for improving outcomes following first-line therapy have shown benefit. First, a study led by the Japanese Gynecologic Oncology Group (JGOG) has shown that administration of 3-weekly carboplatin, in combination with weekly paclitaxel, results in improvement in progression-free and overall survival compared to the current standard of 3-weekly dose scheduling (Katsumata, Yasuda et al. 2009). Currently, these results are being tested in the international ICON8 study (www.clinicaltrials.gov). Second, the addition of bevacizumab, in combination with carboplatin-paclitaxel chemotherapy, followed by maintenance bevacizumab in two phase III trials (GOG218 and ICON7) has also demonstrated improved PFS in comparison to standard carboplatin-paclitaxel, However, the benefit appears to be most marked for patients with sub-optimally debulked stage III, and stage IV EOC (Burger, Brady et al. 2011, Perren, Swart et al. 2011).

1.6 Survival

Patients diagnosed with Stage 1 EOC have a greater than 90% 5-year overall survival. The 5-year overall survival following surgery and adjuvant chemotherapy for Stage 2 disease is approximately 55-65%. At the time of diagnosis, most patients are in the advanced stage of the disease (Stage 3 and 4) in which the 5-year survival rate decreases dramatically to less than 30% (Agarwal and Kaye 2003, Cannistra 2004, Jemal, Siegel et al. 2008).

1.7 Drug Resistance in Ovarian Cancer

Most patients with EOC are initially chemo-sensitive but eventually develop chemo-resistant disease. Understanding the mechanism of resistance and development of novel therapeutic strategies for overcoming drug resistance are, therefore, vital to improving outcomes in this disease.

Chemo-resistance in ovarian cancer is very poorly understood. Drug resistance can arise due to cancer cell-specific genetic and/or cellular abnormalities, as well as pharmacokinetic and tumour micro-environment-derived effects (Agarwal and Kaye 2003). For example, alterations in drug influx and efflux, due to changes in cell membrane transport proteins, can affect intracellular drug concentration and has been shown to correlate with chemo-resistance *in vitro* (Borst, Evers et al. 2000). Tumour cell capacity for drug-induced damage repair can also result in resistance (Dijt, Fichtinger-Schepman et al. 1988). Drug inactivation within the cell and changes in the concentration of the intracellular drug target, as well as alteration in binding affinity of the drug to target proteins, are other potential mechanisms of drug resistance (Giannakakou, Sackett et al. 1997, Kavallaris, Kuo et al. 1997, Sale, Sung et al. 2002). In addition, increased DNA-damage repair mechanism, or prevention of apoptosis, may result in increased viability and therefore resistance to platinum (Dijt, Fichtinger-Schepman et al. 1988, Dabholkar, Bostick-Bruton et al. 1992, Dabholkar, Vionnet et al. 1994, Brown, Hirst et al. 1997). Recently, the role of the microenvironment and specific extracellular matrix (ECM) proteins, has been explored as potential new targets for reversal of drug resistance (Tredan, Galmarini et al. 2007)

It is also important to consider that cytotoxic agents are often more effective against proliferating cells. However, even in rapidly proliferating tumours, there is always a proportion of cells in a quiescent state and these cells show a degree of resistance to cytotoxic agents, compared to proliferating cells (Shah and Schwartz 2001). In 2010, Cooke

et al provided evidence by genomic analysis of cell line series, derived from high-grade serous carcinomas before and after platinum resistance, in support of the model for resistance in ovarian cancer in which pre-existing intra-tumour genetic heterogeneity is a major factor in determining relapse with resistance disease (Cooke, Ng et al. 2010).

1.8 Strategies for Reversing Platinum Resistance

Chemical modifications of DNA bases (adducts) are common types of DNA damage introduced by reactive cytotoxic drugs that covalently bind DNA and interfere with replication fork. These DNA-damaging agents are grouped into two groups; bifunctional agents and monofunctional agents. Monofunctional agents have one active site and modify single bases (Hsiang, Lihou et al. 1989, Helleday, Petermann et al. 2008).

Bifunctional agents such as platinum base agents cisplatin and carboplatin, have two reactive sites and crosslink two DNA bases within the same strand (intra-strand crosslinks) or on opposite DNA strand (inter-strand crosslinks). Inter-strand crosslinks cause more severe damage by blocking the replication fork.

Carboplatin, cisplatin and oxaliplatin are three most commonly used DNA damaging chemotherapeutic drugs used in clinic to treat solid cancers (Rosenberg, VanCamp et al. 1969). The active species of these platinum-containing anti-cancer drugs including is formed by aqueous hydrolysis as these compounds enter the cells. This active form of the drug interacts with DNA, RNA and protein but the cytotoxic effect of the compound is mediated via the formation of DNA intrastrands and interstrands (Lawley and Phillips 1996, Noll, Mason et al. 2006). The major problem associated with using these drugs is the emergence of platinum resistance in patients. This resistance can be either intrinsic or occur during

cyclical treatment (Helleday, Petermann et al. 2008). Many researches are currently focused on finding additional agents to be added to the treatment, to overcome platinum resistance in patients.

1.8.1 Targeting the tumour microenvironment

Interaction between tumour cells and the stroma (ECM) may result in resistance to chemotherapy-induced apoptosis (Elliott and Sethi 2002). Production of collagen VI by tumour cells results in resistance to chemo-therapy (Sherman-Baust, Weeraratna et al. 2003). In addition, many genes up-regulated in resistant cells are related to ECM. For example, COL6A3 encoding for collagen alpha-3(VI) chain, one of the alpha chains of type VI collagen, is one of the most expressed genes in chemo-resistant cells. Moreover, growing platinum-sensitive cells in rich collagen VI condition promotes resistance to platinum-based chemotherapy. Staining of tumours with collagen VI antibody indicates expression of collagen VI in tumour cells and also reorganisation of ECM around tumour cells (Sherman-Baust, Weeraratna et al. 2003). Remodelling of ECM may promote resistance in tumours when exposed to chemotherapy reagents, therefore a component of ECM may represent novel targets for overcoming chemo-resistance in cancer therapy (Shahzad, Lopez-Berestein et al. 2009).

1.8.2 Targeting Pharmacokinetic Factor

1.8.2.1 Dose of Chemotherapy

Chemotherapy is usually given to patients at close to the maximal tolerated dose (MTD) to achieve maximum tumour cell death. There is some correlation between dose and survival in advanced ovarian cancer (Kaye, Paul et al. 1996) In this clinical trial, patients with advanced

ovarian cancer received 6 cycles of cyclophosphamide-cisplatin at a dose of 50 mg/cm^2 and 75 mg/cm^2. The higher dose of cisplatin resulted in a significant increase in median survival initially (Kaye, Paul et al. 1996). However, the survival benefit decreased over time (Vasey 2005).

Alternatively, intraperitoneal (IP) administration of chemotherapy can increase the local concentration of the cytotoxic agent without increasing total dose by direct delivery, into the intraperitoneal cavity, which is the predominant site of tumour in ovarian cancer (Pinato, Graham et al. 2013). However, patients with IP administration of cisplatin and taxane experienced higher rates of grade III/IV gastro-intestinal toxicity in trials and poorer quality of life, compared with patients who received IV (Armstrong, Bundy et al. 2006). Despite this there is a consistent increase in survival in patients treated with IP chemotherapy. These trials were limited by differences in dose and schedule between treated arms. Hence, a phase III trial PETROC is ongoing to determine the relative benefits of IP versus IV chemotherapy in EOC (www.clinicaltrials.gov).

1.8.2.2 Improving drug delivery

Poor drug delivery to the tumour may result in low or no response to chemotherapy in cancer patients. Low bioavailability of drug, high plasma protein binding, high first-pass metabolism and low tissue binding are some of the factors that can result in a drug concentration lower than its effective concentration in the tumour. To date, some novel delivery systems have been introduced to improve the delivery of chemotherapeutic agents to tumour cells such as liposomes and polymer-drug carriers (Duncan 2003, Harris and Chess 2003). Liposomes achieve increased tumour-cell delivery by exploiting the increased permeability of tumour vasculature relative to normal capillaries. Gene-directed enzyme prodrug therapy and antibody-directed enzyme prodrug therapy are other approaches developed to increase tumour cell-specific drug exposure (McNeish, Green et al. 1998, Houba, Boven et al. 2001).

1.8.3 Targeting Cancer Pathways

To date, many small-molecule inhibitors and monoclonal antibodies, targeting key molecules of signalling pathways involved in cell growth, cell survival, metastases, angiogenesis and other cancer hallmarks have been developed and entered clinical trials. This includes small molecule inhibitors of MEK, PARP, VEGF, mTOR, AKT and PI3K (Hainaut and Plymoth).

1.8.3.1 DNA repair pathways and their components

Many DNA-damaging agents currently used in treatment of cancer trigger apoptosis by causing DNA double-strand breaks. The toxicity of DNA-damaging drugs can be reduced by the activity of several DNA-repair pathways. Different DNA-damage agents trigger different mechanisms of DNA repair (Helleday, Petermann et al. 2008).

Mismatch repair (MMR)

The base substitution mismatches and insertion-deletion mismatches generated during DNA replication, are corrected by MMR pathway. Activation of the MMR pathway by platinum-induced DNA damage normally triggers apoptosis (Blommaert, Floot et al. 1998, Perez 1998). One of the causes of drug resistance is Mismatch repair (MMR) deficiency within the cancer cell. Defects in MMR genes which code for hMLH1 and hMSH2 result in the failure of tumour cells to recognise DNA damage. MMR deficiency may result in the resistance of ovarian cancer cells to a wide range of DNA-damaging agents, including doxorubicin and cisplatin (Brown, Hirst et al. 1997). However, sensitivity to oxaliplatin, a platinum analogue, is independent of the MMR status of tumour cells and it has been hypothesised that oxaliplatin can be effective in platinum-resistant patients with MMR deficiency (Fink, Nebel et al. 1996). Results of a Phase II clinical study in platinum-resistant ovarian cancer patients indicated

that oxaliplatin can be comparable with paclitaxel, in terms of efficiency and safety, if given to patients every three weeks (Piccart, Green et al. 2000). In addition, MLH1 promoter methylation has been associated with drug resistance. In a clinical study, plasma DNAs of patients with epithelial ovarian cancer, enrolled in the SCOTROC1 Phase III clinical trial, were examined for methylation of hMLH1 CpG island before carboplatin/taxol chemotherapy and at relapse. It was shown that methylation of hMLH1 is increased in relapsed tumours and 34 of 138 relapse samples had hMLH1 methylation which was not detected before chemotherapy. Those patients had a worse outcome, following further chemotherapy, compared with the patients in whom methylation did not occur (Gifford, Paul et al. 2004). Moreover, demethylating agent DAD sensitised MMR-deficient, drug resistant ovarian and colon tumour xenografts ,that were MLH1 negative because of gene promoter hypermethylation, to temozolomide, carboplatin, cisplatin and epirubicin. However, DAD alone had no effect on tumour growth rate. DAD treatment did not sensitise the same xenografts to taxol and also did not increase chemo-sensitivity in tumour xenografts which had deficient MMR because of hMLH1 mutation (Plumb, Strathdee et al. 2000).

Nucleotide Excision Repair (NER)

NER pathway is responsible for removal of DNA lesions that distorts the DNA double helix or block DNA replication and transcription. NER plays an important role in mediating resistance to platinum-based agents (Rabik and Dolan 2007). The excision repair cross-complementation group 1 (ERCC1) is one of the proteins playing a major role in mediating this pathway (Bergstralh and Sekelsky 2008). *In vitro* association between increased expression of ERCC1 and resistance to cisplatin has been demonstrated in ovarian cancer cells (Ferry, Hamilton et al. 2000). Also, silencing ERCC1 expression using RNA interference resulted in sensitivity to platinum agents in platinum-resistant ovarian cancer cells (Selvakumaran, Pisarcik et al. 2003). Also, the level of ERCC1 is significantly high in

clear cell ovarian cancer tumours which are known to be relatively platinum-resistant and having poor prognosis (Reed, Yu et al. 2003).

Homologous recombination (HR)

Replication associated DSBs are repaired by homologous recombination (HR) and other replication-associated repair pathways. In the homologous recombination process, a DNA sequence from an intact DNA molecule, often the newly-synthesised sister chromatid, is used to repair replication lesions (Arnaudeau, Lundin et al. 2001). BRCA plays an important role in this process. Cancer cells that are deficient in BRCA1 and BRCA2 function show a high degree of chromosome instability. There is a correlation between the BRCA deficiency and platinum sensitivity in EOC (Dann, DeLoia et al. 2012). Ovarian cancer patients with BRCA1/2 mutations who are treated with platinum-based chemotherapy show better response rate and survival compared with ovarian cancer patients with functional BRCA1/2 treated with the same treatment (Ben David, Chetrit et al. 2002, Tan, Rothermundt et al. 2008). Cells lacking wild-type BRCA1 and BRCA2 are highly sensitive to inhibitors of PARP, a nuclear enzyme that facilitates the repair of single stranded DNA breaks. Inhibition of PARP leads to formation of double strand breaks that require functional BRCA1 and BRCA2 for DNA repair. Inhibition of PARP is a promising strategy in treatment of BRCA mutation-associated ovarian cancer (Banerjee and Kaye 2011).

Non-homologous end joining (NHEJ)

Ionising radiation and radiomimetic drugs induce double-strand breaks (DSB) that are mainly repaired by non-homologous end joining (NHEJ). In this process, the two ends of DNA strand break are connected and resealed without the need for sequence homology between the ends. In this process (NHEJ), each broken DNA end is first bound by one Ku 70/80

heterodimer and two heterodimer must come together to bridge matching ends to ensure efficient ligation. This complex is bound by the DNA-dependent protein kinase catalytic sub-unit (DNA-PK_{cs}) which stabilises the protein/DNA binding, therefore enabling the NHEJ DNA-repair process to carry on (Cary, Peterson et al. 1997, Hammarsten and Chu 1998, Gell and Jackson 1999, Singleton, Torres-Arzayus et al. 1999, Yoo and Dynan 1999, DeFazio, Stansel et al. 2002). In addition to the DNA-PK_{cs} and Ku molecules bound to DNA, remaining molecules forming the NHEJ apparatus consist of the DNA ligase IV/XRCC4 (X-ray cross complementation group 4 proteins) complex (Hsu, Yannone et al. 2002).

1.8.3.1.1 DNA-dependent protein kinase (DNA-PK)

DNA-dependant protein kinase (DNA-PK) is a nuclear serine/threonine kinase and a member of the PI3K like kinases (PI3KK), mainly involved in the non-homologous end-joining DNA repair process. Other members of this family include the DNA-repair protein kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR). These three protein kinases (DNA-PK, ATM,ATR) show sequence homology with other members of the phosphatidylinositol-3-kinae (PI3K) family. However, they do not phosphorylate lipids, therefore they are not true PI3K kinases (Hartley, Gell et al. 1995, Smith, Divecha et al. 1999) (Bosotti, Isacchi et al. 2000). Lack of DNA-PK_{cs} in cells causes radio-sensitivity, due to defective DSB repair (Jung and Alt 2004). In addition to the role of DNA-PK_{cs} in DNA DSB repair, DNA-PK_{cs} is critical for telomere capping and protection of chromosomes' ends. (Bailey, Meyne et al. 1999, Gilley, Tanaka et al. 2001, Goytisolo, Samper et al. 2001) .

Autophosphorylation of DNA-PK results in kinase inactivation and dissociation of DNA-PK_{cs} from the heterodimer Ku (Chan and Lees-Miller 1996, Douglas, Moorhead et al. 2001). DNA-PK has two main autophosphorylation clusters ranging from residues 2031-2056 and 2609-

2647. The most conserved site among eukaryotes is thr-2609 which is autophosphorylated following irradiation and has been found to co-localise at sites of DNA damage (Anderson and Lees-Miller 1992, Chan, Chen et al. 2002, Reddy, Ding et al. 2004). Only minutes after exposure to irradiation, phosphorylated kinase is detectable at the site of DNA damage. This suggests that post-translational modification of DNA-PK is necessary for the initiation of the cellular response to DSBs (Ding, Reddy et al. 2003).

Moreover, Involvement of DNA-PK in transcriptional control has been indicated in a number of studies. This includes the involvement of DNA-PK in regulation of RNA polymerase I. DNA-PK inhibits transcription initiation by Pol I. This inhibitory process requires phosphorylation of Pol I (Kuhn, Gottlieb et al. 1995).

Inhibition of components of the NHEJ pathway, particularly DNA-PK is an attractive approach to modulate resistance to DNA DSBs induced by therapeutic agents. The most successful approach to inhibit DNA-PK is targeting the ATP binding site of the kinase domain by small molecules (Allen, Halbrook et al. 2003). Screening of compounds libraries has revealed several small molecules that are able to inhibit DNA-PK efficiently but unfortunately, most of those molecules have poor pharmacokinetic properties.

1.8.3.1.1.1.1 Inhibitors of DNA-PK

Wortmannin was one of the first molecules that was revealed to inhibit DNA-PK. This compound is a non-competitive general inhibitor of PI3 kinases (Stein 2001). Wortmannin inhibits DNA-PK by irreversible alkylation of lysine 802 in the active site of DNA-$PK_{cs.}$ Lysine 802 is required for phosphate transfer (Wymann, Bulgarelli-Leva et al. 1996). Wortmannin is also an effective radio-sensitiser *in vitro* (Sarkaria, Tibbetts et al. 1998, Hashimoto, Rao et

al. 2003), but despite the efficiency of wortmannin *in vitro*, it's *in vivo* toxicity, poor solubility in aqueous solution and lack of specificity limit the application of this DNA-PK inhibitor in the clinic (Collis, DeWeese et al. 2005).

LY294002 is another non-specific DNA-PK inhibitor used in many studies as a PI3K inhibitor. LY294002 binds reversibly to the kinase domain of DNA-PK. Lack of specificity, *in vivo* toxicity and rapid metabolic clearance are some of factors that limit clinical utility of this compound (Collis, DeWeese et al. 2005).

NU7026 is a more specific DNA-PK inhibitor which has a modified structure of LY29002 and is 70-fold more selective for DNA-PK than other PI3Ks, and five-fold more selective for DNA-PK than for ATR and ATM (Hollick, Golding et al. 2003). This compound has been shown to potentiate the growth inhibitory effect of a variety of chemotherapeutic agents including doxorubicin, idarubicin, etoposide and mitoxantrone *in vitro* (Willmore, de Caux et al. 2004). However, poor solubility of NU7026 remains problematic for clinical application of this compound.

NU7441 is a small molecule DNA-PK inhibitor based on the LY294002 backbone with improved potency compared to NU7026 (Willmore, de Caux et al. 2004). NU7441 increased the cytotoxicity of IR and etoposide in colon cancer cells (Zhao, Thomas et al. 2006). In addition, this compound delayed the repair of IR and etoposide-induced DSBs and increased G2/M accumulation associated with IR and etoposide in colon cancer cells (Zhao, Thomas et al. 2006). Although pharmacokinetic properties of NU7441 need to be improved, the ability of this compound to enhance the effect of IR and chemotherapy *in vitro* and *in vivo* has made it

a promising candidate for further development. The effect of NU7441 on platinum resistance has not been reported.

IC86621, IC87102 and IC87361 are other compounds, based on the LY294002 structure, that have been tested *in vitro* as DNA-PK inhibitors. IC86621 caused decreased repair of IR DSBs and increased sensitisation to IR *in vitro* (Kashishian, Douangpanya et al. 2003). In addition, IC87361 showed significant radio-sensitisation and growth delay in xenograft models (Shinohara, Geng et al. 2005). All these three compounds are non-toxic and have therapeutic potential to increase the effects of DNA-damaging agents on cancer cells.

Recently a topoisomerase II alpha inhibitor, NK314, was shown to induce the degradation of DNA-PK. This compound appears to be a dual inhibitor of TII-alpha and DNA-PK (Hisatomi, Sueoka-Aragane et al. 2011). The mechanism by which this compound inhibits DNA-PK is still unknown. NK314 is currently in clinical trial for the treatment of adult T-cell leukaemia-lymphoma (www.clinicaltrials.gov).

1.8.3.1.2 Poly ADP ribose polymerases (PARPs)

Poly ADP ribose polymerases (PARPs) form a large, multifunctional family of enzymes which play an important role in repairing DNA single strand breaks. They are present in the nucleus and facilitate the repair of single strand DNA breaks by signalling the DNA damage presence to the DNA repair machinery. This signalling is performed via addition of ADP – ribose polymers to the DNA. PARP1 is the most abundant enzyme in this group which repairs base excisions. Inhibition of PARP1 results in the accumulation of single strand DNA breaks, eventually leading to double strand breaks (Dantzer, Ame et al. 2006).

BRCA1 and BRCA2 play major roles in double strand break repair process (Powell and Kachnic 2003, Ashworth 2008). Germline BRCA1/2 mutations have been found in about 10-15% of ovarian cancer tumours (Pal, Permuth-Wey et al. 2005). Genetic or epigenetic inactivation of BRCA1/2 genes is frequently found in high grade serous ovarian cancer tumours (Press, De Luca et al. 2008). In addition, recent analysis of ovarian cancer tumours published by the Cancer Genomic Atlas (TCGA) indicates the presence of BRCA1/2 mutations or BRCA1 silencing in 33% of high-grade ovarian cancer tumours (Cancer Genome Atlas Research 2011). Deficiency in BRACA1/2 function are associated with a better response to platinum-based agents in ovarian cancer (Tan, Rothermundt et al. 2008).

PARP inhibition may be beneficial to increasing the efficacy of platinum in patients with BRCA1/2 mutations. To date, several ongoing or completed studies have evaluated the effect of the combination of PARP inhibitors and different chemotherapeutic reagents on the reversal of chemo-resistance. In a phase I clinical trial, Fong et al assessed the anti-tumour activity of the PARP inhibitor olaparib in platinum-sensitive, resistant and refractory ovarian cancer patients with BRCA1/2 deficiency and found a association between clinical benefit rate across the platinum sensitive and resistant subgroups (69% and 45% respectively) (Fong, Yap et al. 2010).

1.8.3.2 Apoptosis pathway

A failed attempt to repair DNA damage, induced by cytotoxic agents, may result in the trigger of programmed cell death (apoptosis). Apoptosis is initiated by various stimuli such as DNA damage, growth factor deprivation, heat shock leading to the activation of apoptotic genes and eventually activation of caspase cascades. Initiation of apoptosis can occur either by the extrinsic or intrinsic pathways (Elmore 2007, Ouyang, Shi et al. 2012).

Intrinsic pathway

The intrinsic pathway is mediated primarily by mitochondrial dysfunction (Khosravi-Far and Esposti 2004, Fulda and Debatin 2006). Signals triggered from mitochondria, activate the initiator caspases, caspase 9 (Villa, Kaufmann et al. 1997). Formation of a large protein complex called the apoptosome is required for activation of caspase 9 (Salvesen and Renatus 2002, Shi 2002, Hill, Adrain et al. 2003). The key components of apoptosome are cytochrome c, which is released from mitochondria and a co-factor known as the apoptotic protease activating factor-1 (APAF-1) (Adams and Cory 2002, Cain, Bratton et al. 2002). Once activated, initiator caspases activate downstream effector caspases through cleavage at asparate residues which leads to separation of the large and small subunits. Activated effector caspases are responsible for cleavage of several downstream protein targets which are involved in vital cellular functions such as DNA repair, replication and transcription (Earnshaw, Martins et al. 1999). The enzymatic function of caspases is inhibited by the inhibitor of apoptosis proteins (IAP) (Bergmann, Yang et al. 2003, Vaux and Silke 2003).

The intrinsic pathway is regulated by B-cell lymphoma 2 (Bcl-2) proteins. Bcl-2 proteins form a large family of polypeptides which contain at least one Bcl-2 homology (BH) domain that mediates protein- protein interaction (Youle and Strasser 2008). The most well-known members of this family are Bcl-2 and Bcl2-assosiated X (BAX) proteins. Bcl-2 is an oncogene that can protect cells from apoptotic response to DNA-damage agents (Hockenbery 1992). BAX on the other hand, is a pro-apoptotic protein and acts as a dominant inhibitor of Bcl-2. Following stimulation of apoptosis, BAX undergoes a conformational shift and integrates into the outer mitochondrial membrane (Korsmeyer, Shutter et al. 1993, Yang and Korsmeyer 1996, Soriano and Scorrano 2011). BAX oligomeric complexes act as channels through which proapoptotic proteins, such as cytochrome c, can be released. The ratio of Bcl-2 to BAX determines whether a cell enters

apoptosis (Gross, McDonnell et al. 1999). Sensitivity to cytotoxic agents can be increased by the inhibition of anti-apototic proteins and introduction of pro-apoptotic genes.

Extrinsic pathway

Induction of apoptosis by the extrinsic pathway occurs when death receptors located on the cell surface, such as FAS, Apo2L and other members of the tumour necrosis factor (TNF) receptor family, are activated by extra cellular ligands. FAS ligand binds to the FAS receptor cause dimerisation of the receptor followed by the binding of adaptor proteins such as the FAS-associated death domain (FADD) protein to the death domains of the both the adaptor and the receptor protein. In addition, procaspase 8 binds to the death-effector domain of FADD. This complex consists of FAS, FASL, FADD and procaspase 8 and is known as the death-inducing signalling complex or DISC. Formation of this complex results in self-activation of procaspase 8 molecule. Activated caspase 8 then activates downstream effector caspase including caspase 3 (Elmore 2007).

Caspase 3

Both intrinsic and extrinsic pathways lead to activation of the effector caspase, caspase 3. Caspase 3 has a vital role in several key events during apoptosis, such as DNA fragmentation and nuclear fragmentation. Caspase 3 has a central role in the regulation of essential apoptotic pathways. For instance, cisplatin-induced cytochrome c release and caspase 8 mediated procaspase-9 process are dependent on caspase 3 in MCF-7 breast cancer cells (Blanc, Deveraux et al. 2000). It has also been indicated that apoptosis in MCF-7 caspase 3 deficient cells is defective in response to cisplatin. Stable transfection of those cells with caspase 3 cDNA restored the deficiency (Blanc, Deveraux et al. 2000).

Tumour suppressor protein P53

TP53 which encodes for the tumour suppressor protein P53, is one of the most frequently mutated genes in human cancers, including ovarian cancer. P53 plays an important role in apoptosis. Following DNA damage, p53 becomes activated either directly or indirectly and up-regulates expression of mitochondrial pro-apoptotic proteins such as BAX, p53 up-regulated modulator of apoptosis (PUMA) and **p53**-regulated apoptosis-inducing protein 1 (p53AIP1), as well as proteins like TRAIL-R1, TRIAL-R2, involved in cell death receptor pathways (Hengartner 2000). Lack of functional p53 in cancer cells can, therefore, result in chemo-resistance as these cells are unable to undergo apoptosis in response to DNA damage (Gasco and Crook 2003).

Several attempts have been made to overcome resistance to cisplatin in tumour cells having dysfunctional p53. This includes the development of a triplatinum analogue BBR3464. This compound is 20-fold more potent than cisplatin *in vitro* with a different pattern of activity in cancer cells. It forms DNA intrastrand cross-links that are not removed by nucleotide excision repair and therefore may persist longer in tumour cells (Kasparkova, Zehnulova et al. 2002). This drug induces apoptosis independent of the p53 status of cells *in vitro* and has also shown good activity in p53 mutant xenografts (Manzotti, Pratesi et al. 2000). Clinical assessment of BBR3464 indicated that some response was demonstrated in platinum-sensitive patients but unfortunately, little activity was seen in platinum-resistant tumours (Sessa, Capri et al. 2000).

1.8.3.3 Angiogenesis

Angiogenesis, the formation of new blood vessels is one of the key factors in tumour growth and metastasis (Verheul and Pinedo 2000). Vascular Endothelial Growth Factor (VEGF) and

its receptors play a crucial role in this process. The VEGF protein family consists of at least five structurally-related family members which transmit their signal via three VEGF receptor tyrosine kinases (VEGFR1, VEGFR2 and VEGFR3). VEGFA (also known as VEGF) is the best known member of the VEGF family and is secreted by a range of tumour cells Binding of VEGF to its receptor leads to dimerisation and auto-phosphorylation of the receptor which causes activation of RAS-RAF-MAP kinase cascade and also PI3K/AKT pathway (Olsson, Dimberg et al. 2006). Activation of these two pathways in endothelial cells leads to angiogenesis and in tumour cells lead to eventually cell invasion, migration, survival and proliferation. In keeping with this, ovarian cancer cells express both VEGF and VEGFR2. It has been suggested that VEGF–mediated signalling acts through the PI3K/AKT pathway and activation of AKT is required for VEGF-mediated endothelial cell survival (Gerber, McMurtrey et al. 1998, Fujio and Walsh 1999). Angionenesis, induced by VEGF, can be inhibited either by targeting the VEGF molecule itself or via its receptor.

Inhibition of angiogenesis pathway in ovarian cancer

Evidence from phase II and III clinical trials using the angiogenesis inhibitor, bevacizumab, indicate that inhibition of angiogenesis is a promising strategy in treating recurrent ovarian cancer (Perren, Swart et al. , Burger, Sill et al. 2007, Cannistra, Matulonis et al. 2007).To date, bevacizumab is the most developed and promising angiogenesis inhibitor in the treatment of ovarian cancer. Bevacizumab is a humanised monoclonal antibody directed against VEGFA (Folkman 1971, Jain 2005, Ellis and Hicklin 2008).

Small molecule inhibitors of tyrosine kinase inhibitors can also inhibit VEGF signalling by inhibiting the activity of VEGF receptors. However, tyrosine kinase inhibitors usually have

multi-targeting activity and have additional activity against other tyrosine kinases. (Morabito, De Maio et al. 2006).

Several VEGFR inhibitors are under evaluation regarding their use in ovarian cancer patients, either as monotherapy or in combination therapy with cytotoxic agents. For example, sorafenib is a VEGFR-2 and VEGFR-3 inhibitor which also inhibits Raf-1, therefore it potentially inhibits tumour proliferation via RAS-ERK pathway as well as angiogenesis. However, activity of sorafenib, in Phase II clinical trials with patients with recurrent ovarian cancer, has been reported to be low (Biagi, Oza et al. 2011). In contrast, cediranib, also an inhibitor of VEGFR, has showed encouraging results in a Phase II clinical trial of ovarian cancer patients, resulting in a clinical response rate of 30% and median progression-free survival of 5.2 months (Matulonis, Berlin et al. 2009). Pazopanib and vandetanib are other TKI of VEGFR under clinical and non-clinical evaluation (Annunziata, Walker et al. 2010, Friedlander, Hancock et al. 2010).

1.8.3.4 Epithelial growth factor receptor pathway

Epithelial growth factor receptor (EGFR) is widely expressed on the surface of mammalian epithelial cells (Miettinen, Berger et al. 1995). EGFR belongs to the human epithelial receptor family (HER), which consists of 4 members: HER1/EGFR, HER2/neu, HER3 and HER4. These 4 receptors share a similar structure consisting of an intracellular tyrosine kinase domain, an extracellular ligand binding domain and a transmembrane lipophilic domain (Yarden and Sliwkowski 2001).

EGFR becomes activated by ligand binding, followed by heterodimerasation or homodimerasation. To date, several molecules have been found to bind to EGFR, including epidermal growth factor (EGF), amphiregulin (AR), transforming growth factor α (TGF-α),

epiregulin (ER) and neuroregulin (Lemmon and Schlessinger 1994). The main pathways activated by EGFR dimerization are the RAS-RAF-mitogen-activated protein kinase (RAS/RAF/MAPK) and PI3K/AKT pathways (Buettner, Mora et al. 2002, Arteaga 2003, Citri and Yarden 2006).

EGFR plays a crucial role in tumourigenesis and its expression has been associated with the outcome of cancer treatment (Sibilia, Kroismayr et al. 2007). Overexpression of EGFR has been detected in 30-98% of epithelial ovarian cancers (Moscatello, Holgado-Madruga et al. 1995, Bartlett, Langdon et al. 1996, Fischer-Colbrie, Witt et al. 1997, Alper, Bergmann-Leitner et al. 2001). Dysregulation of EGFR can contribute to the malignancy of ovarian or other tumours via activation of cell proliferation, migration, angiogenesis or promotion of resistance to apoptosis (Ciardiello and Tortora 2001, Nicholson, Gee et al. 2001, Arteaga 2002, Tsujioka, Yotsumoto et al. 2010). Increased expression of EGFR has been correlated with reduced recurrence-free or overall survival rate in head and neck, ovarian, cervical, bladder and oesophageal cancers (Ciardiello and Tortora 2001, Nicholson, Gee et al. 2001, Arteaga 2002, Tsujioka, Yotsumoto et al. 2010).

EGFR dysregulation in cancer cells is due to three main reasons; mutation in the gene, receptor amplification/overexpression and autocrine/paracrine loop formation. EGFR pathway can be inhibited by either extracellular, in which the ligand binding motif of the EGFR is blocked by monoclonal antibodies, or by using the tyrosine kinase inhibitors which inactivate downstream signalling cascades (Gui and Shen 2012).

To date, two MAbs (panitumumab and cetuximab) and two tyrosine kinase inhibitors (erlotinib and gefitinib) have been approved by the food and drug administration (FDA) for different cancers, although neither have been licenced yet for use in epithelial ovarian cancer (Gui and Shen 2012). Preclinical studies have demonstrated that cetuximab can re-

sensitise various human cancer cell lines and xenograft models (including epithelial ovarian cancer models) to chemotherapy and radiotherapy (Bull Phelps, Schorge et al. 2008). In a phase II clinical study of single agent cetuximab in patients with persistent or recurrent EOC, 25 patients were recruited, all of whom were EGFR positive. Single agent cetuximab showed minimal activity in recurrent epithelial ovarian cancer patients in this phase II clinical study (Schilder, Pathak et al. 2009).

Erlotinib and gefitinib are the most advanced tyrosine kinase inhibitors in clinical development. Erlotinib has been approved for the treatment of pancreatic cancer and non-small cell lung cancer but it has shown minimal response in cell proliferations assays in ovarian cancer cell lines (Bull Phelps, Schorge et al. 2008). Results of a phase II combination therapy study of an unselected population with ovarian cancer has indicated that erlotinib plus carboplatin-paclitaxel does not improve pathological complete response compared with carboplatin-paclitaxel alone (Blank, Christos et al. 2010).

1.8.3.5 RAS/RAF MEK Pathway

Aberrant regulation of the RAS/RAF/MEK cascade has been found in various types of cancer including ovarian cancer. Mutation in BRAF and KRAS characterise the development of low-grade serous ovarian cancer (Singer, Oldt et al. 2003). Therefore, inhibition of RAS/RAF/MEK pathway may be a good target to treat the low-grade invasive disease. Mitogen-activated protein kinase (MAPK) signalling pathway has an important role in the regulation of cell proliferation, differentiation and survival. (Sebolt-Leopold and Herrera 2004).

RAS, which is a small GTPase, becomes activated by the exchange of GDP with GTP. RAS-GTP then contributes to the activation of the serine/threonine kinase RAF by binding to this

protein. The recruitment of RAF to the cell membrane is essential for its activation by RAS-GTP. Activated RAF then activates mitogen-activated protein kinase kinase (MAPKK;MEK) by phosphorylation (Mitin, Rossman et al. 2005). MEK is a dual tyrosine and serine/threonine kinase and once activated, it phosphorylates the mitogen-activated protein kinases (MAPKs), also known as extracellular signal regulated kinase (ERKs) (Gardner, Vaillancourt et al. 1994). MAPKs are a family of serine/threonine kinases. Activated MAPKs enters the nucleus and regulates the activity of many transcription factors involved in cell proliferation, survival and differentiation. There are three distinct but parallel MAPK pathways: MAPK, JNK and p38 (Johnson and Lapadat 2002). MAPK becomes activated by growth factors while JNK and p38 are activated by various environmental stress signals, such as ionising radiation. The JNK and p38 pathways usually trigger apoptosis (Behrens, Sibilia et al. 1999, Kralova, Dvorak et al. 2008).

Cross-talk between the RAS/RAF/MEK and the PI3K/AKT pathways has been shown in several studies (Aksamitiene, Kiyatkin et al.). The PI3K/AKT pathway can interact with the RAS/RAF/MEK signalling pathway. AKT can phosphorylate and inactivate BRAF (Zimmermann and Moelling 1999, Guan, Figueroa et al. 2000). In addition, AKT has been shown to activate RAF1 through a protein kinase C dependent but RAS-independent mechanism leading to inhibition of apoptosis (Majewski, Nieborowska-Skorska et al. 1999). Both AKT and ERK can prevent apoptosis by phosphorylating the BH3-only domain protein, BIM. This phosphorylation causes BIM to become targeted for degradation in proteasome (Gelinas and White 2005). In addition, both pathways can prevent the apoptotic effect of the pro-apoptotic Bcl-2 Homologous domain-3 (BH-3) only domain protein BAD by phosphorylation (Datta, Dudek et al. 1997, Bonni, Brunet et al. 1999, Harada, Becknell et al. 1999).

To date, several small molecule inhibitors of RAS/RAF MEK pathway have been developed (McCubrey, Steelman et al. 2012). Selumetinib (AZD6244) is a MEK inhibitor which is under clinical development. Selumetinib significantly suppressed tumour growth in a clear cell carcinoma (CCC) xenograft model (Bartholomeusz, Oishi et al. 2012). A phase II clinical study in patients with low-grade ovarian cancer carcinoma indicated that selumetinib controlled the disease in 81% of patients. From the fifty-one women who were recruited for the study, 8 patients had complete or partial response to treatment and 34 patients had stable disease. Sixty percent of patients had progression-free survival longer than six months. In addition, selumetinib was well tolerated. DNA analysis of patients showed that RAF/RAS mutation had no effect on clinical response in this study (Farley, Brady et al. 2013). Moreover, a phase II trial studying the side effects of selumetinib in recurrent low-grade ovarian cancer patients is currently ongoing (www.clinicaltrials.gov).

1.8.3.6 PI3K/AKT pathway

The phosphatidylinositol-3-kinase (PI3K) pathway is an important regulator of mammalian cell proliferation, growth, glucose metabolism and survival. These cellular processes are responsible for all aspects of tumourigenesis, so the PI3K pathway and downstream signalling components, such as AKT, are attractive targets for the development of therapeutic strategies for human cancers (Cantley 2002).

The PI3K pathway is activated by direct or indirect binding of receptor tyrosine kinases to adaptor proteins. The PI3Ks are grouped into three classes; class I, class II and class III according to their structure, regulation and lipid substrate specificity. The class I PI3Ks are divided into two classes; class IA and class IB. Class IA catalytic subunits are divided into three isoforms; p110α, p110β and p110δ encoded by *PIK3CA*, *PIK3CB* and *PIC3CD* respectively. The regulatory subunits of class IA PI3Ks are divided into three groups; p85α,

p85β and p85γ, encoded by *PIK3R1*, *PIK3R2* and *PIK3R3,* respectively. The class IB p110 catalytic subunit is encoded by *PIK3CG* and binds the p101 regulatory subunit encoded by PIK3R5 upon activation by G protein-coupled receptors (GPCR) and RAS (Engelman, Luo et al. 2006).

The functions of PI3K class IA subunits are better understood than those of the class IB and class II and class III PI3K proteins. Only class I PI3Ks have been heavily implicated in oncogenesis (Samuels and Ericson 2006). In this chapter, class IA PI3K proteins and their down-stream signalling are discussed.

The class IA regulatory subunits, p85 recruit the p110 catalytic subunit to phosphotyrosine residues in the receptor or adaptor proteins. This leads to localisation of the p110 catalytic subunits of PI3K in the plasma membrane, where they phosphorylate their lipid substrate. Upon activation, the class I PI3K phosphorylates membrane bound phosphatidylinositol-4, 5-bisphosphate (PIP_2) to produce phosphatidylinositol-3, 4, 5-triphosphate (PIP_3), a second messenger that binds to the pleckstrin-homology (PH) domain of AKT, recruiting AKT to the plasma membrane.

AKT, also known as protein kinase B, is a crucial signalling component down-stream of PI3K, and is therefore a very attractive therapeutic target (Manning and Cantley 2007). There are three members of the AKT family: AKT1, AKT2 and AKT3. All three isoforms are very similar in structure and consist of a central catalytic domain, an amino-terminal pleckstrin-homology (PH) domain and a short C-terminal carboxyl domain (Datta, Brunet et al. 1999).

Following the PI3K mediated recruitment to the plasma membrane, AKT is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) on a T regulatory residue (Figure. 1.1) (Bellacosa, Chan et al. 1998). Dependent on the AKT isoform, PDK1 can phosphorylate AKT

on T308/309/305. This phosphorylation is sufficient for stabilising the activation loop of AKT whereas phosphorylation on an S regulatory residue (S473/474/472, dependent on the AKT isoform) by a second kinase is necessary for full activation of the kinase (Alessi, Andjelkovic et al. 1996). Several different kinases have been identified which phosphorylate AKT at Ser473, including the mTOR-Rictor complex 2, integrin-linked kinase (ILK), DNA-dependent protein kinase (DNA-PK) and even AKT itself. Phosphorylated AKT, in turn, regulates a wide range of target proteins that control cell growth, proliferation, survival, and glucose metabolism. These include glycogen synthase kinase 3 (GSK3), proline-rich AKT substrate of 40 kDa (PRAS40), Bcl-2-associated death promoter (BAD) and the Forkhead box O (FOXO) transcription factors (Diehl, Cheng et al. 1998, Brunet, Bonni et al. 1999, Noshita, Lewen et al. 2001, Wang, Harris et al. 2008).

PI3K activity is negatively regulated by the tumour suppressor protein phosphatase and tensin homolog (PTEN). PTEN removes phosphate from phosphatidylinositol-3, 4, 5-triphosphate (PIP_3), converting it back to phosphatidylinositol-4, 5-bisphosphate (PIP_2).

1.8.3.6.1 Aberrations in the PI3K/AKT/mTOR pathway in cancer

Dysregulation and mutation of several components of the PI3K-AKT pathway have been reported in various types of human malignancies, including ovarian cancer, and have been shown to lead to neoplastic transformation in model systems (Vivanco and Sawyers 2002). Genomic and epigenetic alternations of genes encoding AKT and the tumour suppressor protein phosphatase and tensin homolog (PTEN), including loss of heterozygosity and methylation, have been frequently reported in various types of human malignancies, including ovarian, breast and lung cancers (Ali, Schriml et al. 1999, Hanahan and Weinberg 2000, Hanahan and Weinberg 2011).

Moreover, somatic missense mutations in *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K, are commonly detected genetic aberrations in breast cancer (Campbell, Russell et al. 2004, Samuels, Wang et al. 2004). Over-expression of p110 has also been observed in ovarian cancer (Shayesteh, Lu et al. 1999). A frequent mutation in *PIK3CA* (E545K) has been shown to activate AKT signalling and induce cellular transformation (Meyer, Koren et al. 2013). The regulatory subunit of PI3K, p85, has also been shown to be mutated in ovarian and colon cancers, along with the *PIK3R1* gene, which encodes the p85a regulatory subunit and is found to be mutated in 10% of human gliomas (Philp, Campbell et al. 2001). Genetic abnormalities also commonly occur in PI3K related pathways. For example, mutations in the oncogene, RAS, which activates PI3K, is frequently detected in pancreatic cancer (Janku, Lee et al. 2011, Lau and Leung 2011). *PIK3CA* amplification is commonly seen in head and neck, cervical and NSCLC squamous cancers (Janku, Tsimberidou et al.). Consequently, efforts to develop specific and efficient inhibitors against the key components of the PI3K pathway will potentially result in new therapeutics to treat human malignancies.

Overexpression and hyper-activation of AKT has been found in a number of human malignancies. Increased AKT1 activity has been observed in 50% of prostate carcinomas and also in 40% of ovarian and breast tumours (Bellacosa, Chan et al. 1998, Cheng, Lindsley et al. 2005). In addition, overexpression of AKT2 has been found in pancreatic and ovarian cancers, while AKT3 is overexpressed in hormone-insensitive prostate and breast cancers (Altomare and Testa 2005, Bellacosa, Kumar et al. 2005). Moreover, an activating mutation of AKT1 (E17K) has been reported in breast, ovarian and colorectal cancers. This transforming single mutation was found in the PH domain of the *AKT1* gene and results in growth-factor independent membrane translocation of AKT and increased AKT phosphorylation levels (Carpten, Faber et al. 2007). The same mutation has also been detected in *AKT3* in melanoma cells (Davies, Stemke-Hale et al. 2008). Constant overexpression of AKT leads to cell survival and malignant transformation, and in contrast,

inhibition of AKT activity stimulates apoptosis in a range of mammalian cells. There is also evidence suggesting an association between activation of AKT and tumour chemo-resistance (Kim, Dan et al. 2005).

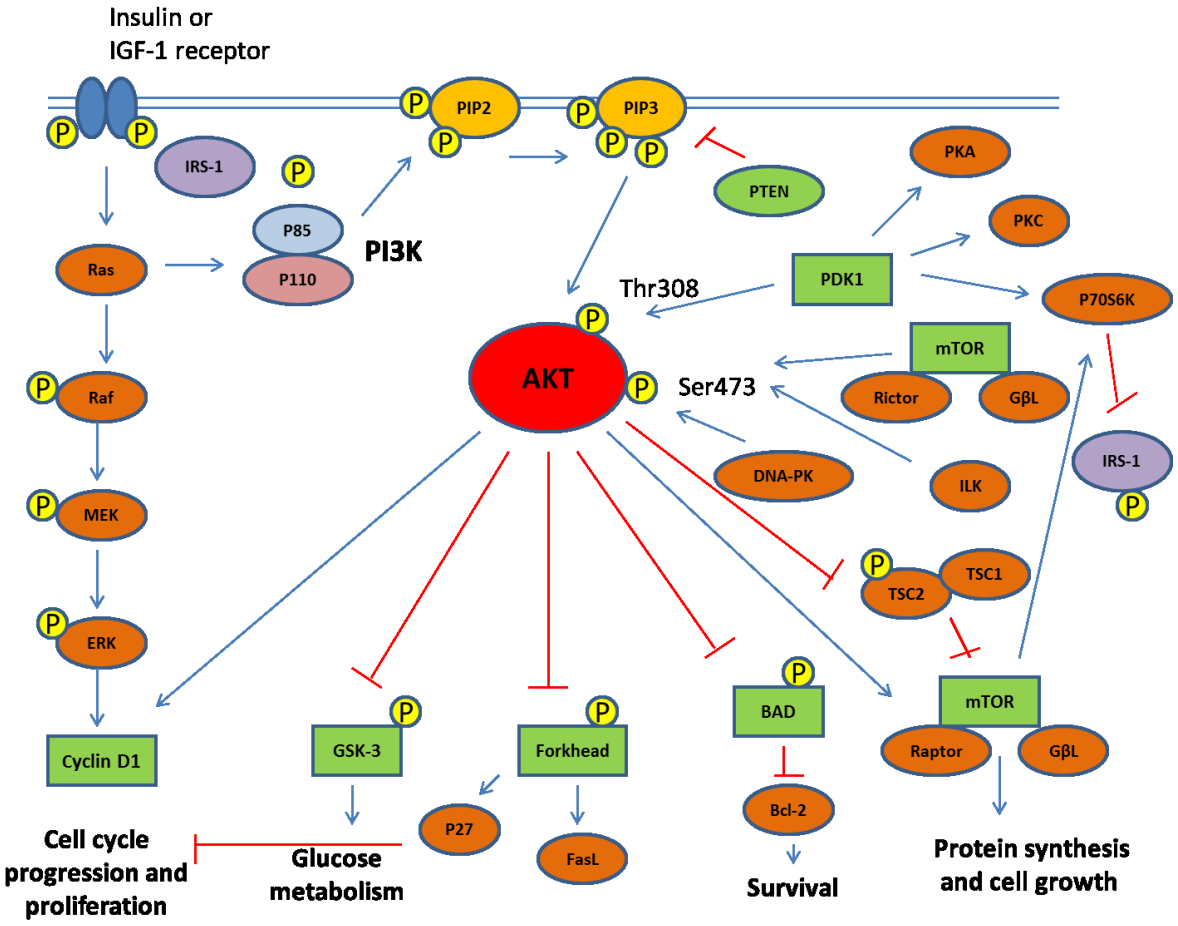


Figure 1-1 PI3K signalling pathway

PI3 kinases are activated downstream of ligand-bound receptor tyrosine kinases. Activated PI3K produces PIP3 which recruits PKB/AKT to the cell membrane where it is activated through dual phosphorylation by PDK1 and the mTOR complex. Activated AKT, in turn, phosphorylates a wide range of downstream target proteins that control cell growth, proliferation, survival and glucose metabolism (Workman, Clarke et al. 2006).

1.8.3.6.2 Cellular functions of AKT

1.8.3.6.2.1 Cellular survival

Activated AKT plays an important role in the regulation of cell survival. AKT phosphorylates the pro-apoptotic Bcl-2 family member BAD on its inhibitory Ser136 site. This phosphorylation decreases the binding of BAD to Bcl-XL which leads to the inhibition of the release of mitochondrial death proteins (Datta, Dudek et al. 1997, Datta, Brunet et al. 1999). AKT also phosphorylates GSK3 isoforms on a highly conserved N terminal regulatory site which leads to inactivation of the kinase (Cross, Alessi et al. 1995). The pro-survival Bcl-2 family member, MCL-1, is one of the direct targets inhibited by GSK3 (Maurer, Charvet et al. 2006). This causes inhibition of the release of cytochrome c from mitochondria and therefore, inhibition of formation of caspase-9 from its pro-caspase form and prevention of apoptosis. In addition, AKT inhibits caspase-9 activity directly through phosphorylation of Ser196 (Cardone, Roy et al. 1998). AKT also inhibits the forkhead transcription factor 1 (FOXO-1) through phosphorylation of Thr32 and Ser253. These phosphorylations facilitate binding of FOXO-1 to 14-3-3 proteins which leads to cytoplasmic sequestration and therefore decreases expression of FOXO1 targets (Tran, Brunet et al. 2003). Two of the most important targets of FOXO-1 in apoptosis pathway, in which their expression is decreased by this process, are the BH3-only protein BIM and the pro-apoptotic cytokine Fas ligand (Dijkers, Birkenkamp et al. 2002). AKT also phosphorylates Mouse double minute 2 homolog (MDM2) on Ser166 and Ser186 leading to translocation of MDM2 to the nucleus. Activated MDM2 then negatively regulates p53 function in the nucleus leading cell survival (Mayo and Donner 2001). Moreover, AKT can promote cell survival through crosstalk with other pathways. For example, it has been reported that PI3K-AKT pathway can inhibit JNK/p38 apoptotic signalling (Ozes, Mayo et al. 1999, Kim, Khursigara et al. 2001).

1.8.3.6.2.2 Cellular Metabolism

AKT stimulates glucose uptake in response to insulin. AKT-2 is the primary isoform of AKT in insulin-responsive tissues. In response to insulin, AKT-2 binds to the glucose transporter-4 (GLUT4) -containing vesicles and phosphorylate their component proteins (Calera, Martinez et al. 1998). AKT activation, in response to insulin, results in translocation of the glucose transporter 4 (GLUT4) into the plasma membrane (Kohn, Summers et al. 1996). Translocation of GLUT4 to the plasma membrane is dependent on phosphorylation of Rab-Gap TBC1 domain family member 4, also known as AS160. In response to insulin, AKT phosphorylates AS160, leading to inhibition of its GAP activity. This leads to allowing a Rab-family GTPase to become GTP loaded and stimulate GLUT4 vesicle translocation (Sano, Kane et al. 2003, Eguez, Lee et al. 2005). There are five phosphorylation sites on AS160, known to be phosphorylated by AKT, of which S588 and T642 are the most crucial. Mutations on these two sites block insulin-stimulated GLUT4 translocation (Sano, Kane et al. 2003). AKT phosphorylates combination of these five phosphorylation sites in response to insulin (Bai, Wang et al. 2007). However, recent studies suggest an AS160-independent mechanism of regulation of AKT-mediated translocation of GLUT4 to plasma membrane and other candidates. AKT substrates have been identified to be involved in this process, including PIKfyve (Berwick, Dell et al. 2004).

GLUT1 is the main glucose transporter in most cell types. GLUT1 is regulated through alternation in expression level. AKT phosphorylates TSC2 and PRAS40 leading to the activation of mTORC1. Activation of mTORC1 is required for both HIFα-dependent transcription of the GLUT1 gene and cap-dependent translation of GLUT1-mRNA. AKT activation can also alter glucose metabolism within cells (Zelzer, Levy et al. 1998, Taha, Liu et al. 1999). Once glucose enters the cell, it is converted to its active form; glucose 6-phosphate. Glucose phosphorylation and conversion to its active form is mediated by hexokinases in mitochondria. AKT has been found to stimulate the association of

hexokinase isoforms with mitochondria (Robey and Hay 2006). Glucose 6-phosphate can be either catabolised through glycolysis or be stored by conversion to glycogen. AKT can regulate both of these processes. AKT activation can enhance glycolysis by promoting the expression of glycolytic enzymes through HIFα (Lum, Bui et al. 2007). On the other hand, AKT can also stimulate glycogen synthesis by mediating the phosphorylation and inhibition of GSK3. Inhibition of GSK3 prevents GSK3 from phosphorylating its substrate glycogen synthesis (Elstrom, Bauer et al. 2004). In addition, AKT contributes to glucose homeostasis through phosphorylation and inhibition of FOXO1 (Accili and Arden 2004).

1.8.3.6.2.3 Cell proliferation

AKT can stimulate proliferation through affecting various downstream targets which are involved in cell cycle regulation. AKT phosphorylates the p27 cyclin-dependent kinase inhibitor on T157 and prevents its localisation to the nucleus. Phosphorylation of p27 on T157 leads to cytosolic sequestration of the kinase inhibitor via 14-3-3 (Liang, Zubovitz et al. 2002, Shin, Yakes et al. 2002). AKT also inhibits expression of p27 through phosphorylation and inhibition of the FOXO1 transcription factor (Medema, Kops et al. 2000). AKT has also been shown to phosphorylate the cyclin-dependent kinase inhibitor p21 on T145. This phosphorylation leads to p21 cytosolic localisation (Zhou, Liao et al. 2001).

AKT also regulates proliferation through phosphorylation of downstream targets which are involved in cell cycle entry. For example, GSK3 mediated phosphorylation of the G1 cyclin D and cyclin E and the transcription factors c-myc and c-jun leads to proteasomal degradation of these proteins which play an important role in the G1 to S phase cell cycle transition (Diehl, Cheng et al. 1998, Welcker, Singer et al. 2003, Yeh, Cunningham et al. 2004, Wei, Jin et al. 2005). Phosphorylation and inhibition of GSK3 by AKT increases the stability of these proteins and therefore, the transition of cells from G1 to S phase. AKT activation is increased during the G2/M phase of cell cycle and this activation promotes cell progression

through mitosis (Shtivelman, Sussman et al. 2002). Even in the presence of DNA damage, activated AKT overcomes the cell cycle checkpoint and progresses the cell through mitosis (Kandel, Skeen et al. 2002). AKT directly phosphorylates the DNA damage checkpoint kinase ChK1 on S280, which leads to translocation of ChK1 to the cytosol, therefore blocking its checkpoint function (Puc, Keniry et al. 2005). In addition, activity of mTOR1, which is a key regulator of cell proliferation, is controlled by AKT through phosphorylation of TSC2 and PRAS40. AKT mediated cell proliferation and oncogenic transformation is dependent on mTORC1 activation (Skeen, Bhaskar et al. 2006).

1.8.3.6.2.4 Cell Growth

One of the important roles of AKT is promoting cell growth. This regulation is mostly through the activation of mTOR complex 1 (mTORC1) in response to both growth factor and nutrients and signalling. MTORC1 has an evolutionary conserved role in cell growth control. It controls a wide range of growth-related processes including protein and lipid synthesis and ribosome biogenesis (Wullschleger, Loewith et al. 2006). The two best characterised targets of mTORC1 are the eukaryotic initiation factor 4E binding protein (4E-BP1) and the ribosomal protein S6 kinase (P70S6K). Phosphorylation of 4E-BP1 by mTORC1 leads to its dissociation from the translation repressor eIF4E, allowing the interaction of eIF4G to eIF4E which initiate protein translation (Gingras, Gygi et al. 1999, Rhoads 2009). AKT was believed to directly phosphorylate mTOR on S2448, which is often associated with mTOR1 activation, but recent studies suggest S6K1 rather than AKT to be responsible for phosphorylation of this site (Chiang and Abraham 2005). AKT activates mTORC1 indirectly through phosphorylation and inhibition of the tuberous sclerosis complex 2 (TSC2) which is a critical negative regulator of mTORC1 signalling (Gao and Pan 2001, Goncharova, Goncharov et al. 2002). AKT phosphorylates two sites on TSC2 directly (S939 and T1462). TSC2 with its binding partner TSC1 form a complex which acts as a GTPase-activating protein (GAP) for the Ras-related small G protein Rheb. Rheb activates mTORC1 when in its GTP-bound

active form (Manning and Cantley 2003). More recently, another substrate of AKT has been identified to be a regulator of mTOR1 complex. The proline-rich AKT substrate of 40 kDa (PRAS40) was initially identified for its ability to bind to 14-3-3 protein in a growth factor regulated manner (Harthill, Pozuelo Rubio et al. 2002). AKT was demonstrated to directly phosphorylate PRAS40 on T246. Phosphorylation of PRAS40 on T246 was later found to be important for 14-3-3 binding (Kovacina, Park et al. 2003, Vander Haar, Lee et al. 2007). PRAS40 is a negative regulator of mTORC1 (Sancak, Thoreen et al. 2007) and inhibits the activity of mTORC1 by preventing its binding to S6K1 and 4E-BP1. Phosphorylation of PRAS40 at T246 by AKT leads to the release of PRAS40 from the complex, therefore stimulation of mTORC1 signalling (Wang, Harris et al. 2007, Wang, Harris et al. 2008). Furthermore, it has been suggested that the phosphorylated P70S6K protein downstream of mTORC1 exerts negative feedback on insulin and insulin-like growth factor signalling through proteasomal degradation of Insulin receptor substrate 1(IRS-1) and IRS-2, resulting in down-regulation of PI3K/AKT signalling (Tremblay and Marette 2001).

1.8.3.6.3 PI3K/AKT pathway and chemo-resistance

Increased activation of PI3K/AKT pathway can lead to drug resistance in cancer. Resistance can arise from extended cell survival or AKT dependent blockage of the pro-apoptotic signalling cascades. In addition, increased activation of PI3K/AKT pathway can lead to overexpression of multidrug resistance associated protein-1, a transmembrane drug reporter that functions as a drug efflux pump. Overexpression of this protein leads to chemo-resistance in cancer (Tazzari, Cappellini et al. 2007).

The role of PI3K/AKT pathway in development of drug resistance to conventional anti-cancer therapies such as tyrosine kinase inhibitors, monoclonal antibodies against RTKs, radiation

therapy, hormone therapy and chemotherapy has been suggested in several studies (Burris 2013).

For example, increased activation of PI3K/AKT pathway has been associated with resistance to the anti-HER2 antibody, trastuzumab, in patients with breast cancer. Clinical studies have indicated that activation of PI3K/AKT pathway due to genetic alteration is linked with significantly shorter progression-free survival and was found to be a significant independent risk factor for disease progression in breast cancer patients receiving trastuzumab therapy (Berns, Horlings et al. 2007). Experiments in trasuzumab-resistant breast cancer cells has shown that inhibition of the PI3K/AKT pathway with pan PI3K inhibitors GDC-0941 and LY294002 and the dual PI3K/mTOR inhibitor BEZ235 significantly inhibited cell proliferation in a dose-dependent manner (Burris 2013). In addition, a combination of the PI3K inhibitor LY294002 with trastuzumab significantly decreased cell viability in trastuzumab-resistant breast cancer cell lines (Nagata, Lan et al. 2004). Moreover, activation of the PI3K/AKT pathway has been associated with a poorer response to treatment with lapatinib, a duel HER2/EGFR TKI in breast cancer patients, compared to those patients without oncogenic activation of the PI3K/AKT pathway (Wang, Zhang et al. 2011). Inhibition of the PI3K/AKT signalling with BEZ235 reversed lapatinib resistance in PIK3CA activated of PTEN-deficient breast cancer cells (Eichhorn, Gili et al. 2008).

The potential for PI3K/AKT pathway inhibitors to restore sensitivity to EGFR inhibitors has been assessed in several pre-clinical studies (Kim, Kim et al. 2010, Donev, Wang et al. 2011, Burris 2013). Inhibition of PI3K/AKT pathway reversed resistance to cetuximab in non-small cell lung cancer (NSCLC) cells (Kim, Kim et al. 2010). Moreover, inhibition of the PI3K signalling by PI-103, a pan PI3K inhibitor, resulted in the reversal of resistance to gefitinib in EGFR-mutant NSCLC cell lines (Donev, Wang et al. 2011).

RAS can activate both RAF/MEK and PI3K/AKT pathways and extensive cross-talks have been seen between the RAS/RAF/MEK and PI3K/AKT pathways. Increased activation of PI3K due to activating mutations in *PIK3CA* or loss of PTEN function has been shown to significantly reduce the response to MEK inhibitors in colorectal cancer cell lines (Wee, Jagani et al. 2009).

Activation of PI3K/AKT pathway can lead to resistance to ionising radiation, due to promotion of cell survival even after radiation-induced DNA damage. Inhibition of PI3K/AKT signalling has been evaluated as a potential to increase sensitivity to radiation therapy. For example, inhibition of PI3K activity with LY294002 has been shown to sensitise glioblastoma cells to radiation therapy (Kao, Jiang et al. 2007). In addition, inhibition of the p110α isoform of PI3K was efficient to sensitise glioblastoma cancer cells to radiation, both *in vitro* and *in vivo* models (Chen, Zhou et al. 2008).

Several studies have linked the PI3K/AKT pathway to platinum and taxol resistance in ovarian cancer and it has been demonstrated that constitutively active AKT2 leads to cisplatin resistance in previously sensitive A2780S ovarian cancer cells, whereas PI3K inhibition by LY294002 restored the sensitivity in this cell line. In addition, in the cisplatin-resistant A2780CP ovarian cancer cell line, expression of the dominant negative AKT2 or treatment with the PI3K inhibitor LY294002 reversed resistance resulting in cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway (Yuan, Feldman et al. 2003). Furthermore, expression of a constitutively active PI3K catalytic subunit induced taxol resistance in ovarian cancer xenograft models. This effect could also be reversed with LY294002 (Hu, Hofmann et al. 2002). Similarly, resistance to paclitaxel can be reversed in both *in vitro* and *in vivo* ovarian cancer models by inhibition of PI3K (Hu, Hofmann et al. 2002).

It has been shown, in chemo-sensitive ovarian cancer cells, that treatment with cisplatin induces apoptosis by reducing intracellular levels of X-linked inhibitor of apoptosis protein (XIAP), an inhibitor of apoptosis, and inducing AKT cleavage. Conversely, it has been shown that XIAP overexpression blocks cleavage of caspase-3 and caspase-7 and increases phosphorylation of AKT. This effect is associated with a decrease in cisplatin sensitivity, and has also been shown to be reversed by treatment with LY294002 (Fraser, Leung et al. 2003). These findings suggest an involvement of the PI3K/AKT pathway in XIAP-mediated chemo-resistance of ovarian cancer cells.

PTEN is overexpressed in chemo-sensitive ovarian cancer cell lines compared to chemo-resistant cells, and reductions in levels of PTEN expression by shRNA in sensitive cells resulted in an up-regulation of phospho-AKT and a resistance of the cells to cisplatin (Yan, Fraser et al. 2006). Furthermore, it has been hypothesised that decreased AKT activity could increase cisplatin-induced apoptosis in transfected cells.

PIK3CA genomic alternations are more frequent in ovarian cancer patients who relapsed with platinum resistance disease within 6 months (platinum resistant) than in those with more than 6 months of progression-free survival (platinum sensitive), or in patients with complete response to platinum. For examples, ovarian clear cell carcinoma (CCC) which is one of the most lethal types of ovarian cancer and often resistant to platinum based chemotherapy is characterised by frequent activating mutations of PIK3CA (35 – 45 %) (Kuo, Mao et al. 2009)

In 2010, Wallin et al. explored the use of a potent PI3K inhibitor in combination with the DNA-damage agent doxorubicin (Wallin, Guan et al. 2010). They found that PI3K inhibition increased apoptosis and enhanced the anti-tumour effects of doxorubicin in a subset of ovarian and breast cancer cell lines. They reported that in most cell lines where combination synergy was observed, there was an increase in the expression of nuclear p-AKT S473 in

response doxorubicin treatment alone, suggesting that inhibition of AKT pathway in combination with cytotoxic agents is mostly effective in the set of tumour cells that rely on the PI3K/AKT pathway for survival (Wallin, Guan et al. 2010).

Altogether, inhibition of the PI3K/AKT/mTOR signalling pathway is an attractive strategy to overcome resistance to standard treatments in cancer therapy.

1.8.3.6.4 Inhibition of the PI3K/AKT pathway

The AKT pathway can be inhibited either by targeting AKT itself, by blocking upstream activators such as PI3K or RTKs or by inhibiting downstream effectors of AKT activity, such as mTOR. AKT pathway inhibition holds great promise for a variety of tumour types and hence much of the progress to date is not specific to ovarian cancer. As the area continues to develop, the successful compounds will be tested more broadly, including in ovarian cancer.

1.8.3.6.4.1 AKT inhibitors

AKT inhibitors can be categorised into four main groups based on their mechanism of action and chemical composition; ATP competitive inhibitors, allosteric inhibitors, peptide-based inhibitors and lipid-based inhibitors (Stronach, Cheraghchi-Bashi et al. 2011).

1.8.3.6.4.1.1 Perifosine

The lipid-based AKT inhibitor, perifosine, is one of the first generation of AKT inhibitors and the most advanced, with respect to clinical studies. Perifosine prevents AKT translocation into cell membrane (Kondapaka, Singh et al. 2003). The safety and efficacy of periposin as a

single agent, or in combination with other anti-cancer agents, has been evaluated in a number of phase I, II and III clinical trials in several types of cancer. The first phase I clinical study with prifosine was carried out, in 2002, in patients with advanced solid tumours. This study was incomplete due to the gastrointestinal toxicity caused by high daily doses of the drug (Crul, Rosing et al. 2002). Applying a different loading dose/maintenance dose schedule in a following phase I study, minimised the toxicity and improved the efficacy, resulting in reduced gastrointestinal toxicity and rapid achievement of steady plasma concentration (Van Ummersen, Binger et al. 2004). Single agent perifosine has been evaluated in several phase II studies in different types of cancer including prostate cancer, head and neck cancer, breast cancer and melanoma. Only a few objective responses were reported in these trials and gastrointestinal toxicities were seen (Ernst, Eisenhauer et al. 2005, Posadas, Gulley et al. 2005, Argiris, Cohen et al. 2006, Bailey, Mahoney et al. 2006, Knowling, Blackstein et al. 2006, Marsh Rde, Rocha Lima et al. 2007, Leighl, Dent et al. 2008). In these trials, using single agent perifosine showed poor results and gastrointestinal and constitutional toxicities were reported. In addition, perifosine has been used in combination with radiation, chemotherapy and other targeted agents in several clinical trials. The safety of daily perifosine in combination with fractionated radiotherapy has been demonstrated in a phase I clinical trial in patients with different types of advanced cancers including colon, bladder and prostate cancers (Vink, Schellens et al. 2006). Moreover, combination of perifosine with a number of traditional cytotoxic agents, such as taxanes and gemcitabine, has been shown to be safe in a phase I clinical (Bailey, Mahoney et al. 2006). However, in 2013 the discontinuing of phase III clinical studies of perifosine was announced (www.clinicaltrials.gov).

1.8.3.6.4.1.2 MK2206

MK2206 (Merck) is an orally active small molecule allosteric inhibitor of AKT, targeting all

three isoforms of AKT. MK2206 has shown broad preclinical anti-tumour activity. It inhibits both T308 and S473 phosphorylation. MK2206 also inhibits the downstream effects of insulin on Glut4 translocation and glucose transport (Tan, Ng et al. 2011). MK2206 is or has been in several clinical trials, either as single agent or in combination with other small molecule inhibitors or chemotherapeutic drugs. It has been evaluated in phase I studies in patients with advanced solid tumours. MK-2206, as a single agent, is demonstrating good efficacy and tolerance level in phase I clinical trials (Yap, Yan et al. 2011). Currently, safety and efficacy of MK2206 in combination with different anti-cancer drugs is evaluated in several clinical studies (www.clinicaltrials.gov). In a phase I clinical study, combination of MK2206 with weekly paclitaxel was shown to be safe in patients with solid tumours (Chien, Truong et al. 2013). Also MK2206 plus carboplatin and paclitaxel, docetaxel, or erlotinib was well-tolerated in patients with solid advanced tumours (Molife, Yan et al, 2014).

1.8.3.6.4.1.3 API-2

Triciribine (API-2) is a small molecule AKT inhibitor which was synthesised in 1980s. API-2 is a synthetic tricyclic nucleoside initially known as a DNA synthetic inhibitor demonstrating anti-tumour and anti-viral activities (Schweinsberg, Smith et al. 1981). API-2 has been tested as a cytotoxic agent in several phase I and II clinical trials in patients with advanced malignancies but, despite the efficacy of this compound in inhibiting the growth of several cell lines *in vitro*, clinical studies with this compound were discontinued due to poor efficacy and poor toxicity profile of this drug, including hyperglycemia, thrombocytopenia and hypertriglyceridemia at high doses in clinic (Schweinsberg, Smith et al. 1981, Feun, Blessing et al. 1993). In 2004, API-2 was rediscovered as an effective AKT inhibitor following screening a chemical library of 1992 compounds in the National Cancer Institute Diversity Set (Yang, Dan et al. 2004). API-2 suppresses phosphorylation of AKT on both S473 and T308 residue. It inhibits kinase activity of all three AKT isoforms. The mechanism of action of

API-2 is poorly understood. There is evidence, however, that it may act via the PH domain of AKT. It has been suggested that following entering the cells, API-2 converts into the active TCN-P by adenosine kinase. Active TCN-P then interacts with the PH domain of AKT. This binding inhibits interaction of PIP3 with AKT, leading to prevention of phosphorylation of AKT (Yang, Dan et al. 2004).

1.8.3.6.4.1.4 GSK690693

GSK690693 is an ATP competitive, nanomolar pan-AKT kinase inhibitor, which belongs to the aminofurazan family of compounds (Rhodes, Heerding et al. 2008). It is selective for all isoforms of AKT. However, it is also selective towards 13 other kinases including other members of the AGC kinase family and members of the group II PAK kinase family. GSK600693 reduced phosphorylation of down-stream target of AKT including PRAS40, GSK3β and p70S6K in a dose-dependent manner in tumour cells (Rhodes, Heerding et al. 2008). In pre-clinical studies, GSK690693 has shown moderate activity in ALL tumours resulting in a reduction of phosphorylation of down-stream substrates of AKT (Carol, Morton et al. 2010). In addition, daily administration of GSK690693 inhibited tumour growth in BT474 and HCC-1954 breast cancer, SKOV3 ovarian cancer and LNCaP prostate cancer xenogafts (Rhodes, Heerding et al. 2008). The first time in human (FTIH) phase I clinical study for safety, tolerability, pharmacodynamics and pharmacokinetics of GSK690693 was terminated before completion of the study and a following planned phase I study in refractory haematological malignancies was withdrawn prior to enrolment. The reasons for the withdrawal of GSK690693 have not been made public (www.clinicaltrials.gov). Treatment with GSK690693 resulted in hyperglycemia in mice (Crouthamel, Kahana et al. 2009). Hyperglycemia following the treatment with AKT inhibitors is due to the role of AKT in insulin signalling. AKT2 has been suggested to profoundly affect insulin signalling, compared to

other isoforms of AKT (Yang, Tschopp et al. 2004), suggesting that isoform selective compounds may be required to minimise the toxicity of AKT inhibitors.

1.8.3.6.4.1.5 GSK2141795

GSK2141795 is a novel member of the N-alkyl pyrazole class of orally available kinase inhibitors. It is a potent, pan-AKT inhibitor, with potency (Ki) values for human AKT 1, 2 and 3 kinases of 0.066nM, 1.4nM and 1.5 nM, respectively (GlaxoSmithKline unpublished data). The safety and efficacy of oral GSK2141795 was evaluated for the first time in human in 2009, in a phase I clinical study in patients with solid tumours or Lymphomas. This study showed that GSK2141795 is well tolerated at the dose of ≤ 75 mg (H. A. Burris 2011). GSK2141795 is currently evaluated in several phase I clinical trials in combination with the MEK inhibitor Trametinib (www.clinicaltrials.gov).

1.8.3.6.4.2 PI3K inhibitors

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1.8.3.6.4.2.1 LY294002 and wormannin

LY294002 and wormannin are the two most well-known isomer non-specific PI3K inhibitors. LY294002 is a classical reversible, ATP competitive PI3K inhibitor while wormannin is an irreversible inhibitor which forms a covalent bond with a conserved lysine residue involved in the phosphor-binding reaction. Both inhibitors have unfavourable pharmaceutical properties. In addition, they have poor selectivity for PI3K and have crossover inhibition activity for other lipid and protein kinases, including mTOR and DNA-PK. In spite of these properties, both compounds have served important roles as research tools for better understanding the role

of PI3K in human cancers (Vlahos, Matter et al. 1994, Marone, Cmiljanovic et al. 2008, Cleary and Shapiro 2010).

1.8.3.6.4.2.2 PX-866

PX-866 is a modified analogue of wortmannin which has been evaluated in phase I and II clinical trials. PX-866 has been tested in a Phase I clinical trial in combination with docetaxel for solid tumours and in Phase I clinical trial in combination with cetuximab for colorectal cancer. Only mild side effects such as diarrhoea and nausea have been observed in those two clinical trials (Bartholomeusz and Gonzalez-Angulo 2012). PX-866 is currently being evaluated in about five clinical trials for melanoma, CRC, prostate cancer, NSCLC and other advanced cancers (www.clinicaltrials.gov).

1.8.3.6.4.2.3 GDC-0941

GDC-0941 is a more selective inhibitor of PI3K developed by Genetech (Folkes, Ahmadi et al. 2008). It targets both PI3K and hypoxia-induced factor-1 alpha (HIF-1-alpha) (Burrows, Babur et al. 2011). GCD-0941 synergised with the MEK inhibitor UO126 in inhibiting the growth of NSCLC (Zou, Zhang et al. 2012). This compound is currently being evaluated in a clinical trial for metastatic breast cancer or advanced cancers (www.clinicaltrials.gov).

1.8.3.6.4.2.4 XL-147

XL-147 is a PI3K inhibitor developed by Exelixis/Sanofi-Aventis. It is currently being evaluated in approximately 11 clinical trials, either as a single agent or in combination with chemotherapy, hormonal therapy, and erlotinib for various cancers (www.clinicaltrials.gov).

1.8.3.6.4.2.5 NVP-BKM120

NVP-BKM120 is a pan-class I PI3K inhibitor developed by Novarits. NVP-BKM120 behaves synergistically when combined with cytotoxic agents such as docetaxel or temozolomide (Maira, Pecchi et al. 2012). It is currently in at least 36 clinical trials, either as a single agent or in combination with other drugs, with patients having advanced cancers such as breast, prostate, endometrial, CRC, NSCLC, melanoma and advanced leukaemia (www.clinicaltrials.gov).

Some have questioned whether PI3K inhibitors will be effective in cancer therapy due to the complicated feed-back loop which results in the activation of certain receptor molecules (Tan and Yu 2013).

1.8.3.6.4.3 mTOR inhibitors

1.8.3.6.4.3.1 Rapamycin

Rapamycin (Rapamune, Pfizer) is an allosteric mTORC1 inhibitor and doesn't directly affect the mTOR catalytic site. Rapamycin associates with the FK506 binding protein 12 (FKBP-12). This association leads to the disassembly of mTORC1, leading to repression of its activity (Sabatini 2006). Rapamycin has been examined in various clinical trials for different cancers including breast, brain, prostate, lymphoma, pancreatic, NSCLC and leukaemia (Faivre, Kroemer et al. 2006, Liu, Cheng et al. 2009).

1.8.3.6.4.3.2 Afinitor

Afinitor is a rapamycin analogue and an inhibitor of mTORC1 which has been approved to treat patients with pancreatic neuroendocrine tumours (Yao, Phan et al. 2013).

1.8.3.6.4.3.3 OSI-027

OSI-027 is a pan mTOR kinase inhibitor developed by OSI Pharmaceuticals/Astellas Pharma Inc. Pan-mTOR inhibitors effectively inhibit both mTOR complexes therefore block signalling cascades down-stream of both mTORC1 and mTORC2. Using pan-mTOR inhibitors has advantages over using inhibitors of mTORC1 only. Inhibition of mTORC2 prevents increased AKT phosphorylation which is a result of inhibition of mTORC1 and loss of the S6K1-mediated negative feedback loop to IRS1. OSI-027 has been shown to be 100-fold more selective for mTOR relative to PI3K and DNA-PK. This mTOR inhibitor has effectively induced apoptosis in different types of cancer including breast cancers (Bhagwat, Gokhale et al. 2011). OSI-027 has been evaluated in clinical trials with patients with lymphoma and advanced solid tumours (www.clinicaltrials.gov).

1.8.3.6.4.3.4 AZD8055 and AZD2014

AZD8055 and AZD2014 (Astra Zeneca) are other potent pan mTOR inhibitors (Pike, Malagu et al. 2013) which have been evaluated in glioma patients who had not responded to standard treatments (www.clinicaltrials.gov).

1.8.3.6.4.3.5 PP-242 and INK-128

PP-242 (Intellikine) is a potent inhibitor of both mTORC1 and mTORC2. PP-242 but not rapamycin caused cell death in acute leukemia cells (Janes, Limon et al. 2010). INK-128 is a derivative of PP-242 which has been shown to prevent prostate cancer invasion and metastasis (Hsieh, Liu et al. 2012). INK-128 is been evaluated in phase I clinical trials for patients with relapsed MM or patients with solid malignancies (www.clinicaltrials.gov).

Despite promising pre-clinical and early clinical results, feed-back PI3K activation as a result of mTOR inhibition limits the application of mTOR inhibitors in clinic.

1.8.3.6.5 AKT/DNA-PK interaction as a mediator for platinum resistance

Activation of AKT upon induction of DNA-damage leads to platinum resistant in cancer cells. Several studies have reported activation of AKT in response to cisplatin in several cancer cell types (Winograd-Katz and Levitzki 2006).

Cisplatin induces mitochondrial release and nuclear translocation of apoptosis-inducing factor (AIF), leading to AIF-dependant apoptosis in chemo-sensitive ovarian cancer cells but not in chemo-resistant cells. AKT activation was found to confer resistance to cisplatin induced AIF-dependant apoptosis by blocking this pathway (Yang, Fraser et al. 2008). In addition, AKT activation is important for survival after UV irradiation (Feng, Tamaskovic et al. 2004). All these findings place AKT as an important mediator of DNA-damage signalling.

In 2008, Bozulic et al reported direct interaction between AKT and DNA-PK in response to DNA-damage. They demonstrated the presence of active AKT in the nucleus of γ-irradiated cells adjutant to DNA-double strand breaks and suggested phosphorylation of AKT by DNA-PK at S473, in response to DNA damage leading to pro-survival signal for the cell by affecting DNA damage (Bozulic, Surucu et al. 2008). However, kinase activity of DNA-PK for AKT had been already proposed by Feng el al (Feng, Park et al. 2004).

In 2011, it was reported by this lab that DNA-PKcs phosphorylates at S473 in response to cisplatin induced DNA damage in platinum resistant high-grade serous ovarian cancer cells but not in clinically matched platinum sensitive cells. Furthermore, co-localisation and binding of DNA-PKcs and AKT in response to cisplatin induced DNA-damage was demonstrated in nuclei of platinum resistant cells. This observation was not found in platinum sensitive cells (Stronach, Chen et al. 2011).

In addition, increase in the amount of nuclear phospho-AKT (S473), in response to doxorubicin in breast and ovarian cancer models, has been demonstrated in breast and ovarian cancer models. This increase is well correlated with synergy between inhibitors of PI3K pathway and doxorubicin (Wallin, Guan et al. 2010).

Studies on DNA-PK deficient mice have revealed that DNA-PK is not required for insulin and growth factor-induced AKT activation. In addition, DNA-PK is not essential for the maintenance of glucose metabolism. In contrast, DNA-PK is required for activation of AKT upon DNA-damage induced by γ-irradiation. These findings reveal stimulus-specific regulation of AKT activation by specific up-stream kinases (Surucu, Bozulic et al. 2008).

AKT/DNA-PK interaction is an attractive therapeutic target for reversal of platinum resistance.

1.9 Pharmacodynamic (PD) biomarkers in drug development

Despite technological progress and the development of 'targeted agents' in oncology, drug advancement still remains a slow, expensive and high risk process. Toxicity, inadequate therapeutic activity, poor bioavailability and pharmacokinetics (PK) and also mimicking of the tumour microenvironment *in vitro* have been reported as main reasons for the poor success in drug development(Neidle 2008). Hence, paying greater attention to optimising these properties in pre-clinical development is crucial. One of the other critical elements in improving the success rate in drug discovery is the identification of pre-clinical pharmacodynamic (PD) biomarkers of drug effects that will correlate with clinical effects. PD biomarkers may include cellular, molecular, histopathological and imaging parameters (Sarker, Pacey et al. 2007, Sarker and Workman 2007). PD biomarkers are used to assess the engagement of the drug with the target and the effect of the drug on down-stream pathways during the process of treatment and should not be confused with predictive biomarkers which are used to predict how well a patient will respond to treatment before initiation of the treatment. PD biomarkers are used to characterise functional and molecular effects of a drug, ideally in a quantitative manner. PD endpoints can be used at all stages of drug development from the *in vitro* assessment of action on the target, through to preclinical development and clinical trials. PD biomarkers can be used to provide proof of mechanism of action and to select the optimal dose and schedule of administration for the drug. They also have other potential applications in various levels of drug development, such as designing effective combination therapies and predicting the outcome of therapy in selected individuals. In addition, they can provide us with better understanding of response/resistance mechanisms. Incorporation of PD biomarkers enables sensible 'Go/No-Go' decisions to be

made regarding a drug candidate, and so facilitate drug development to proceed in a rational, efficient, hypothesis-testing manner (Sarker, Pacey et al. 2007, Sarker and Workman 2007) .

1.9.1 PET imaging as a PD biomarker

In recent years, the application of metabolic imaging with positron emission tomography (PET) has increased dramatically in oncology. PET measures the three-dimensional (3D) distribution of a positron emission-labelled compound within the living system in a surgically non-invasive manner. The most frequently used radiotracer in oncology is 2-deoxy-2-(^{18}F)fluoro-D-glucose ([^{18}F]FDG): a glucose analogue that is 'trapped' in high metabolic activity cells, such as malignant cells. [^{18}F]FDG PET imaging is therefore a useful technique for functional imaging of glucose turnover in cells and thus [^{18}F]FDG uptake can be used as a marker of the glucose metabolism in malignant cells. This information can be used to assess the number of viable cells in a malignant tissue and the proliferation capacity of those cells. Therefore, quantification of changes in [^{18}F]FDG uptake, following drug treatment, can provide a sensitive and early PD marker of the cytostatic or cytotoxic activity of anti-cancer agents in pre-clinical and clinical level studies, and can therefore be applied to monitor the tumour response to the anti-cancer drug more rapidly and effectively (Aboagye and Price 2003, Boss, Olmos et al. 2008). In the case of development of AKT inhibitors, [^{18}F]FDG PET is particularly suited to assessing the activity of the inhibitor within the malignant tissue as AKT is, itself, involved in regulation of glucose metabolism (LoPiccolo, Blumenthal et al. 2008, Ma, Jacene et al. 2009) . Therefore [^{18}F]FDG uptake measured by PET can be used as a PD biomarker through the development processes of AKT and PI3K inhibitors. Also, as mentioned above, [^{18}F]FDG is increased in most of malignant tumours. Alterations in [^{18}F]FDG uptake can also be used as a marker of tumour response to anti-cancer drugs (Young, Baum et al. 1999).

1.10 Aims of the thesis

The aim of this thesis was to explore the effect of inhibition of AKT, PI3K or DNA-PK on the reversal of platinum resistance in ovarian cancer *in vitro* and *in vivo* and also to determine the impact of those inhibitions on downstream signalling pathways, and whether 3D *in vitro* models are more predictive models than traditional 2D monolayer models in determining dose and schedule optimisation, prior to *in vivo* experiments.

The specific aims of this thesis were:

1. To examine the effect of the AKT inhibitor GSK2141795 on re-sensitisation of platinum resistant tumour cells to cisplatin in 2D and 3D *in vitro* models, and *in vivo,* to determine the potential utility of GSK2141795 for clinical development. (Chapter 3)

2. To evaluate the utility of FDG-PET imaging as a PD biomarker, for the clinical development of the AKT inhibitor GSK2141795. (Chapter 4)

3. To study the effect of AKT inhibition by GSK2141795 as a single agent or in combination with cisplatin on the broader AKT pathway, and other cellular signaling pathways, to identify canonical or non-canonical PD biomarkers. (Chapter 6).

4. To understand the role of PI3K in activation of AKT in response to DNA damaging agents and to investigate whether the nuclear DNA-PK mediated activation of AKT, in response to cisplatin, is dependent on activation of PI3K. (Chapter 5)

5. To explore the effectiveness of DNA-PK inhibition using NU7441 to restore platinum-sensitivity in platinum-resistant ovarian cancer cells *in vitro* and *in vivo*. (Chapter 5)

6. To explore the effects of DNA-PK inhibition with NU7441, alone or in combination with cisplatin, on cellular protein signalling cascades in *vitro* and *in vivo,* to identify candidate PD biomarkers for clinical development. (Chapter 7)

2 Materials and Methods

2.1 Human samples, cell lines and drugs

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Cell lines

High grade serous ovarian cell line PEO1, PEO4, PEA1 and PEA2 was obtained from Dr. Simon Langdon (Edinburgh, UK). SKOV3 cells were obtained from ECACC. All cancer cell lines were originated from patients with high grade serous (HGS) ovarian cancer. PEO1/4 and PEA1/2 cells were isogenically derived from two HGS ovarian cancer patients before and after platinum resistant relapse. PEO1 and PEA1 were platinum sensitive and PEO4 and PEA2 were platinum resistant. IC_{50} of each sensitive cell line and resistant cell line for cisplatin was around 2 μM and 10 μM respectively. Additional information regarding mutation status of each cell line is shown in table 2.1. All cell lines were maintained in RPMI1640 (Sigma) supplemented with 10% foetal calf serum, 50U/ml penicillin, 50 μg/ml streptomycin and 0.2 mM L-glutamine (all from Sigma), at 37°C/5% CO_2 in a humidified atmosphere.

Table 2-1 Summary of know mutations found in cell lines included in this thesis

Cell line	P53 status	ERBB2 amplification	Other mutations
PEO1	mutant	No	ABCB1 VEGF
PEO4	mutant	No	ABCB1 VEGF
PEA1	mutant	No	VEGF
PEA2	mutant	No	ABCB1
SKOV3	mutant	Amplified	MLH1 PIK3CA CDKN2A

Mutational analysis of PEO1/4 and PEA1/2 was performed by Katherine Stemke-Hale at the ‘ Characterised Cell Line Core’ at the MD Anderson Cancer Centre, Texas, USA, in

collaboration with the group of Robert Bast using the same method as described in (Stemke-Hale, Gonzalez-Angulo et al. 2008). Mutation data for SKOV3 cell line was obtained from catalogue of somatic mutations in cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Copy number data retrieved from the Cancer Genome Project website (https://www.sanger.ac.uk/research/projects/cancergenome/).

Drugs

The AKT inhibitor GSK2141795 and the PI3K inhibitor GSK2126458 were obtained from GlaxoSmithKline (Collegeville, PA and Research Triangle Park, NC, USA). Cisplatin was obtained from Hammersmith Hospital's pharmacy as 1mg/ml sterile saline solution. The DNA-PK inhibitor NU7441 was purchased from Selleck Chemicals LLC (Houston TX, USA). Chemicals used in making buffers or gels were purchased from Sigma (UK).

Human samples

All human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.

Clinical tumour biopsies from patients treated on a Phase I trial of GSK2141795 in patients with gynaecological malignancies, described in Gungor *et al* (Gungor H 2011) were used to determine signalling changes using RPPA. In summary 12 patients with gynaecological malignancies were enrolled in three cohorts. Patient's characteristics are shown in Table 2.2. Eligible patients had a histologically or cytologically confirmed diagnosis of FDG-PET-positive recurrent or persistent ovarian cancer or endometrial cancer. Eligible

subjects also had measurable disease of at least 2 cm. Patients received ≤ 75 mg GSK2141795 daily. The efficacy of the compounds was assessed by dynamic FDG-PET scans and serial PK sampling performed at baseline, Week 2, and Week 4. Tumour Biopsies were taken pre-treatment and week 4 after initiation of the treatment. Protein lysates were extracted and analysed as described in 2.5 for xenograft samples.

Table 2-2 characteristics of patients whose tumour samples were used for RPPA analysis

Trial ID	cohort	Dose GSK795	RECIST	CA125 (4wk)	Response group (4wk)	Histology	FIGO	grade	Mutations
100	1	50mg	PD	9.402138	poor	Serous	3c	3	none detected
101	1	50mg	SD	20.726496	good	Adeno	4	3	(BRCA1)
102	1	50mg	SD	36.482334	good	Serous	4	3	PHLPP2_L1016S
103	1	50mg	SD	-18.07622	poor	Endo	4	unkown	PIK3R1_M326I
1200	2	75mg	SD	58.536587	good	Clear Cell	2a	3	PIK3CA_H1047RL, PIK3R1_M326I
1201	2	75mg	SD	39.88289	good	Serous	3b	3	none detected
1202	2	75mg	PD	26.38889	good	Serous	3c	3	KRAS_G12SRC
1203	2	75mg	SD	-15	poor	Serous	4	1	none detected
1206	3	25mg 2wk->75mg 2wk	PD	7.6826196	poor	Serous	3c	3	MET_N375S
1207	3	25mg 2wk->75mg 2wk	PD	32.65306	good	Endo (endometrial)	4b	2	PIK3CA_H1047RL
1205	3	25mg 2wk->75mg 2wk	SD	-25	poor	Clear Cell	3c	3	KRAS_G12DAV, PIK3CA_H1047RL, PIK3R1_M326I
1208	3	25mg 2wk->75mg 2wk	SD	11.875	poor	Serous	3	2	PHLPP2_L1016S

2.2 *In vitro* methods

2.2.1 Apoptosis, cell viability and combination assays

Cells were seeded in white-walled 96 well plates for caspase 3/7 apoptosis assays and in parallel in clear 96 well plates for 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT cell viability assays (3×10^3 – 10×10^3 in 100 µl per well) and incubated at 37°C / 5% CO_2. After 24 hours, cells were treated with AKT, PI3K or DNA-PK inhibitors diluted in RPMI 1640. Cells were pre-incubated with the inhibitor at 37°C / 5% CO_2 for 1 hour, prior to addition of cisplatin (cddp). After 24, 48 or 72 hours, caspase 3/7 activity was measured by luminescence using the Caspase-Glo 3/7 apoptosis assay (Promega, UK) as per the manufactures instructions. Briefly, equal volume of a luminogenic caspase 3/7 substrate (100 µl) was added to each well and plates were incubated at room temperature for 1 hour. Plates were read on a LUMIstar optima micro-plate reader (BMG LABTECH). Relative cell viability across treatments was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, UK). Media was aspirated from wells and was replaced by 100 µl MTT at the concentration of 10 µg/ml. Plates were incubated at 37°C/ 5% CO_2. After 2.5 hours formazan was dissolved in 10% sodium dodecyl sulphate in 0.01% hydrochloric acid (HCl) solution. Plates were read at 570 nm on a SPECTRA MAX 190 plate reader (Molecular Devices). Data obtained from the caspase assays were normalised to the MTT cell viability data following the same treatment. Each treatment included three replicates, and each experiment was performed in triplicate.

2.2.2 Apoptosis and Cell cycle analysis using propidium iodide

Cells were treated with GSK2141795 (1 µM or 5 µM) or cisplatin (25 µM) as either single agents or in combination. Control cells were treated with PBS alone. In the combination treatment, cells were pre-treated with GSK2141795 1 hour prior to addition of cisplatin. After

24 hours of cisplatin treatment, cells were harvested with 0.25% trypsin, and combined with floating cells in the media, prior to centrifugation. Cells were fixed in 70% ethanol, at a density of 1 x 10^6, and stored at –20°C overnight. The next day, cells were washed once with PBS, and then incubated with propidium iodide (PI) (0.05 mg/ml PI, 0.2 mg/ml RNase A in PBS) for 30 minutes at 4°C in the dark. Samples were analysed with a FACScalibur flow cytometer (Becton Dickinson). Ten thousands events per sample were collected and DNA content was measured based on the PI fluorescence (excitation at 488 nm, emission 585 nm; FL2). Data was analysed using the Flowjo software (Tree Stan. Inc). All cells were included in the analysis and cells were not gated to include the apoptotic sub-G1 population into the cell cycle profile. Sub-G1 cells were included in the cell cycle analysis as the apoptotic population.

2.2.3 Small interfering RNA (siRNA) transfection

Cells were grown to 60% confluency in 6 well plates prior to transfection with On-Target Plus siRNA (Thermo Scientific, UK) directed to *PIK3CA* (L-003020), *PIK3R1* (L-003028), or non-targeting control (D-001810), as per manufactures protocol. SiRNAs in 1x siRNA buffer were mixed with 2 µL transfection-reagent #1 (Dharmacon, USA) per transfection in a total volume of 400 µL with OptiMEM media. The transfection reaction was incubated at room temperature for 30 minutes. The siRNAs were then added to cells in 1600 µL antibiotic free RPMI1640/10%FCS.

For western-blot analysis, siRNA transfected cells were treated with cisplatin or kinase inhibitor, combination of both or were left untreated. Protein lysates were collected 2 hours after the treatment.

For MTT cell viability and caspase 3/7 assays, 48 hours after transfection, cells were re-seeded into opaque and clear 96 well plates, for apoptosis and cell viability assays, respectively and were treated identically with cisplatin and/or kinase inhibitors. For each transfection condition, 24 hours after seeding, 3 replicate wells were treated with 25 μM cisplatin or kinase inhibitor or combination of both. Also three wells were left untreated. After 24 hours, caspase activation was measured by caspaseGlo 3/7 (Promega, UK) assay and cell viability was assessed by MTT assay (Sigma, UK). Caspase activity was normalised to cell density for each treatment.

2.2.4 Protein Extraction and Western Blot analysis

Cells were grown in 35 mm × 10 mm tissue culture dishes (Corning, NY, USA) for protein extraction for western blotting. Cells were washed twice with ice-cold PBS prior to lysis on ice in 250 μl of protein lysis buffer (2.2% SDS, 68 mM Tris, 11% glycerol and 1x complete protease inhibitor cocktail (Roche, UK) and 1X phosphatase inhibitor (Pierce, USA). Lysates were cleaned by centrifugation for 10 minutes at 4ºC at 10000 x g and quantified by BCA protein assay according to the manufactures protocol (Pierce, USA). Samples were stored at -80ºC Dithiothreitol (DTT) (100mM final concentration) was added prior to denaturation at 95°C for 5 minutes. Twenty micrograms of cleared lysate was electrophoresied at 20 mA, 100 V for 2 hours through 8-12% SDS polyacrylamide gels (Table 2.1 and 2.2) then transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked with 5% Bovine serum albumin (BSA) or non-fat milk in PBS/0.1% TritonX100 at room temperature for 2 hours. Membranes were probed using a 1:1000 dilution of primary antibody (in 5% Bovine serum albumin (BSA) or non-fat milk in PBS/0.1% TritonX100) (Table 2.3) overnight at 4°C, and then were washed for 10 minutes in PBS/0.1% TritonX100 three times . Membranes were incubated with secondary antibodies (1:2000, Dako Cytomation, Denmark) for 2 hours at room temperature and again washed with PBS/0.1%

TritonX100 three times. After washing, bound antibodies were visualized by Immobilon enhanced chemiluminesence (Millipore, UK) and photographic film.

2.2.5 Stripping western blot membranes for re-probing

Western blot membranes were stripped to re-probe for additional proteins. A stripping buffer containing 20 ml SDS 10%, 12.5 ml 0.5 M Tris HCl pH 6.8 (in 100 ml) and β-mercaptoethanol (0.8 ml/100 ml buffer) warmed to 50ºC, was used. The membrane was stripped for 45 minutes, and then washed in PBST for 40 minutes.

Table 2-3 Separating gel components for western blotting

Stock Solutions	8%	10%	12%
30% acrylamide/0.8% bisacrylamide	4 ml	5 ml	6 ml
4X Tris-Cl/SDS, pH=8.8 (1.5 M Tris-Cl, pH 8.8 0.4% SDS)	3.75 ml	3.75 ml	3.75 ml
H_2O	7.25 ml	6.25 ml	5.25 ml
10% ammonium persulfate	50 µl	50 µl	50 µl
TEMED	10 µl	10 µl	10 µl

Table 2-4Stacking gel (3.75%) components for western blotting

Stock Solutions	
30% acrylamide/0.8% bisacrylamide	0.65 ml
4X Tris-Cl/SDS, pH=6.8 (0.5M Tris-Cl, pH 6.8, 0.4% SDS)	1.25 ml
H_2O	3.05 ml
10% ammonium persulfate	25 µl
TEMED	5 µl

Table 2-5 list of antibodies used in western blot analysis

Antibody	Dilution	MW	Isotype	Manufactory
PI3 Kinase p110α	1 in 1000 in 5% dry milk, 1% TBS,0.1%Tween	110 KDa	Rabbit, polyclonal	Cell Signalling Technology, Cat. No: 4255
PI3 Kinase p85 α	1 in 1000 in 5% BSA, 1% TBS, 0.1% Tween-20	85 KDa	Rabbit, polyclonal	Cell Signalling Technology, Cat. No : 4292
AKT Total	1 in 1000 in 5% BSA, 1% TBS, 0.1% Tween-20	60 KDa	Rabbit, monoclonal	Cell Signalling Technology, Cat. No: 9272
AKT pS473	1 in 1000 in 5% BSA,1% TBS,0.1%Tween-20	60 KDa	Rabbit, monoclonal	Cell Signalling Technology, Cat. No : 4060
β-tubulin	1 in 10000 in 5% dry milk, 1% TBS,0.1%Tween	55 KDa	Rabbit, Polyclonal	Cell Signalling Technology, Cat. No: 2146
Anti-Rabbit Immunoglobulins	1 in 20000 in 5% BSA,1% TBS,0.1%Tween-20	-	Goat. Polyclonal	Dako, Cat. No: P044801-2

2.2.6 Enzyme-linked immunosorbent assays (ELISA)

Levels of phospho-PRAS40 (pT246) and total PRAS40 were assessed using ELISA kits (Invitrogen, KHO0421) according to the manufacturer's instructions. SKOV3 monolayers and MTS's were treated with GSK2141795 (0.01 – 5 μM) or GSK2126458 (0.1 – 40 nM) for 24 – 72 hrs. After treatment, cells were lysed in cell extraction buffer (Invitrogen FNN0011) according to the manufacture's instruction, and samples were diluted 1 in 50 in diluent buffer provided in the kit. Absorbance was measured at 450 nm using a SPECTRA MAX 190 plate reader (Molecular Devices). PRAS40(pT246) levels were normalised to total PRAS40 levels (Invitrogen, KHO0411). The level of pAKTS473 and pAKTT308 compared to total AKT were also assessed using similar ELISA kits (Invitrogen, KHO0111, KHO0101, KHO0201).

2.2.7 Multicellular tumour spheroids (MTS)

SKOV3 tumour cells were trypsinised from monolayer culture, and cell suspensions seeded at a density of 10000 cells per well in 24-well (1% agarose-coated) plates. Spheroids were grown at 37°C/5% CO_2 for 7 days, in 200 μl of culture media.

To make the 1% agarose plates, 5 mg agarose was dissolved in 500 ml ddH2O and the solution was autoclaved. While still warm, 0.5 ml agarose solution was dispensed in each well of 24-well tissue culture plate in a tissue culture cabinet and plates were allowed to set at room temperature. Plates were stored at 4ºC and before use the plates were incubated with 500 ml warm culture media and were incubated at 37ºC incubator for 1 hour prior to cell seeding.

2.2.8 *In vitro* radioligand binding studies

For time-course studies, SKOV3 monolayers and spheroids (MTS) were treated with two concentrations of GSK2141795 (1 and 5 μM) for 48 hours and incubated at 37°C, 5% CO_2. Twenty four hours into the incubation, SKOV3 monolayers and MTS's were incubated with culture media (0.5 ml per well), containing [^{3}H]FDG (296 kBq) for between 0 – 24 hours in 24 well plates. For dose-response studies, SKOV3 monolayers and MTS's were treated with a range of concentrations of GSK2141795 (0.001 - 5 μM) for different lengths of time (monolayers for 24 hours; MTS's for 48 hours). Two or six hours prior the end of incubation, monolayers and MTS's were incubated with 0.5 ml medium per well containing [^{3}H]FDG (296 kBq).

Following incubation with [^{3}H]FDG, monolayers and MTS's were washed twice with PBS, and cells solubilised in 1ml 0.1M NaOH at 37°C overnight prior to transfer to 5ml tubes and addition of 4ml scintillation fluid (4ml; Packard Ultima Gold MV). Radioactivity was determined by liquid scintillation counting (Perkin Elmer, TriCarb2900). For each MTS, 100 μl of the last wash was measured as a background. Data were analysed by iterative non-linear regression curve fitting using Prism v5.0 (GraphPad Software, San Diego, California, USA). Each experiment was done in triplicate and analysed individually.

2.3 *In vivo* xenograft experiments

All animal studies were ethically reviewed and carried out in accordance with Animal (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of animals.

2.3.1 Mouse strain

Female Mice strain Crl: Nu/Nu – foxn1, age approximately 11 weeks weighing 25 – 30 g were utilised in this study.

2.3.2 Establishment of xenografts

Two tumour xenografts were established in each animal, by injecting $10x10^7$ SkOV3 tumour cells / 100µL sub-cutaneously (sc) in each flank, using a 25-G needle, under light isoflurane anaesthesia. The cell suspensions for inoculation were prepared in PBS, warmed to 37^oC following trypsinisation of cells in monolayer culture.

2.3.3 Tumour Growth and Toxicity Monitoring

Tumour size and tumour volume were calculated by measuring two perpendicular tumour diameters, and using the 3D ellipsoid tumour volume formula below.

Volume = $(width^2 \times length)/2$

From the day after inoculation, mice were monitored for tumour size, and twice a week thereafter. Monitoring of each animal's well-being occurred daily**,** based on body weight and food/water consumption from the day of inoculation.

2.3.4 Treatment Procedure

Treatments were started when tumour xenografts reached approximately $80mm^3$. Animals were then randomly assigned to one of 4 treatment groups, with 5 animals per group. The 4

groups were: vehicle only, GSK2141795 or NU7441 only, Cisplatin (cddp) and cddp + GSK2141795 or NU7441.

Group 1: Control (vehicles):

Conscious animals received 10 ml/kg 20% PEG / 1% DMSO daily for 14 days at noon. Animals were sacrificed by exsanguination on day 14 at 5 pm.

Group2: GSK2141795 or NU7441 only:

Conscious animals received 30 mg/kg (10 ml/kg) of GSK2141795 by oral gavage or 10 mg/kg NU7441 by intraperitoneal injection (IP) daily, at noon for 14 days. Animals were sacrificed by exsanguination on day 14, at 5 pm, 5 hours following administration of the final dose of the drug.

Group 3: cddp:

Conscious animals received single IP injection of cisplatin in saline (1.5 mg/kg in AKT study or 1 mg/kg in DNA-PK study (10 ml/kg), twice a week (Tuesdays and Fridays) at 2 pm, for 14 days. Animals received an extra dose of cisplatin on day 14 before being sacrificed at 5 pm.

Group 4: cddp + GSK2141795 or NU7441

Conscious animals received 30 mg/kg of GSK2141795 by oral gavage or 10 mg/kg NU7441 by IP injection daily for 14 days at noon and single IP injection of cisplatin in saline (1.5 mg/kg in AKT study or 1 mg/kg in DNA-PK study), at 2 pm (2 hours following oral administration of GSK2141795 or NU7441), on Tuesdays and Fridays. Animals were

sacrificed by exsanguination on day 14 at 5 pm and blood was collected. The glucose level in blood was measured using a glucose meter. Tumour and muscle tissues were harvested and frozen at -80 for further studies.

2.3.5 *In vivo* PET imaging studies

For time-course studies, following overnight fasting mice were treated with GSK2141795 (30 mg/kg) for 1 - 72 hours, prior to [^{18}F]FDG administration (~8 MBq) via a jugular vein cannula. For dose-response studies, following overnight fasting, mice received either vehicle (20% PEG / 1% DMSO) or GSK2141795 (10, 20 or 30 mg/kg) by oral gavage, 5 hours prior to [^{18}F]FDG (~8 MBq) administration.

All animals were subsequently scanned 120 minutes after FDG administration, on a dedicated small animal PET scanner (Siemens Inveon PET/CT module, Siemens Molecular Imaging Inc. UK). Animals were kept under isofluorane anaesthesia throughout the imaging period with constant monitoring of vital signs. Whole blood was drawn immediately before sacrificing the animals and variable tissues were collected for pharmacokinetic analysis. Dynamic emission scans were acquired in list mode format over 60 minutes. Following completion of the scanning period, animals were sacrificed by exsanguination. Standardised uptake values (SUV) of radioactivity were calculated for the tumour region of interest (ROI) for each image of a dynamic series using the formula below:

$$SUVmean_{(30\text{-}60\ min)} = \frac{Tissue\ radioactivity\ concentration\ at\ time\ (Ct)}{Injected\ dose\ at\ t_0\ (Bq)\ /\ body\ weight\ (g)}$$

Tumour time radioactivity curves (TAC) were generated by calculating a ratio of the tumour to plasma uptake and by calculating the SUV at 60 minutes post administration of the [^{18}F]FDG for each animal. [^{18}F]FDG uptake in GSK2141795 treated xenografts were subsequently compared to the untreated control animals.

2.4 Pharmacokinetics

All blood and tumour samples taken for pharmacokinetic analysis were stored at -80°C prior to analysis. Samples collected at the same nominal times were pooled for analysis. Prior to bio analysis, blood samples were mixed 1:1 (v:v) with HPLC grade water, and tissue samples were mixed 1:4 with HPLC grade water (w:v; tissue: water) and homogenised using a probe-type homogeniser. Mouse tissue homogenates, plasma and blood samples were analysed for GSK2141795 using LC/MS/MS. Tissue homogenate concentrations (ng/mL homogenate) were converted to tumour tissue concentrations (ng/g tissue) by calculating the product of the measured homogenate concentration and the dilution factor incurred during sample processing. This conversion assumes complete extraction of the analyte from the tissue homogenate samples during bioanalytical sample preparation. The in-life portion of this study was performed at the GSK Clinical Imaging Centre, UK, and the mass spectrometry (MS) analysis of this study was carried out by GSK, USA.

2.5 Reverse Phase Protein Array (RPPA)

SKOV3 and PEO4 cells after drug treatments were washed twice with cold PBS and lysed using 100 µL RPPA lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na3VO4, 10% glycerol, containing freshly added protease and phosphatase inhibitors (Roche Applied

Science) in 6-well plates. Tumours recovered from *in vivo* experiments or clinical biopsies were crushed in 100 µl cold RPPA lysis buffer using an electric pellet pestle (Sigma, UK). Lysates were centrifuged at 14000 rpm for 15 minutes and incubated on ice for 30 minutes. Supernatants were collected and pellets discarded. Protein concentration was determined using the BCA assay (Invitrogen, UK). Lysates were analysed by RPPA as described at MD Anderson Cancer Centre (Tibes, Qiu et al. 2006) (Figure. 2.1). RPPA data was normalised, median centred and analysed in Microsoft Excel. For proteins regulated by direct phosphorylation, in addition to the total and phospho-proteins, phospho/total normalised level was also assessed as an individual expression. Proteins with significantly expression levels (t-test; $p<0.05$) between sample group were identified. Proteins showing less that 1.3 fold difference between groups were discarded on the basis of unlikely biological relevance despite statistical significance. Venn diagrams were used to compare protein expression levels from different treatment groups and Venn groups were determined as shown in Figure. 2.2.

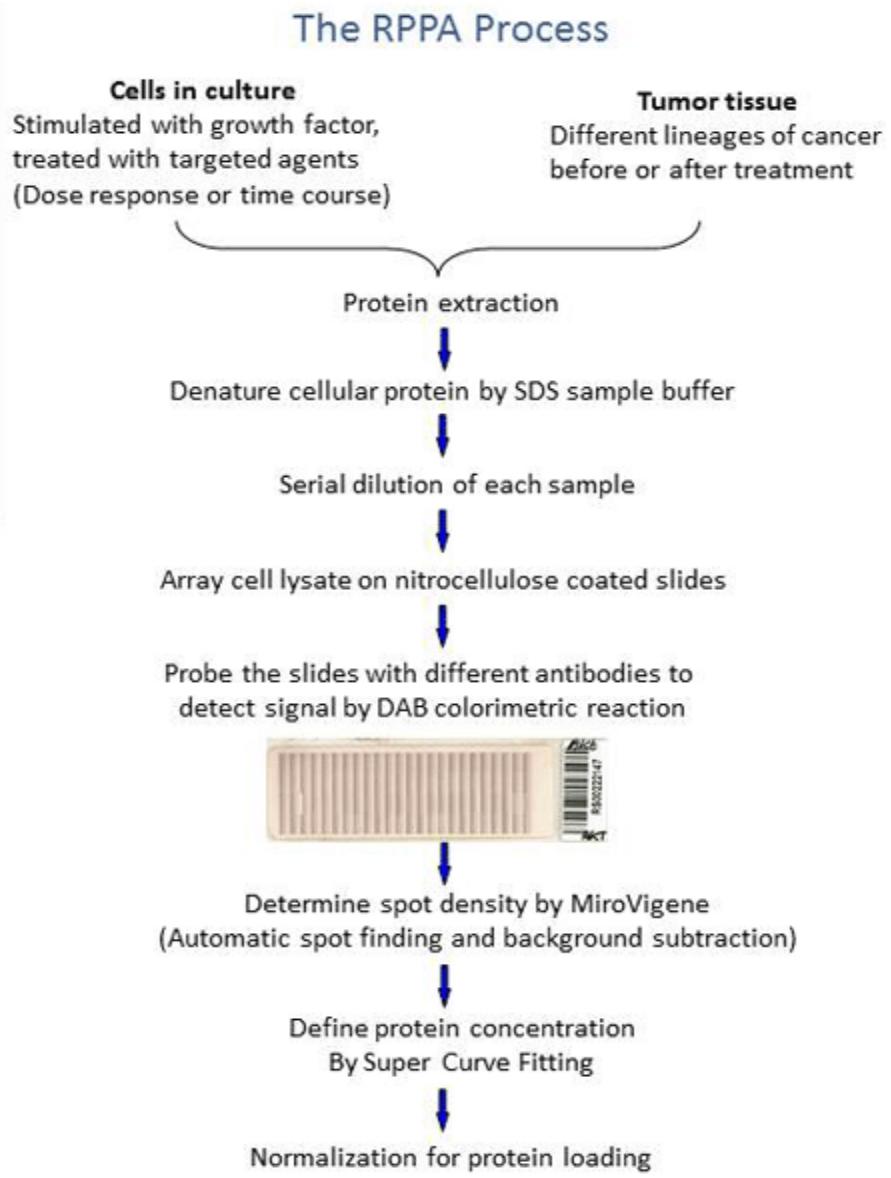


Figure 2-1 RPPA Process

Following the protein extraction, cellular protein will be denatured by SDS sample buffer. Cell lysates are arrayed on nitrocellulose coated slides followed by being probed with different antibodies. Figure from www.mdanderson.org

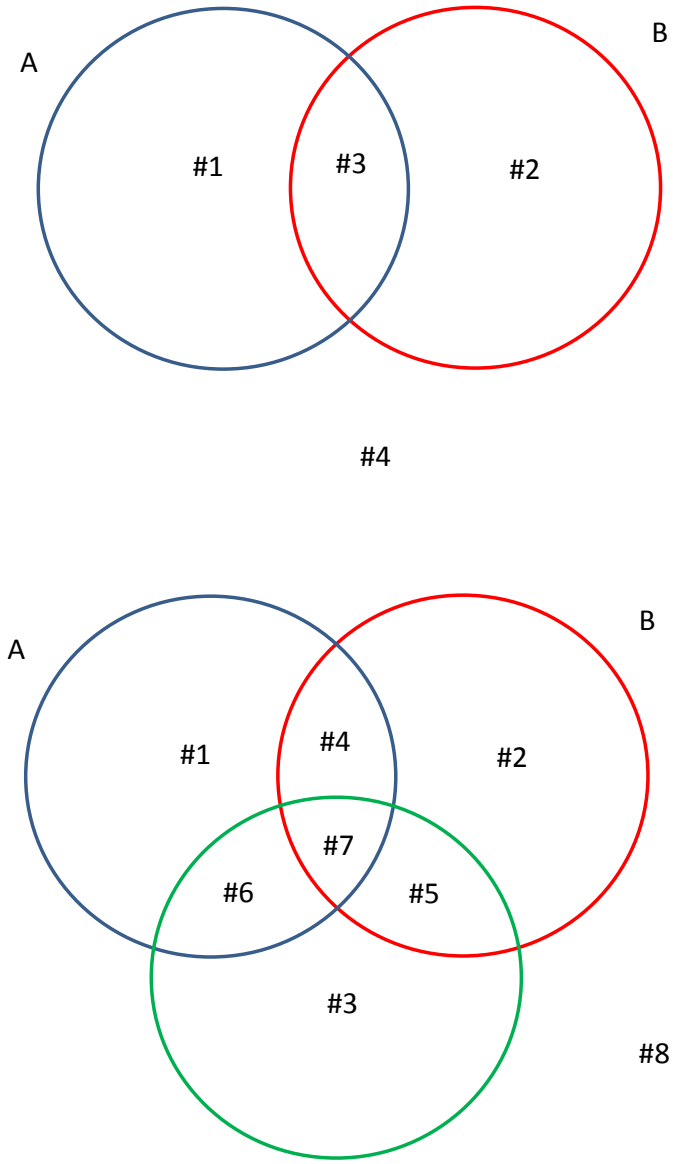


Figure 2-2 Venn groups used for analysis of the RPPA data.

2.6 Statistical analysis

RPPA data were processed using the Microsoft Excel. Raw data were log transformed, normalised and median centred. Significant changes in protein levels were tested by student's t-test, two-sample unequal variance (heteroscedastic). The significant changes were defined as more than 1.6-fold with adjusted $P < 0.05$. For non-parametric tests, Mann-Whitney U test was used. Levene's test was used to assess the equality of variances for two or more groups.

Normalisation to an experimental control, normally un-treated or vehicle, was done by dividing the mean of replicates to the mean of controls. Caspase 3/7 data were further normalised to cell density.

The standard deviation (SD) was calculated to measure of how widely values are dispersed from the average value (the mean). Standard error of mean (SEM) was calculated to measure how distant the sample mean is from the population mean and to indicate the standard deviation of sampling distribution. Both SD and SEM were calculated in Microsoft Excel.

IC_{50} values were calculated) with 95% confidence interval using as the GraphPad Prism software (GraphPad Software, Inc). Relative IC_{50} values (the concentration required to bring the dose-response curve down to point half way between the top and bottom plateaus of the curve) were calculated by selecting 'non-linear regression (curve-fit), log [inhibitor] v response, (variable slop, four parameter)' option in Graph-pad Prism. Significant changes in IC_{50} values were tested by student's t-test. One-way ANOVA was used to test significant differences between more than two groups of samples. If there were two independent

variables in the experiment, two-way ANOVA was used to test differences between groups of samples. The significant changes were defined as $P < 0.05$.

3 Utility of the pan AKT inhibitor GSK2141795 in overcoming platinum resistance in ovarian cancer

3.1 Introduction

One of the most frequently activated signalling pathways in EOC is the PI3K/AKT axis. PI3K, KRAS and PTEN mutations underlie activation in Type I tumours, whereas Type II tumours exhibit frequent copy number changes and over-expression of PI3K/AKT pathway components (Bast and Mills 2012). Work in our laboratory has shown activation of the PI3K/AKT pathway in platinum-resistant cells (as evidenced by increased phosphorylation of AKT at serine 473 by DNA-dependent protein kinase (DNA-PK) (Stronach, Chen et al. 2011) in response to DNA damage, which directly links the established role of AKT in chemo-resistance to the DNA damage caused by platinum agent therefore AKT inhibition represents a potentially useful strategy for platinum sensitisation in ovarian cancer.

GSK2141795 is a novel member of the N-alkyl pyrazole class of orally available kinase inhibitors. It is a potent, pan-AKT inhibitor, with potency (Ki) values for human AKT 1, 2 and 3 kinases of 0.066nM, 1.4nM and 1.5 nM, respectively (GlaxoSmithKline unpublished data).

In this chapter, the effect of inhibition of AKT using GSK2141795 as single agent and in combination with cisplatin on tumour growth and apoptosis, using both *in vitro* and *in vivo* ovarian cancer models will be assessed, to determine the potential utility of this compound for clinical implementation. *In vitro* assays were performed using both 2-dimensional (2D) and 3-dimensional (3D) spheroid cell culture models to determine if 3D models better model in vivo results of GSK2141795. Efficacy was assessed using both apoptosis and growth assays. *In vivo* assays also enabled a degree of toxicity assessment.

3.2 Results

3.2.1 Effect of AKT inhibition using GSK2141795 as single agent or in combination with cisplatin on platinum resistance *in vitro*

3.2.1.1 Single agent 2D activity

In order to examine the effect of the inhibition of AKT by GSK2141795 on apoptosis, platinum resistant SKOV3 cell monolayers were treated with a range of clinically relevant concentrations of GSK2141795 (0.075-5.0 μM) for 24, 48 and 72 hours. The level of caspase 3/7 activation was used to measure apoptosis induction after GSK2141795 treatment. GSK2141795 as a single agent did not induce caspase activation greater than vehicle controls over the selected range of concentrations (0.1 μM-5 μM) and incubation times (24 – 72 hours)(Figure.3.1a and 3.2). Similar results were obtained with single agent GSK2141795 in the platinum resistant PEO4 ovarian cancer cell line (Figure. 3.1b).

3.2.1.2 GSK2141795 in combination with cisplatin

By contrast, treatment with GSK2141795 in combination with cisplatin increased the induction of caspase 3/7 activity in both SKOV3 and PEO4 monolayers after 24 hours (Figure 3.1a and b. respectively). With a combination of GSK2141795 (5 μM) and cisplatin (25 μM) for 24 hours, there was a 3-fold and 8-fold increase in induction of caspase 3/7 activity in PEO4 and SKOV3 cells respectively (Figure. 3.1a and b).

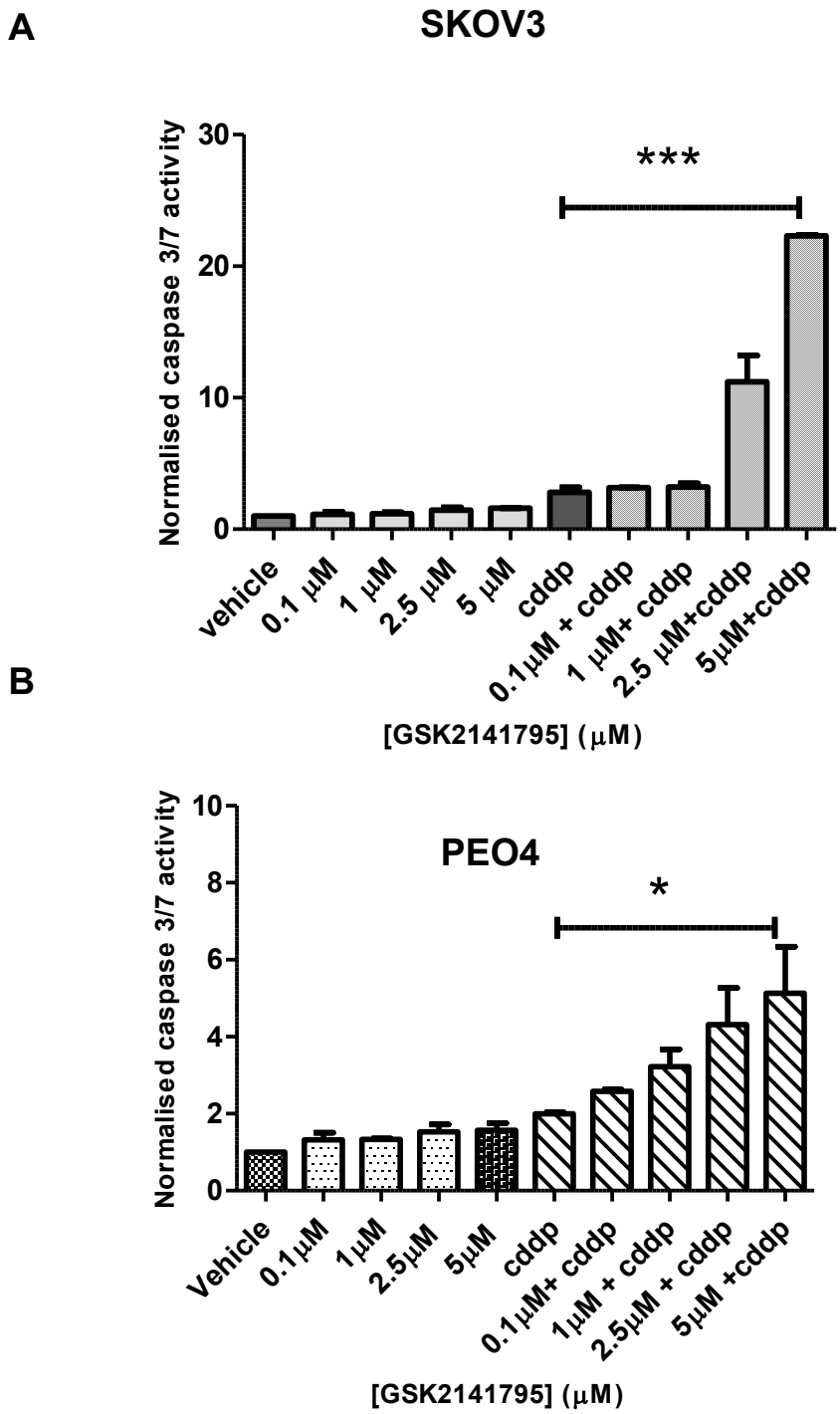


Figure 3-1 Caspase 3/7 activity in SKOV3 (a) and PEO4 (b) monolayers exposed to GSK2141795 as a single agent or in combination with cisplatin (cisplatin) for 24 hours

SKOV3 and PEO4 monolayers were pre-treated with a range of concentrations of GSK2141795, 1 hour prior to treatment with 25 μM cisplatin . Induction of caspase 3/7 activity was assessed at different time points following the initiation of the treatment. Combination of GSK2141795 with cisplatin increased apoptotic response to cisplatin treatment in a dose dependent manner. Treatment with GSK2141795 as a single agent for the same length of time did not result in any significant increase in the apoptotic activity. Data shown are the means ± SEM of 3–4 experiments performed in triplicate. *P < 0.05 **P < 0.01. ***P < 0.001.

The effect of GSK2141795 on platinum sensitisation in SKOV3 cells was also assessed after treatment for 48 and 72 hours. GSK2141795 re-sensitised SKOV3 monolayers to cisplatin in a time and dose dependant manner, with maximal induction of caspase 3/7 after 72 hours of treatment (fold change ~ 4, $P < 0.001$)(Figure 3.2).

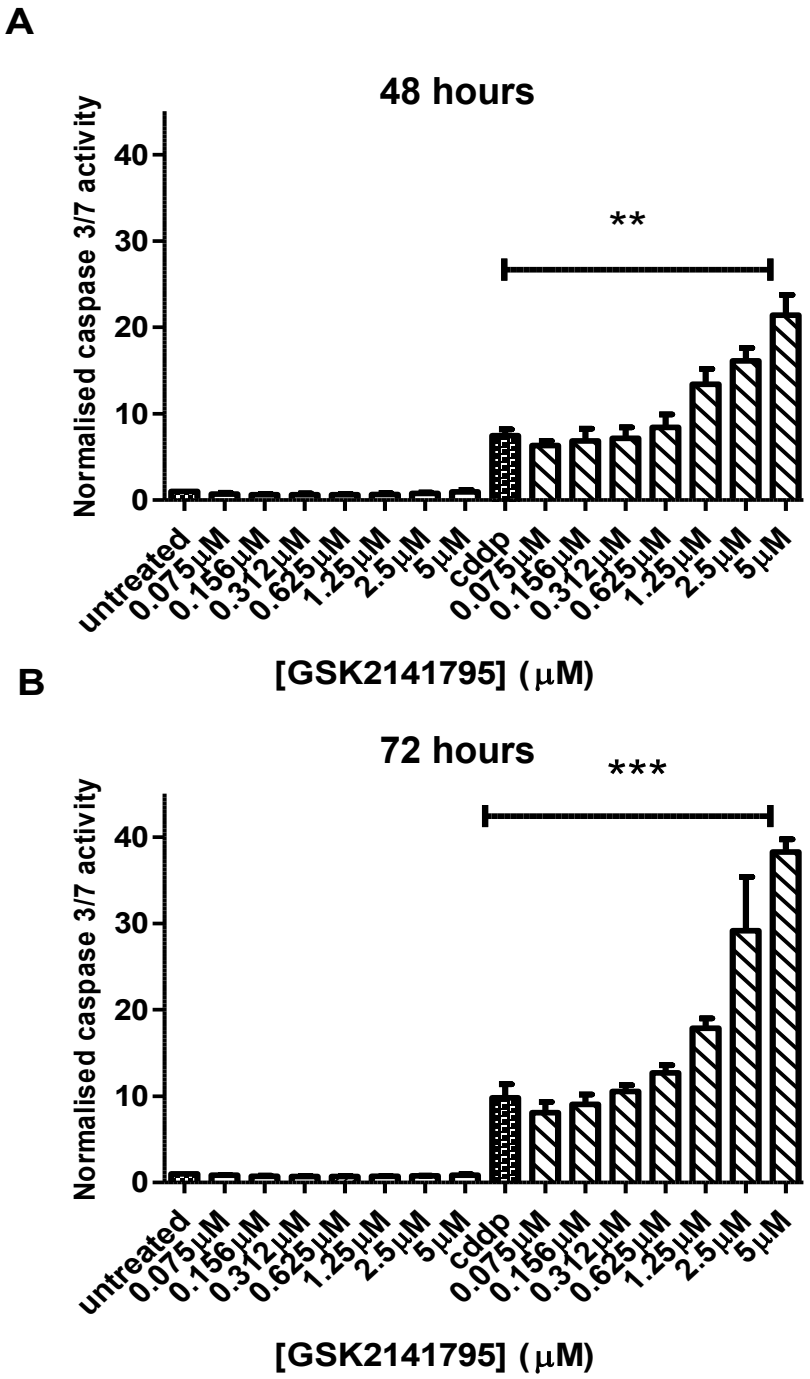


Figure 3-2 Caspase 3/7 activity in SKOV3 monolayers exposed to GSK2141795 as a single agent or in combination with cisplatin (cddp) for 48 (a) and 72 (b) hours

SKOV3 monolayers were pre-treated with a range of concentrations of GSK2141795, 1 hour prior to treatment with 25 μM cisplatin. Induction of caspase 3/7 activity was assessed at different time points following the initiation of the treatment. Combination of GSK2141795 with cisplatin increased apoptotic response to cisplatin treatment in a dose dependent manner in SKOV3 cells at 48 and 72 hours. Treatment with GSK2141795 as a single agent for the same length of time did not result in any significant increase in the apoptotic activity.). Data shown are the means ± SEM of 4 experiments performed in triplicate. *P < .05 **P < .01. ***P < .001.

In contrast in platinum sensitive PEO1 cells (isogenically matched to PEO4 resistant cells), treatment with GSK2141795 for 24 hours in combination with cisplatin, resulted in only a ~ 1.5 -fold increase in caspase 3/7 activity at a GSK2141795 concentration of 5 μM, which was not significant compared to the cisplatin only treated cells (Figure 3.3).

The effect of AKT inhibition using GSK2141795, on platinum re-sensitisation was further assessed in another isogenically matched cell line pair, PEA1 and PEA2. Treatment with GSK2141795 and cisplatin for 24 hours increased the sensitivity to cisplatin in the sensitive PEA1 line, by ~ 1.5 -fold at 10 μM compared to cisplatin only, but this increase was not significant ($p = 0.26$) (Figure 3.4a). In contrast treatment with GSK2141795 at 10 μM re-sensitised the platinum resistant pair, PEA2, with 2.3 -fold induction in caspase 3/7 activity compared to cisplatin alone ($p=0.01$) (Figure 3.4.b).

Furthermore, to test if the increase in apoptosis with combination treatment was additive or synergistic, isobologram analysis was performed. This demonstrated that the effect of combining GSK2141795 with cisplatin was synergistic in both the SKOV3 and PEO4 cells (Figure.3.5).

3.2.1.3 3-D spheroid culture

To further determine the activity of GSK2141795 in 3D culture, SKOV3 tumour cell spheroids were treated with a range of concentrations of GSK2141795 (1-10 μM) as a single agent, or in combination with 25 μM cisplatin for 72 hours. Combination of GSK2141795 and cisplatin significantly increased the induction of caspase 3/7 activity, in comparison to cisplatin-only treated controls ($P< 0.01$) (Figure 3.6). PEO4 cells could not be grown as spheroids.

As the AKT inhibitor GSK2141795 was mostly effective on reversal of platinum resistance in SKOV3 platinum resistant cells, this cell model was chosen for further pre-clinical investigation of the compound.

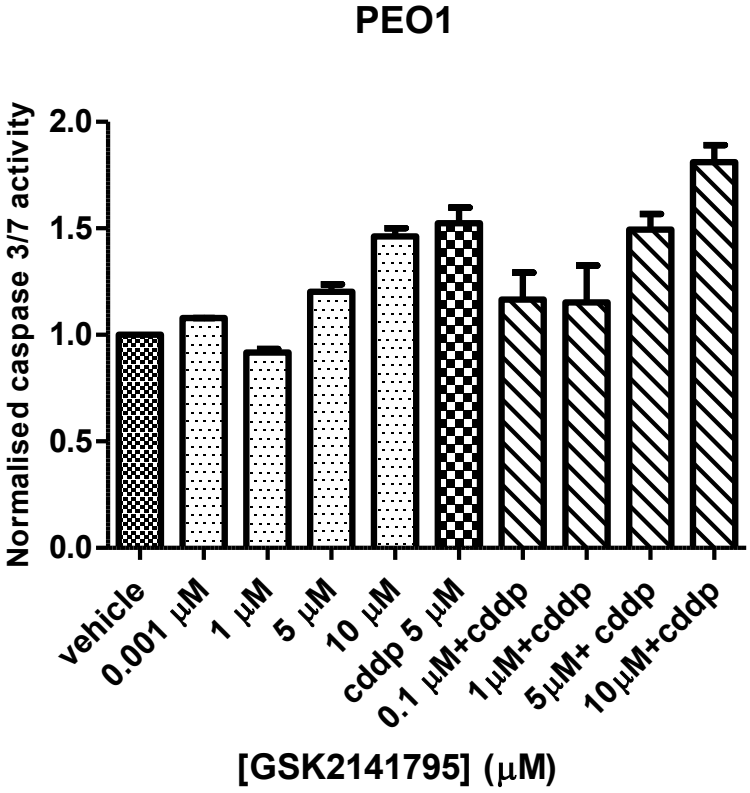


Figure 3-3 Caspase 3/7 activity in PEO1 monolayers exposed to GSK2141795 as a single agent or in combination with cddp (cisplatin) for 24 hours

Platinum sensitive PEO1 monolayers were pre-treated with a range of concentrations of GSK2141795, 1 hour prior to treatment with 5 μM cddp. Induction of caspase 3/7 activity was assessed 24 hours after the initiation of the treatment. Combination of GSK2141795 with cddp did not result in any significant increase in the apoptotic activity in PEO1 cells. Data shown are the means ± SEM of 3 experiments performed in triplicate.

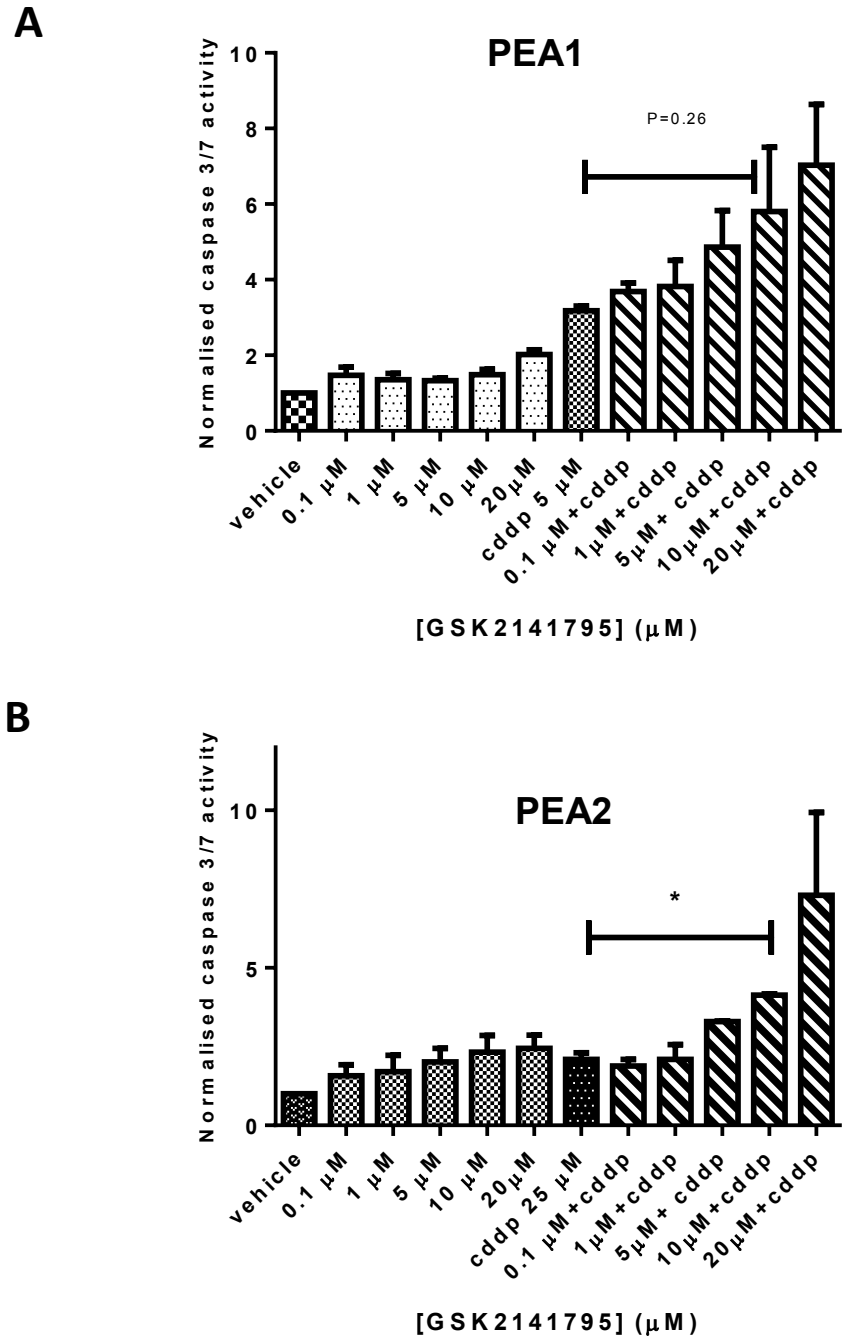


Figure 3-4 Caspase 3/7 activity in platinum sensitive PEA1 monolayers (A) and their isogenically matched pair platinum resistant PEA2 cells (B) exposed to GSK2141795 as a single agent or in combination with cisplatin (cddp) for 24 hours.

PEA1 and PEA2 monolayers were pre-treated with a range of concentrations of GSK2141795, 1 hour prior to treatment with 5 and 25 µM cddp respectively. Induction of caspase 3/7 activity was assessed 24 hours following the initiation of the treatment. Combination of GSK2141795 with cddp increased apoptotic response compared to cddp only treatment in a dose dependent manner in PEA2 cells but not in PEA1 cells. Treatment with GSK2141795 as a single agent for the same length of time did not result in any significant increase in the apoptotic activity in neither PEA2 nor PEA1 cells. Data shown are the means ± SEM of 4 experiments performed in triplicate. . *P < .05

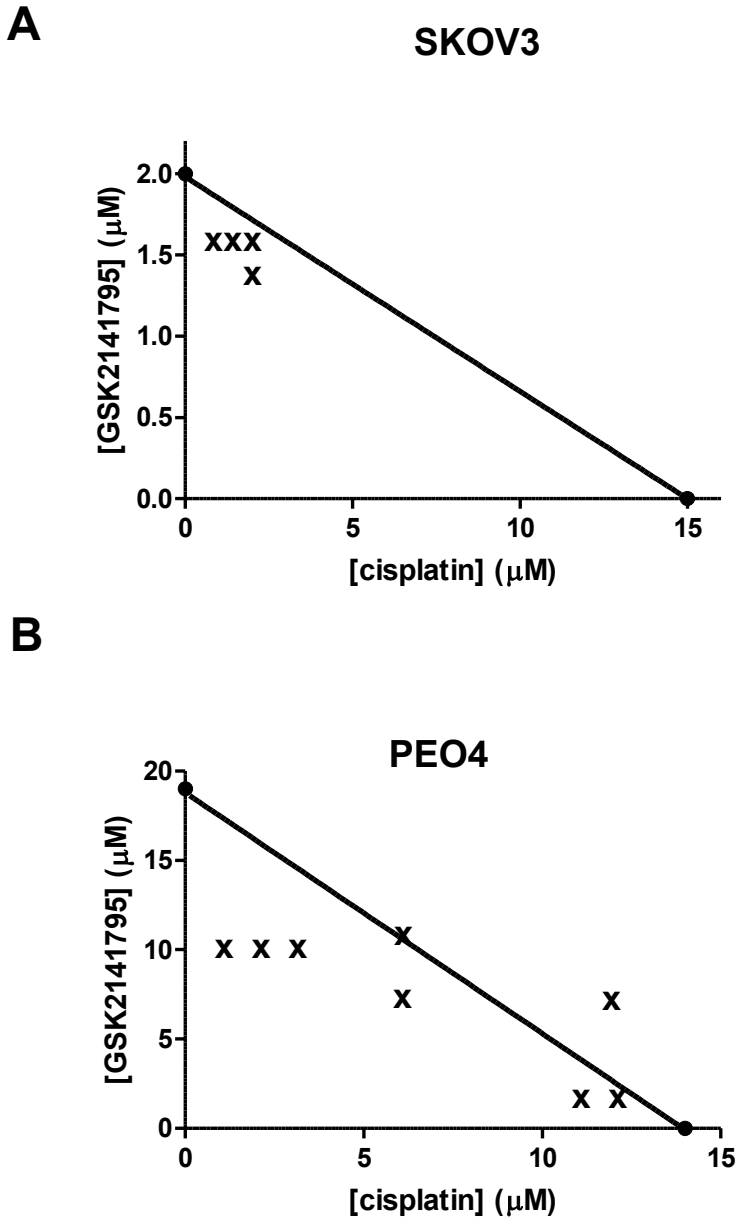


Figure 3-5 GSK2141795 and cisplatin (cddp) interaction in SKOV3 (A) and PEO4 (B) cells

Isobologram generated from GSK2141795 and cddp IC_{50} values taken from the 2D monolayer studies. The IC_{50} values of combinations were determined with the 95% confidence limit and plotted. The x and y intercepts represent the IC_{50} values of either cddp or GSK2141795 as single agents. The line connecting the IC_{50} values represents the additivity line. Combination drug points occurring on or around the line represent additivity whereas points above the line represent antagonism. Points below the line represent synergy. All of the IC_{50} values for the GSK 2141795 and cddp combination in SKOV3 and most of the values in PEO4 were below the line suggesting synergy between these two compounds in these two cell lines.

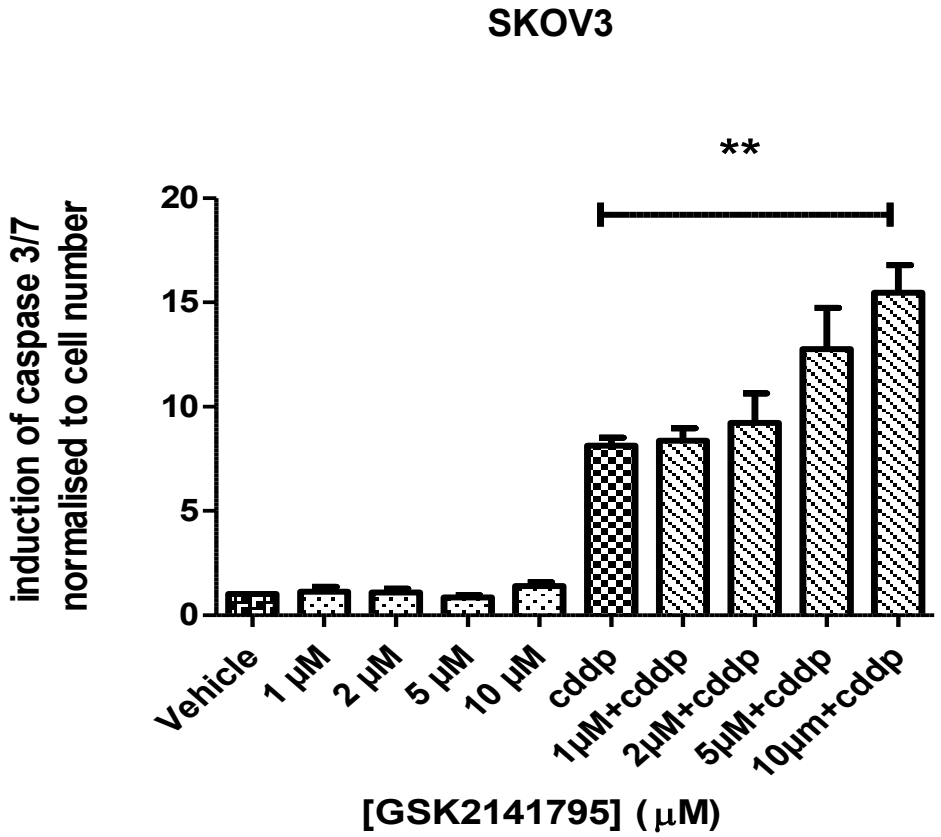


Figure 3-6 Caspase 3/7 activity in SKOV3 multi-tumour spheroids (MTS's).

SKOV3 MTS's were treated with a range of concentrations of GSK2141795 either as a single agent or in combination with 50 µM cddp for 72 hours. Combination of GSK2141795 with cddp resulted in a dose dependent increase in the apoptotic activity in response to cddp whereas treatment with GSK2141795 as a single agent had no effect on the caspase activity in SKOV3 MTS's.). Data shown are the means ± SEM of 3 experiments performed in triplicate. **P < .01.

3.2.2 Growth-inhibitory effect of GSK2141795 in ovarian cancer cells

Based on the above activity of GSK2141795 on apoptosis, the effect of GSK2141795 as a single agent on cell growth and viability of SKOV3 and PEO4 cells was also assessed using the MTT assay. Treatment with GSK2141795 reduced cell growth in a dose-dependent manner over 72 hours of treatment (approx. 3 X cell doubling time). For combination studies, a range of concentrations of GSK2141795 (0.075 – 5 μM) was combined with cisplatin (25 μM). Cell viability was found to be reduced in SKOV3 cells treated with single agent GSK2141795, and this was found to be further decreased in a GSK2141795 dose-dependent manner when the drugs were combined (Figure. 3.7). The half-maximal effective concentration of GSK2141795 in the combination with cisplatin (25 μM) treatment was 3 μM.

3.2.3 Effect of GSK2141795 on cell cycle progression in ovarian cancer cells

In view of the role of the PI3K-AKT pathway on regulation of the cell cycle via p16, p21 and CCND1 phosphorylation, cell cycle analysis by flow cytometry following treatment of cells with GSK2141795 was performed. This revealed cell cycle arrest at G1 in SKOV3 cells treated with 5 μM GSK2141795 for 24 hrs. This arrest was abrogated when the cells were co-treated with 25 μM cisplatin. In contrast, a 6% increase in sub-G0 cell population was observed in cells following the combination treatment, compared to single agent cisplatin treatment (Figure. 3.8) suggesting augmentation of cisplatin induced apoptosis by GSK2141795.

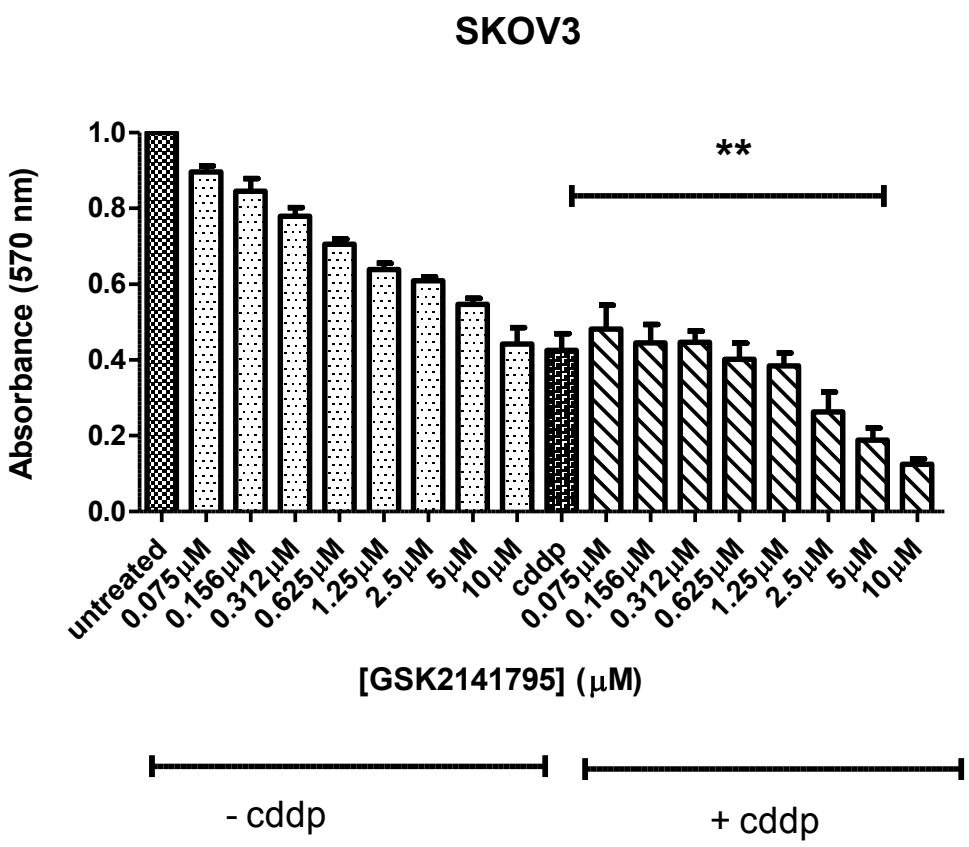


Figure 3-7 Cell viability effect of GSK2141795 as a single agent or in combination with cddp in SKOV3 monolayers

SKOV3 monolayers were treated with a concentration range of GSK2141795 as either a single agent or in combination with 25 μM cddp for 72 hours. Treatment with GSK2141795 resulted in a dose-dependent reduction in cell viability either as a single agent or in combination with cddp). Data shown are the means ± SEM of 3 experiments performed in triplicate. **P < .01.

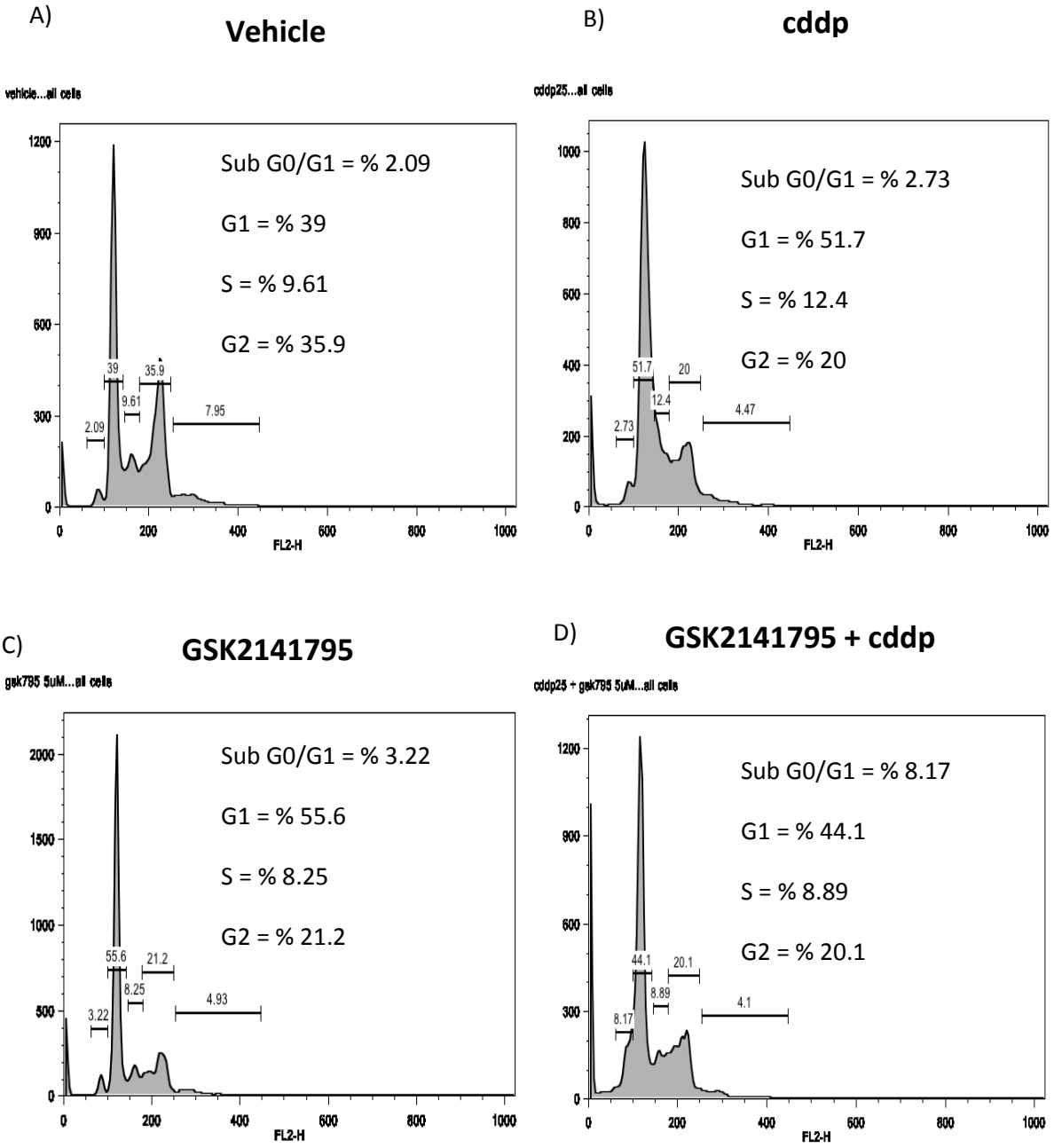


Figure 3-8 Cell cycle analysis of SKOV3 cells after treatment with DMSO (A) cddp 25 µM (B) GSK2141795 5 µM (C) or combination of GSK2141795 (5 µM) and cddp (25 µM) (D)

After 24 hours treatment, cells were labelled with propidium iodide PI and analysed by flow cytometry. The data shows the percentage of cells in each phase of the cell cycle. Treatment with GSK2141795 resulted in a G1 cell cycle arrest. This growth inhibitory effect was reduced when GSK2141795 was combined with cddp. An increase in sub-G0 population was observed in cells treated with the combination treatment compared to the single agent cisplatin treatment. Cells were not gated to include the apoptotic sub-G1 cell population into the analysis therefore small size debries in samples were also detectable (first peak in profiles)

3.2.4 *In vivo* xenograft studies

3.2.4.1 Efficacy of GSK2141795

The combination of GSK2141795 and cisplatin was investigated *in vivo* using a human SKOV3 ovarian cancer xenograft model. After implantation of cells into mice, tumours were allowed to reach a mean volume of 50 – 100 mm^3. Cisplatin (1.5 mg/kg) was administrated twice weekly intraperitoneally and GSK2141795 orally daily at 30 mg/kg for 14 days. On the days in which animals received both drugs, GSK2141795 was administrated 2 hours before cisplatin. Single agent GSK2141795 was found to inhibit tumour growth (Figure. 3.9). The combination of cisplatin with GSK2141795 reduced tumour sizes below that achieved with cisplatin alone. The combination therapy resulted in 90% reduction in tumour sizes in 14 days compared to the vehicle control ($p<0.01$). Single agent GSK2141795 and cisplatin also reduced tumour growth relative to the time-matched vehicle control tumours by 63% and 70% respectively. Of note, single agent GSK2141795 treatment did not result in tumour shrinkage relative to day 0 but prevented the tumours from growing larger.

3.2.5 Toxicity of GSK2141795

GSK2141795, cisplatin and the combination were well tolerated, and mice body weights were not significantly different among treatment groups (Figure 3.10).

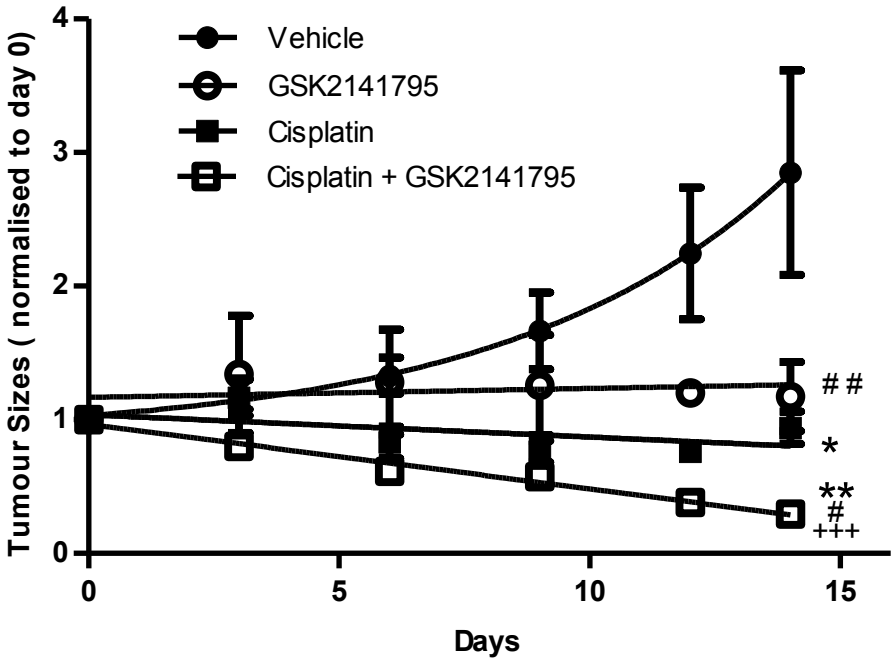


Figure 3-9 Effect of GSK2141795 either alone or in combination with cddp on tumour growth of SKOV3 cells.

SKOV3 tumour-bearing mice were dosed daily with GSK2141795 (30 mg/Kg; oral) or vehicle ± biweekly cddp (1.5 mg/Kg; intraperitonal) for 14 days. Data shown the mean ± SEM for n = 8 tumours/treatment, *p<0.05, **p<0.01, ***p<0.001, where the symbols *, # and + represent significant differences when compared to vehicle, cisplatin and GSK2141795 data, respectively.

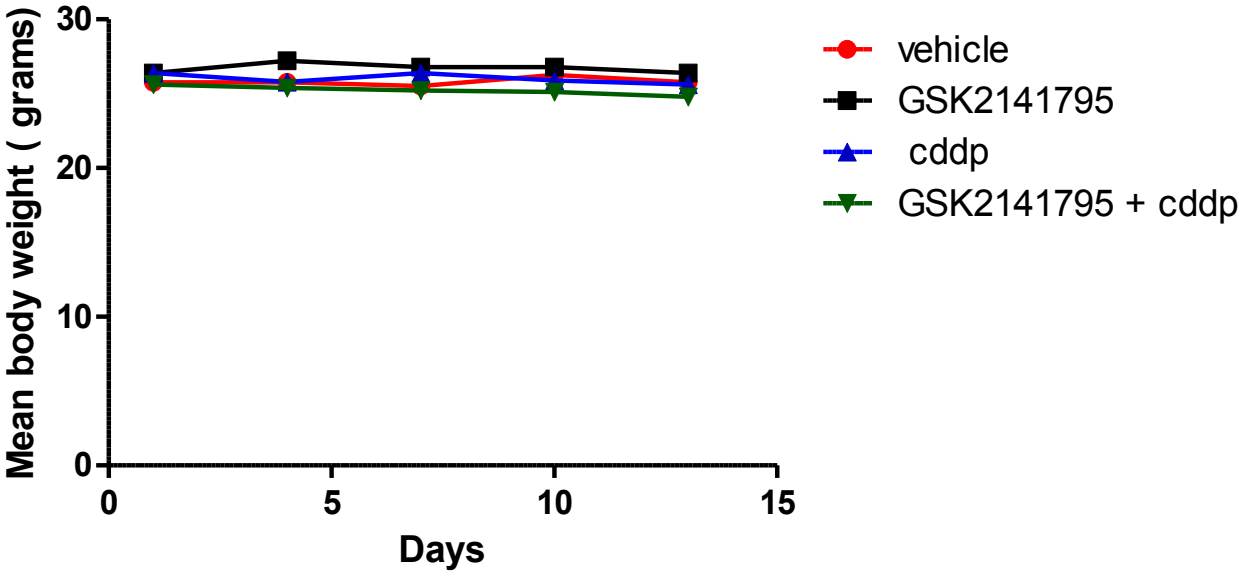


Figure 3-10 Mean body weights from efficacy study.

The doses of GSK2141795 (30 mg/kg), cddp (1.5 mg/kg) and their combination were well tolerated, and body weights were not significantly different among the treated groups. Data shown the mean ± SEM for n = 8 animals.

3.3 Discussion

Several studies have linked the PI3K/AKT pathway to platinum and taxol resistance in ovarian cancer. Constitutive activation of AKT-2 leads to cisplatin resistance in some ovarian cancer cell models. Moreover, treatment with the PI3K inhibitor, LY294002 reverses resistance in cisplatin-resistant ovarian cancer cell lines (Yuan, Feldman et al. 2003). In addition, constitutively active PI3K induces taxol resistance in xenograft models and reversed by LY294002 (Hu, Hofmann et al. 2002). PIK3CA genomic alternations are more frequent in ovarian cancer patients who relapsed with platinum resistance disease within 6 months (platinum resistant) than in those with more than 6 months of progression-free survival (platinum sensitive), or in patients with complete response to platinum. For examples, ovarian clear cell carcinoma (CCC) which is one of the most lethal types of ovarian cancer and often resistant to platinum based chemotherapy is characterised by frequent activating mutations of PIK3CA (35 – 45 %) (Kuo, Mao et al. 2009). Moreover, PTEN expression is associated with chemo-sensitivity in ovarian cancer cell lines (Stronach, Cheraghchi-Bashi et al. 2011). In 2010, Wallin et al. explored the use of a potent PI3K inhibitor in combination with the DNA-damage agent doxorubicin (Wallin, Guan et al. 2010). They found that PI3K inhibition increased apoptosis and enhanced the anti-tumour effects of doxorubicin in a subset of ovarian and breast cancer cell lines. They reported that in most cell lines where combination synergy was observed, there was an increase in the expression of nuclear p-AKT S473 in response to doxorubicin treatment alone suggesting that inhibition of AKT pathway in combination with cytotoxic agents is mostly effective in the set of tumour cells that rely on the PI3K/AKT pathway for survival (Wallin, Guan et al. 2010).

In this chapter, the efficacy of a novel specific ATP-competitive pan-AKT inhibitor agent, GSK2141795, to restore platinum-sensitivity in clinically derived platinum resistant ovarian cancer models was investigated.

GSK2141795 as a single agent had a strong growth inhibitory effect both *in vitro* and *in vivo*. In addition, flow cytometric analysis indicated G1 cell cycle arrest following treatment of the SKOV3 cells with this compound. These results are consistent with the reported growth inhibitory effects and G1 arrest induced by AKT inhibitors by others. For example Mandal et al reported that a small molecule inhibitor of AKT, KP372-1, induced a G1 cell cycle arrest in thyroid tumour cells *in vitro* and *in vivo* (Mandal, Kim et al. 2005). Hirai el al also reported inhibition of cell proliferation by another allosteric inhibitor of AKT in lung and ovarian tumour cells both *in vitro* and in xenograft models (Hirai, Sootome et al. 2010). In addition, growth inhibitory effect of GSK690693, a pan AKT inhibitor with the same mechanism of action of GSK2141795, has been reported in acute lymphoblastic leukaemia cell lines (Levy, Kahana et al. 2009, Altomare, Zhang et al. 2010). In addition, inhibition of AKT pathway by API-2, another small molecule AKT pathway inhibitor, resulted in inhibition of cell growth and induction of apoptosis in several types of AKT-overexpressing human cancer cells (Yang, Dan et al. 2004).

The *in vivo* data suggest that GSK2141795 as a single agent may have pre-dominantly cytostatic effect, limiting its clinical utility. This is consistent with the *in vitro* data of the lack of apoptosis induction by GSK2141795 as single agent in the examined cell lines in this study, including SKOV3 ovarian cancer cells which express a high level of constitutively activated p-AKT (Arboleda, Lyons et al. 2003).

GSK2141795 as a single agent also did not induce apoptosis in either platinum sensitive and platinum resistant ovarian cancer cells in 2D and 3D models. In contrast GSK2141795 combined with cisplatin increased apoptosis in platinum-resistant 2D and 3D ovarian cancer cell models in a dose and time dependent manner.

Multi-tumour cell spheroid models resemble *in vivo* tumour cell microenvironment more closely than 2D models. Many factors could reduce the sensitivity of cells within the 3D environment to certain drugs or treatments compared to cells grown in a monolayer culture. These factors include hypoxia, cell cycle differences, drug penetration, micro-environmental variations and PH in cells grown in a 3D microenvironment compared to cells grown as a monolayer. Drug penetration is thought to be a major factor (Knuchel, Hofstadter et al. 1989). MTS mimics *in vivo* tumour proliferative heterogeneity, hypoxic and necrotic conditions. These conditions can alter expression of numerous genes *in vivo* and in 3D cultures compared to 2D monolayers. In this study the effect of GSK2141795 as single agent and in combination with cisplatin on cell viability and apoptosis was assessed in MTS prior to *in vivo* xenograft experiments. The purpose of experiments performed in 3D cultures was to assess if GSK2141795 can increase apoptosis in combination with cisplatin in platinum resistant cells in a 3D micro-environment, as it was seen in 2D monolayers, before testing the combination treatment in animals. This step was done to avoid unnecessary use of animals if the combination treatment was not effective in a 3D micro-environment. GSK2141795 increased platinum sensitivity in platinum-resistant MTS, as was seen in 2D monolayers.

Treatment with GSK2141795 in combination with csiplatin resulted in tumour shrinkage in the platinum-resistant SKOV3 xenograft model. In the cell models examined, the enhanced apoptotic effect of cisplatin and GSK2141795 in combination was found to be synergistic. Notably, combination of GSK2141795 with cisplatin did not increase apoptotic response to cisplatin in platinum sensitive cell lines which were derived from the same patients as the resistant cells but before the onset of the platinum resistant relapse.

To date several signalling pathways have been reported to mediate resistance of cancer cells to AKT inhibitors. For example, Sommer et al have suggested that elevation of SGK1 expression represents one mechanism resulting in resistance to distinct AKT inhibitors (AZD5363 and MK-2206) in breast cancer cells (Sommer, Dry et al. 2013). The authors have shown that SGK1 can promote survival of AKT inhibitor resistant breast cancer cells independently of AKT. SGK is closely related to AKT and is regulated by the same up-stream regulators of AKT; PI3K, PDK1 and mTORC2. In addition, AKT and SGK show analogous substrate specificities and are likely to phosphorylate overlapping substrates (Kobayashi and Cohen 1999, Kobayashi, Deak et al. 1999, Murray, Cummings et al. 2005, Pearce, Komander et al. 2010).

It should be noted that mechanisms other than SGK1 activity are also likely to contribute to the resistance to AKT inhibitors. Sera et al have demonstrated that overexpression of ribosomal S6 kinase RPS6KA2 (RSK3) and RPS6KA6 (RSK4) mediates resistance to PI3K pathway inhibitors in breast cancer cells *in vitro* and *in vivo* in part through the decrease of the apoptotic response and regulation of protein translation. They have demonstrated that addition of MEK or RSK inhibitors restored responsiveness of RSK-expressing cells to PI3K inhibitors (Serra, Eichhorn et al. 2013). Several studies have also shown that an elevated Extracellular signal regulated kinase (ERK) activation signal either through the activation of compensatory feedback loops observed following PI3K inhibition or through intrinsic KRAS mutations, limits the effectiveness of PI3K pathway inhibitors in clinic (Engelman, Chen et al. 2008, Di Nicolantonio, Arena et al. 2010, Janku, Lee et al. 2011). It has also been suggested in a recent study that nuclear β-catenin confers resistance to the FOXO3a-mediated apoptosis induced by AKT and PI3K inhibitors in primary colon cancer cells (Tenbaum, Ordonez-Moran et al. 2012).

Overall, the findings in this chapter suggest that AKT inhibition alone is not sufficient to trigger apoptosis in the ovarian cancer *in vitro* and *in vivo* models used in this study. As suggested in several studies cancer cells may depend on several pathways parallel to PI3K/AKT pathway such as RAF/MEK/ERK for survival in absence of PI3K/AKT signalling (Dai, Chen et al. 2009, Yan, Dai et al. 2010, Aksamitiene, Kiyatkin et al. 2012). Further investigation is required to find the mechanism that mediates resistance to apoptosis by AKT inhibition in platinum resistant ovarian cancer cells. In conclusion it was demonstrated that AKT inhibition is sufficient to sensitise platinum resistant ovarian cancer cells to cisplatin *in vitro* and *in vivo*. These findings are in agreement with previous findings that resistance to platinum is mediated by AKT in ovarian cancer cells (Stronach, Chen et al. 2011).

In summary, these findings suggest the potential ability of GSK2141795 to be used in the clinic specifically to restore sensitivity to platinum in ovarian cancer patients who have relapsed with platinum resistant disease. Therefore, GSK2141795 was selected as a candidate compound to be further developed in combination with platinum-based chemotherapy in a clinical trial in patients with relapsed resistant ovarian cancer. The pre-clinical development of this compound prior to a phase I clinical trial in patients with ovarian cancer is described in the next chapter.

4 Utility of FDG-PET imaging as a pharmacodynamic marker of AKT inhibition using GSK2141795

4.1 Introduction

The development of drugs for treatment of cancer remains a slow and high-risk process with many compounds failing due to unfavourable pharmacokinetic profiles, toxicity and and/or lack of therapeutic efficacy (Neidle 2008). The high failure rate contributes to the high cost in both financial and human terms of drug development. The reasons for the high failure rates have been extensively reviewed by Workman et al, and a 'Pharmacological audit trail' (PHAT) suggested to minimise failure risks (Workman 2002, Workman 2003) .

A key area of increased focus aimed at improving the probability of success in the drug discovery process, is the identification and validation of reliable pharmacodynamic (PD) biomarkers of target modulation by the drug, that can be used both during pre-clinical and clinical development, and ideally can also be correlated with clinical outcome. One approach clinically is to use tumour biopsies taken before and after drug treatment to measure modulation of PD makers. PD biomarkers may include cellular, molecular, histopathological and/or imaging parameters (Sarker, Pacey et al. 2007, Sarker and Workman 2007). It is anticipated that such PD biomarkers should clearly enable efficient and scientifically-driven "Go/NoGo" decisions allowing expediency of the drug development process.

In the case of AKT inhibitors, AKT substrates such as phospho-PRAS40, are being used as PD biomarkers (Yan, Serra et al. 2013). However, the invasive nature of serial tumour biopsies in patients limits its application and recruitment in clinical trials. In addition, intra-patient tumour heterogeneity can also limit the utility of tumour biopsies from single sites. The development of non- invasive PD biomarkers therefore has the potential to aid clinical drug development.

Positron emission tomography (PET) is a non-invasive imaging technology that measures the three dimensional spatial and temporal uptake of a radioligand *in vivo*, and is widely used in the field of oncology, with fluorine-18 radiolabelled fluorodeoxyglucose ([^{18}F]FDG) being the most commonly used PET tool for diagnostic imaging of tumours. [^{18}F]FDG is a glucose analogue. The hydroxyglutl group at the 2' of glucose is substituted by radioactive isotope fluorine-18 in this molecule. Both molecules are taken up by glucose transporters and follow the same path to pass into the cell. Inside the cell, glucose and FDG are both phosphorylated by hexokinases (HK) to glucose-6-phosphate and FDG-6-phosphate. The 2'-hydroxyl group is needed for further metabolisation of glucose and as [^{18}F]FDG lacks this hydroxyl group, it is trapped within the cell. As a result, the distribution of [^{18}F]FDG is a good reflection of distribution of cellular glucose uptake (Aboagye and Price 2003). [^{18}F]FDG is trapped in highly metabolically active cells, such as malignant cells which have high glucose uptake, thereby facilitating functional imaging and distinction between malignant and healthy tissue. However, some normal tissues with high glucose uptake such as the brain also display high [^{18}F]FDG uptake. [^{18}F]FDG uptake in tissues following drug treatment can provide a sensitive, qualitative and quantitative early PD readout of the cytostatic or cytotoxic action of cytotoxic drugs *in vivo* and in clinical practice. Given the role of AKT in the regulation of glucose metabolism, [^{18}F]FDG-PET could also serve as a powerful tool in assessing the action of AKT inhibitors on tumour metabolism *in vitro* and *in vivo* (Aboagye and Price 2003, Boss, Olmos et al. 2008). AKT has an important role in cellular glucose uptake and metabolism regulation through the regulation of transcription and plasma membrane localisation of glucose transporter 1, the principle glucose transporter expressed in most cell types. AKT also mediates membrane translocation of GLUT4. PI3K/AKT pathway also regulates glycolysis by regulating hexokinase localisation into mitochondria and glucose phosphorylation. AKT also promotes glycolysis within the cell by phosphorylation of GSK3-β, therefore inhibition of glycogen synthesis.

In this chapter the utility of FDG-PET imaging as a non-invasive PD marker for AKT inhibition using GSK2141795 will be explored. The goal was to correlate changes in glucose metabolism (as measured by changes in FDG uptake) with changes in downstream biomarkers (phospho-PRAS40) of AKT inhibition in cell lines. If FDG-PET could be established as a marker of AKT inhibition in cell lines and this relationship confirmed *in vivo*, then FDG-PET could be used to guide individualised dose selection, thereby optimising the risk/benefit profile of the drug in clinical trials and clinical practice.

Most pre-clinical approaches to PET tracer development in oncology use 2D monolayer cultures prior to *in vivo* studies. 2D monolayers lose their three dimensional cell contact and only communicate along a small proportion of their membrane therefore they may not be a very useful tool in predicting the efficacy of drug or radio-tracer up-take by cells in three dimensional *in vivo* micro-environment. Over the last two decades, 3D cultures (spheroids) have been used in studies involving angiogenesis, multicellular drug resistance, drug penetration and many other aspects of tumour biology (Frankel, Buckman et al. 1997, Desoize and Jardillier 2000, Oudar 2000, Sonoda, Kobayashi et al. 2003, Wartenberg, Schallenberg et al. 2003, Wrobel, Debnath et al. 2004). Nevertheless to date only one study has evaluated the use of 3D spheroid assays for optimisation of several PET tracers including FDG in the breast cancer cell line MCF7, exposed to doxorubicin, paclitaxel, docetaxel, tamoxifen or imatinib (Monazzam, Josephsson et al. 2007).

The aim of this chapter was to access the potential utility of FDG-PET as a PD biomarker of AKT inhibition using GSK2141795 *in vivo* and the relative merits of *in vitro* optimisation using 2D and 3D MTS. A translational imaging approach was used to provide pertinent translational information regarding the AKT inhibitor, GSK2141795, prior to its clinical assessment. Such data was essential in the design of clinical studies incorporating this drug.

Multi-cellular ovarian tumour spheroids (MTS; 3D) as well as 2D cell monolayers were used, initially, to determine the optimum dose and scheduling for GSK2141795, and also to predict the *in vivo* response. Subsequently, pre-clinical [^{18}F]FDG PET studies using ovarian cancer mouse xenograft models were conducted, and the results of these studies were used to translate the does and optimal time to scan into the clinical study design.

4.2 Results

4.2.1 Effect of inhibition of AKT by GSK2141795 on phosphorylation of the pharmacodynamic biomarker PRAS40 in 2D and 3D *in vitro* models

To confirm the ability of GSK2141795 to inhibit AKT signalling in SKOV3 cells, 2D monolayers and 3D MTS were treated with GSK2141795 for 48 and 72 hours and analysed for phosphorylation of its downstream substrate PRAS40 with enzyme-linked immunosorbent assays (ELISA). Treatment with GSK2141795 resulted in a concentration-dependent reduction in the ratio of phosphorylated PRAS40 (Thr246) to total PRAS40 in both SKOV3 monolayers and 3D MTS (Figure. 4.1 and 4.2). Phosphorylation of PRAS40 at Thr246 was reduced to <1% pre-treatment level in SKOV3 monolayers treated with GSK2141795 (5 μM) for both 72 hours (Figure. 4.1, b) and 48 hours (Figure. 4.1, a) but this reduction in phosphorylation was only partial (~50% of the pre-treatment level) in the 3D model at 5 μM, perhaps reflecting incomplete penetration of the compound into the 3D spheroids (Figure. 4.2).

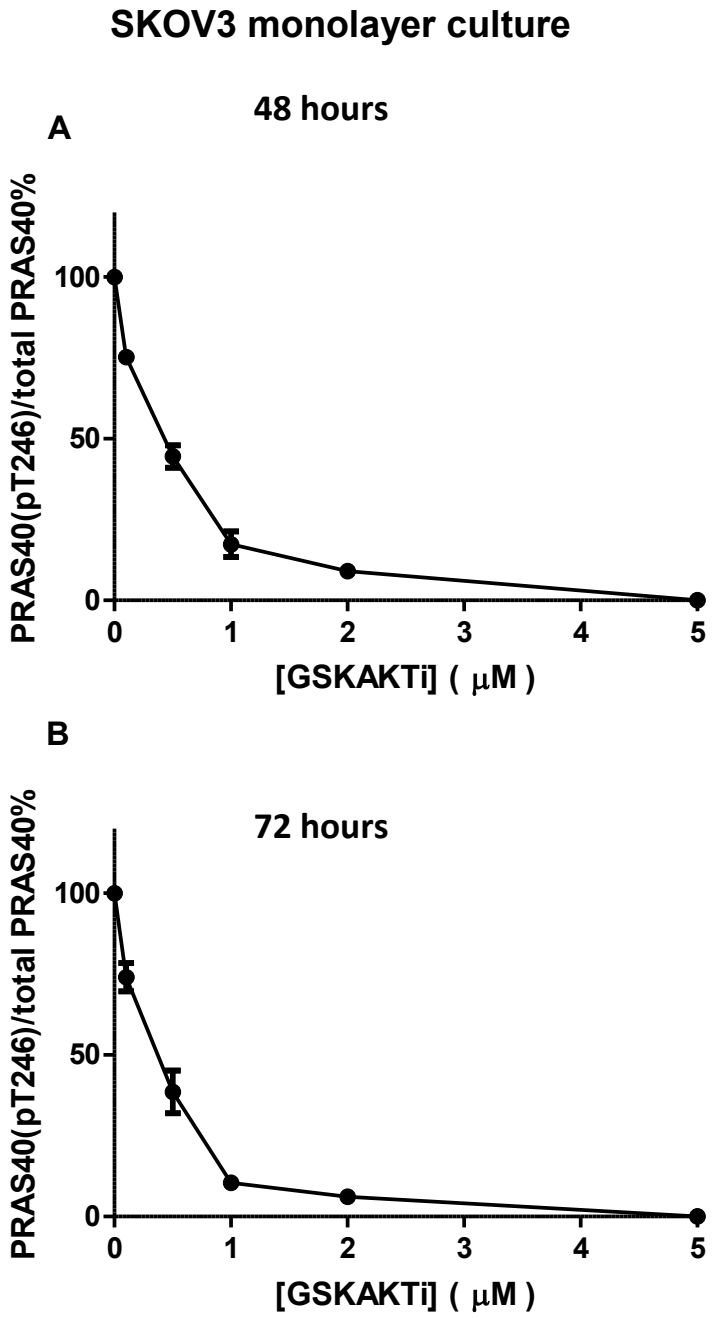


Figure 4-1 Concentration-dependent effect of AKT inhibition by GSK2141795 on phospho-PRAS40 (Thr246) expression in SKOV3 monolayers following 48 (A) and 72 (B) hours exposure

Protein concentration of phospho-PRAS40 (Thr246) and total PRAS40 was determined by enzyme-linked immunosorbent assay (ELISA) after treatment with a range of concentrations of GSK2141795 (0.01 – 5 μM) in monolayers, where treatment resulted in a concentration-dependent decrease in expression of phospho-PRAS40 (Thr246). Data are presented as a phospho-PRAS40 / total PRAS40 decrease relative to untreated samples. Data are the means ± SEM of n = 2 experiments performed in triplicate

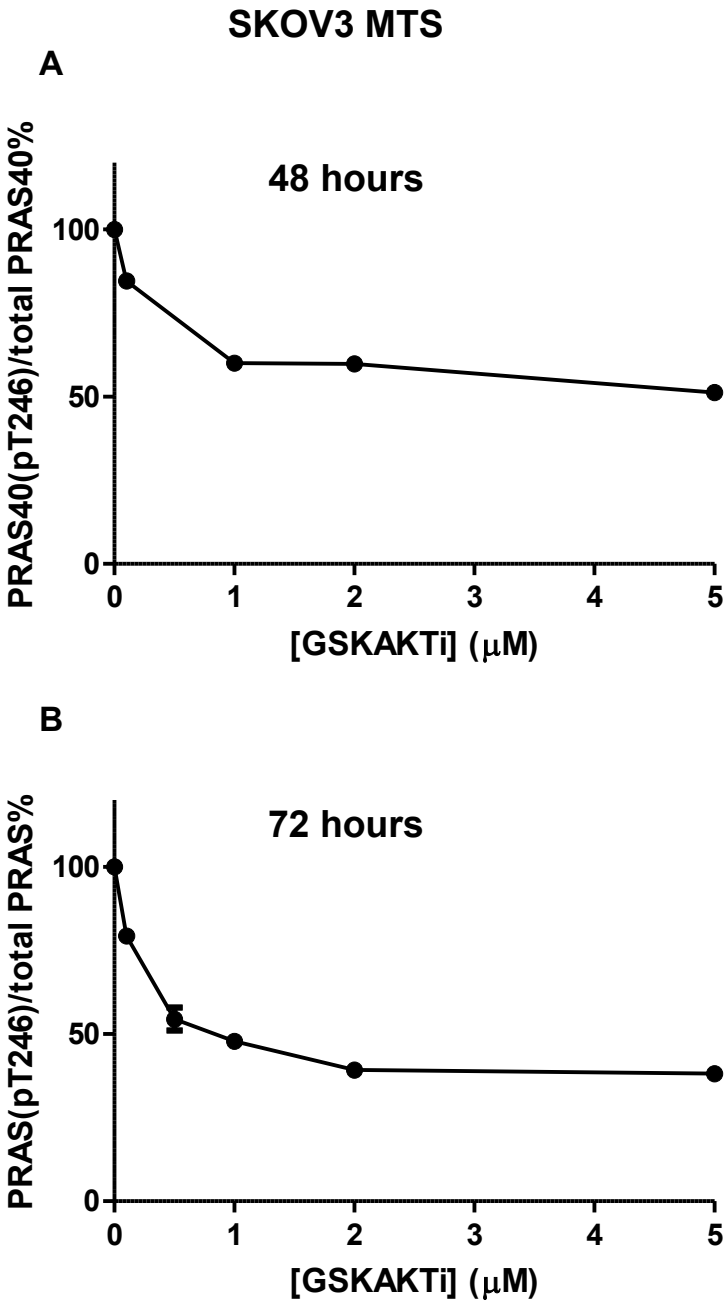


Figure 4-2Concentration-dependent effect of AKT inhibition by GSK2141795 on phospho-PRAS40 (Thr246) expression in SKOV3 MTS's following 48 (A) and 72 (B) hours exposure

Protein concentration of phospho-PRAS40 (Thr246) and total PRAS40 was determined by enzyme-linked immunosorbent assay (ELISA) after treatment with a range of concentrations of GSK2141795 (0.01 – 5 μM) in MTS's, where treatment resulted in a concentration-dependent decrease in expression of phospho-PRAS40 (Thr246). Data are presented as a phospho-PRAS40 / total PRAS40 decrease relative to untreated samples. Data are the means ± SEM of n = 2 experiments performed in triplicate (B) and n=1 experiment performed in triplicate (A).

4.2.2 Effect of AKT inhibition by GSK2141795 on [^{3}H]FDG uptake in 2D and 3D *in vitro* models of ovarian cancer

The influence of GSK2141795 on [^{3}H]FDG uptake in SKOV3 2D monolayers and 3D MTS was assessed. In order to define the optimum incubation time with [^{3}H]FDG *in vitro*, SKOV3 MTS were grown for 7 days and then treated with GSK2141795 (1μM or 5μM) for 48 or 72 hours. MTS were then incubated with [^{3}H]FDG for between 30 minutes to 24 hours prior to the measurement of the [^{3}H]FDG levels in each spheroid. The same experiment was done using SKOV3 2D monolayers except cells were treated with GSK2141795 for 24 hours prior to the measurement of [^{3}H]FDG levels in cells instead of 48 hours due to the greater growth inhibitory effect of GSK2141795 on 2D monolayers compared to MTS.

[^{3}H]FDG uptake into vehicle treated monolayers and MTS reached a plateau at 6 and 10 hours, respectively (t½ = 0.52 hours (monolayers) and 3.04 hours (MTS) (Figures 4.3 and 4.4). Treatment with GSK2141795 resulted in a dose-dependent decrease in the rate of FDG uptake with treatment (t½ = 0.41 and 0.53 hours for 1 and 5 μM, respectively (monolayers); and 5.79 and 4.30 hours for 1 and 5 μM, respectively (MTS) (Figures 4.3 and 4.4).

Although maximal uptake of [^{3}H]FDG into SKOV3 monolayers and MTS occurred at approximately 6 and 10 hours respectively, it is not possible to use these time points when scanning patients in the clinic, i.e. they are not clinically feasible due to the short half-life of ^{18}F (110 minutes), Therefore, in addition, concentration-response studies utilising GSK2141795 were repeated in SKOV3 monolayers at 2 hours (optimum time for clinical scanning; Figure 4.5) and MTS at both 2 and 6 hours (6 hours = optimum time for incubation of the tumours with radio-tracer; Figure 4.6). A small 25% concentration-

dependent decrease in [^{3}H]FDG uptake was observed in the SKOV3 monolayers (ED_{50} = 0.22 μM; Figure 4.5) which plateaued at ~1 μM GSK2141795. In contrast, a 78% - 80% dose-dependent decrease in FDG uptake was observed in the SKOV3 MTS at both the 2 and 6 hour time-points, again reaching a plateau at ~1 μM. The ED_{50} values were similar for monolayer (EC_{50} = 0.22 μM) and MTS culture (ED_{50} = 0.42 μM (2 hours) and 0.18 μM (6 hours); (Figure 4.6).

4.2.3 Decrease in FDG uptake with GSK2141795 correlates with the decrease in PRAS40 phosphorylation – a biomarker of AKT inhibition

The relationship between decreases in FDG uptake in cells and changes in downstream markers of AKT pathway inhibition (phospho-PRAS40/total PRAS40) induced by GSK2141795 were evaluated in SKOV3 monolayers and MTS. The decreases in FDG uptake and phospho-PRAS40/total PRAS40 were strongly positively correlated over the range of GSK2141795 concentrations in both SKOV3 monolayers (R^2=0.910) (Figure 4.7 A) and MTS's (R^2=0.979, Figure 4.7 B). Given that PRAS40 phosphorylation is a validated PD biomarker of AKT inhibition by GSK2141795, as shown in figures 4.1 and 4.2, this suggests that FDG uptake may also be used as a PD biomarker for GSK2141795 mediated AKT inhibition.

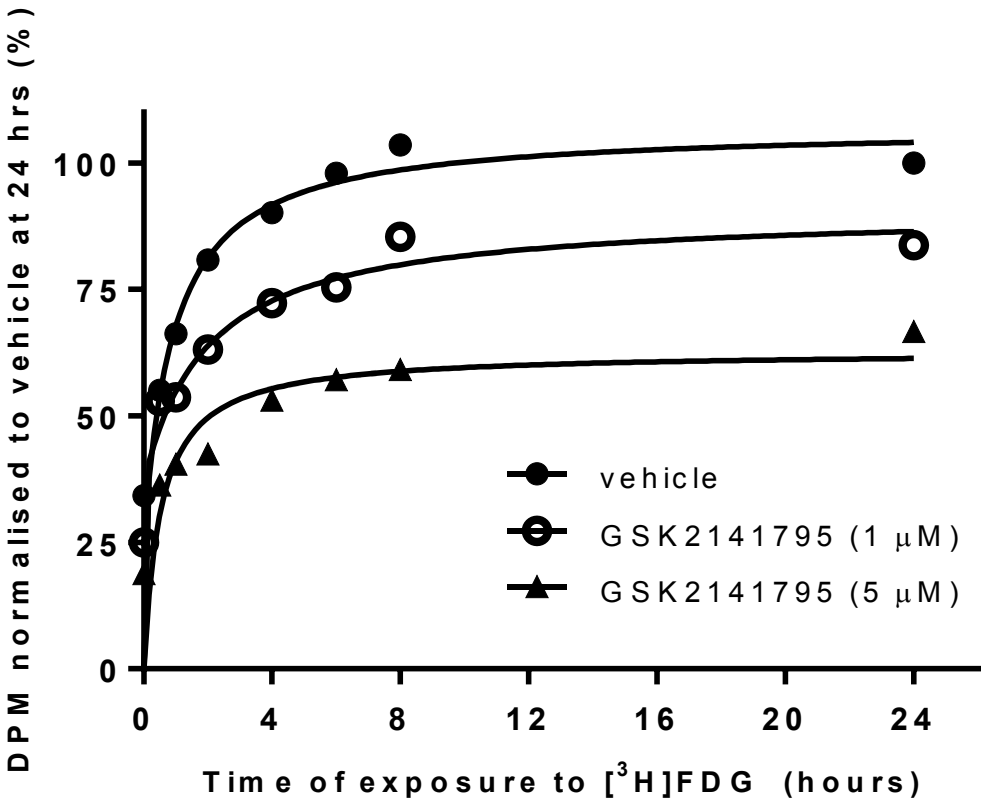


Figure 4-3 Time-course of [^{3}H]FDG uptake in SKOV3 monolayers pre-incubated with either vehicle or GSK2141795 (1 and 5 μM) for 24 hours.

SKOV3 monolayers were pre-incubated with either vehicle or GSK2141795 (1 and 5 μM) for 24 hours and were exposed to [^{3}H] FDG for a range of time intervals (1 – 24 hours). Treatment with GSK2141795 reduced the amount of FDG uptake in SKOV3 monolayers in a dose and time dependent manner. DPM = decays per minute. n=1.

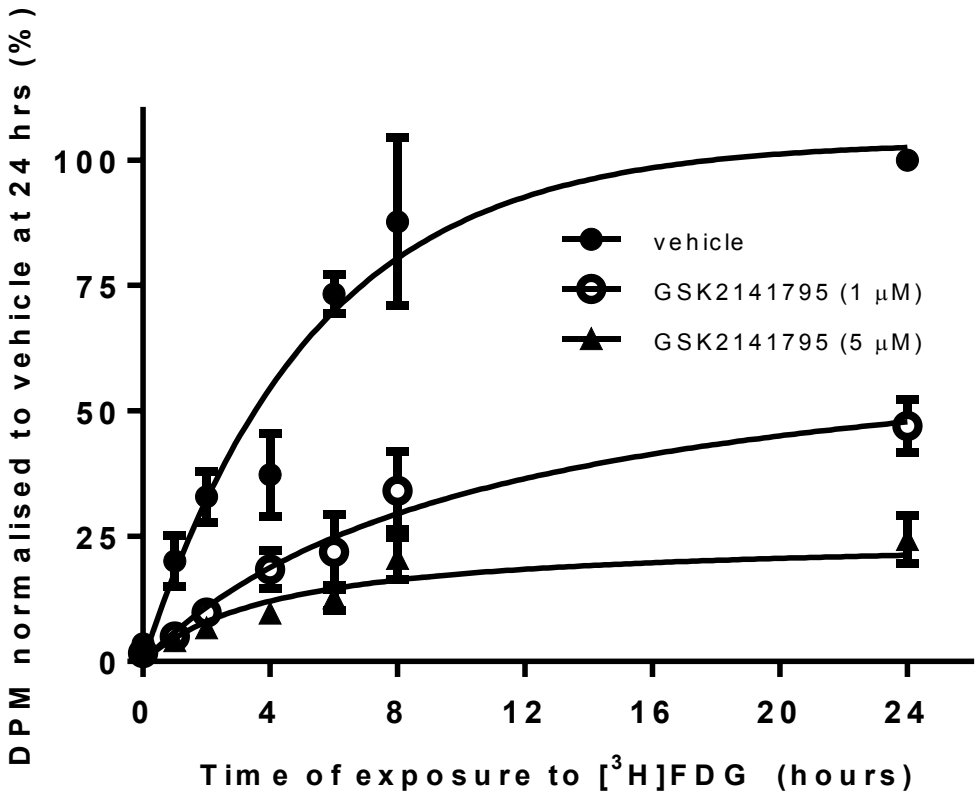


Figure 4-4 Time-course of [^{3}H] FDG uptake in SKOV3 MTS pre-incubated with GSK-2141795 for 48 hours.

SKOV3 MTS were pre-treated with GSK2141795 (1 or 5 μM) and were exposed to [^{3}H] FDG for a range of time intervals. [^{3}H]FDG uptake into SKOV3 MTS reduced following exposure of SKOV3 spheroids to GSK2141795 in a concentration-dependent manner. The optimum *in vitro* incubation time with [^{3}H]FDG was determined to be from 6 hours . Data are the means ± SEM of n = 3 experiments performed in triplicate.

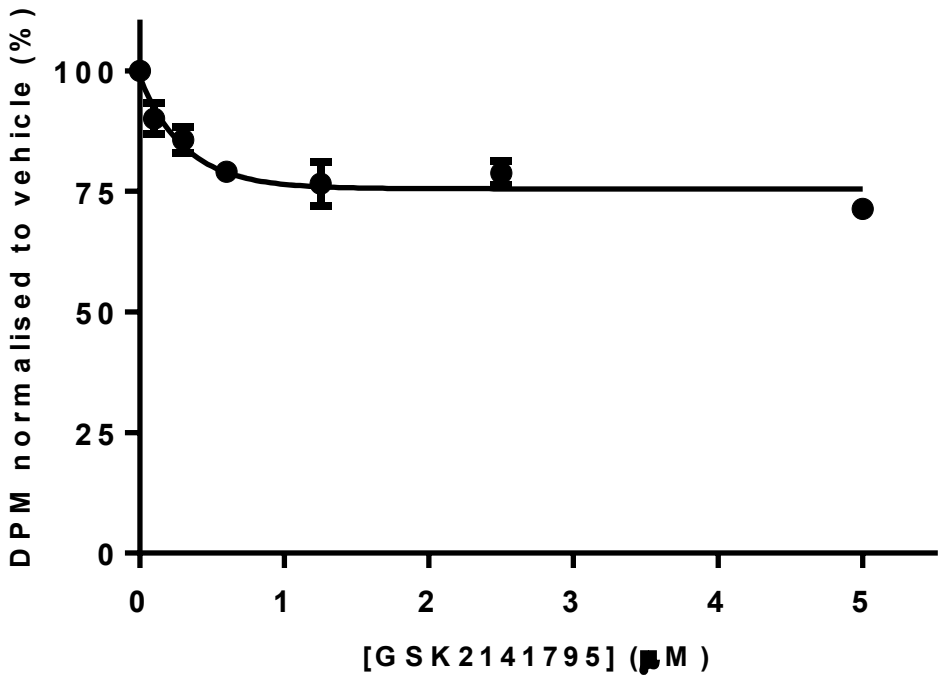


Figure 4-5 Dose response decrease in [^{3}H]FDG uptake in SKOV3 monolayers treated with GSK2141795.

SKOV3 monolayers were exposed to GSK2141795 for 24 hours and incubated with [^{3}H]FDG for 2 hours. Treatment with GSK2141795 reduced the amount of FDG uptake in SKOV3 monolayers in a dose and time dependent manner. Data are the means ± SEM of n = 3 experiments performed in triplicate.

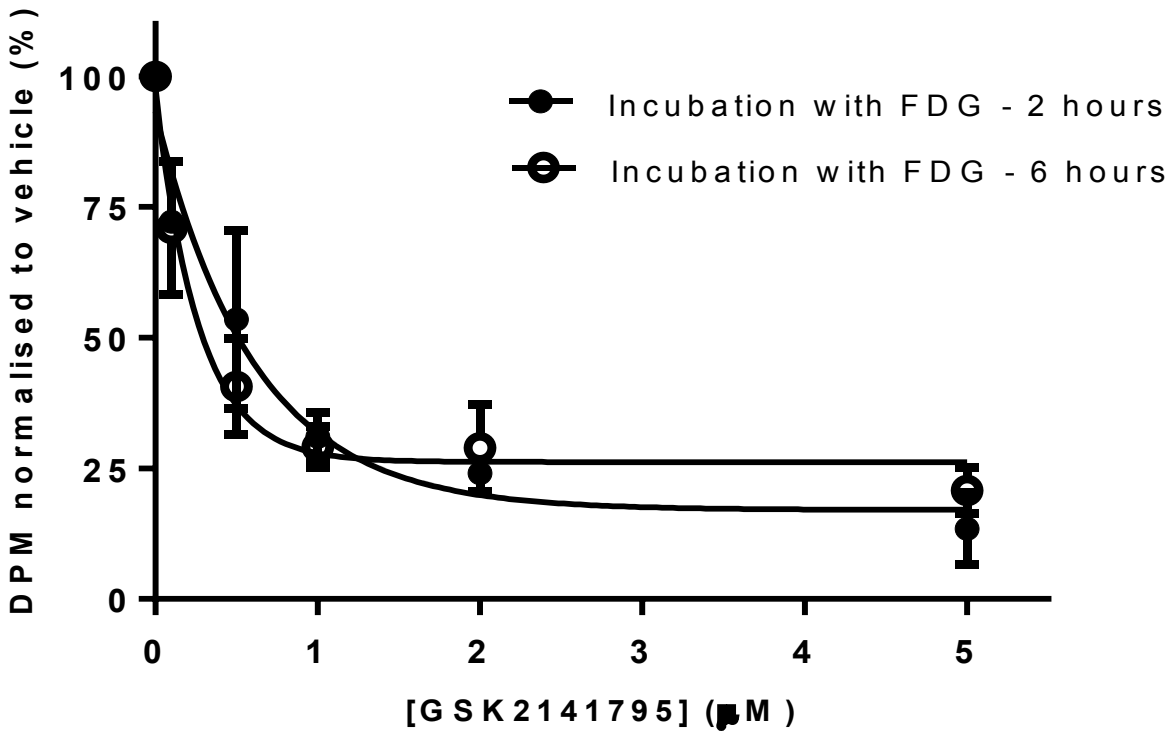


Figure 4-6 Concentration-dependent decrease in [^{3}H]FDG uptake in SKOV3 MTS's treated with GSK2141795.

SKOV3 MTS's were exposed to GSK2141795 for 48 hours and incubated with [^{3}H]FDG for 2 hours and 6 hours . Data shows a clear dose-response in MTS incubated with [^{3}H]FDG for either 2 hours (clinical scan time following FDG administration) or 6 hours (favourable *in vitro* time-point for optimum signal to noise). Data are the means ± SEM of n = 3 experiments performed in triplicate.

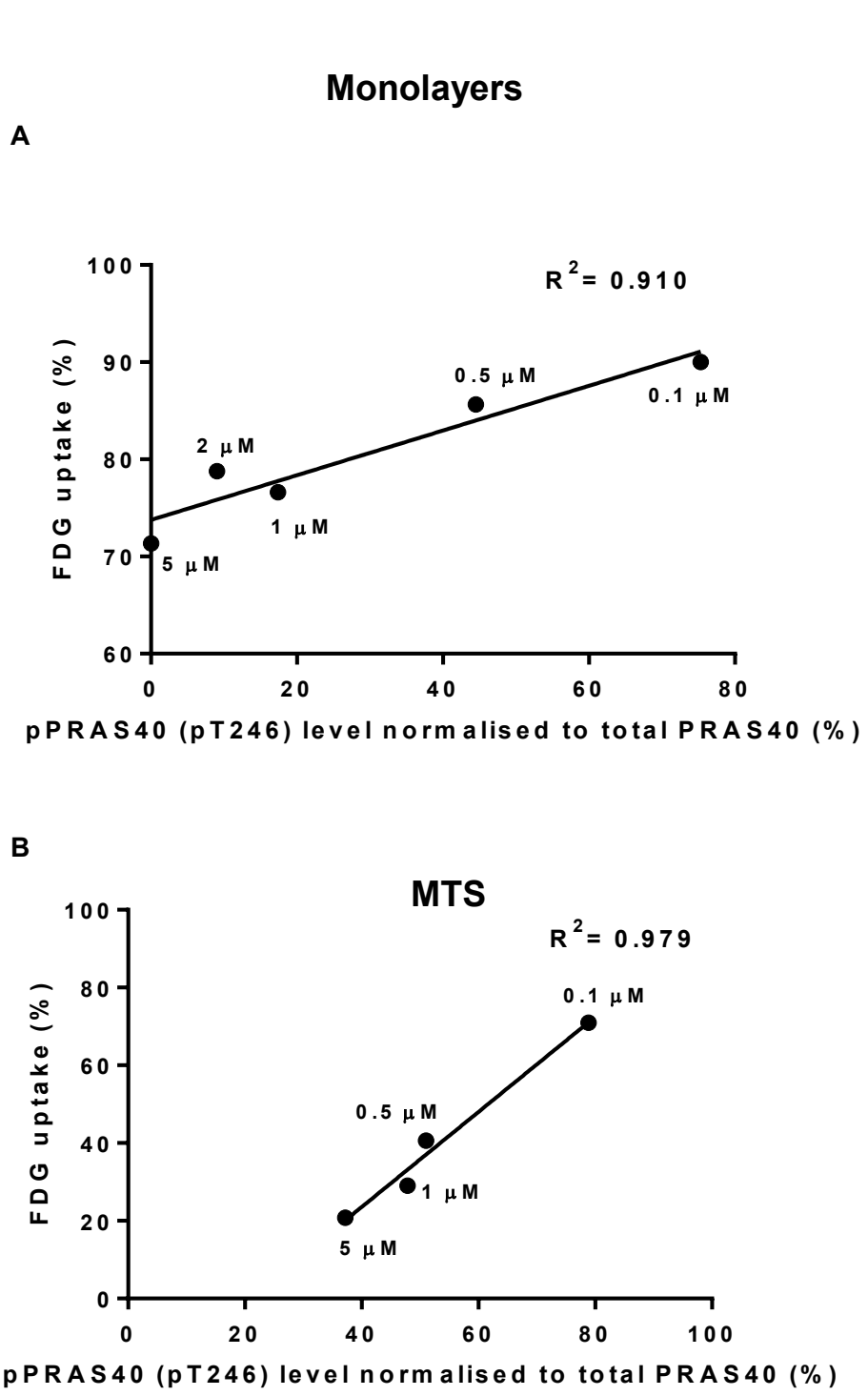


Figure 4-7 Correlation between expression levels of phospho-PRAS40 (Thr246) and [^{3}H]FDG uptake into SKOV3 monolayers (A) and MTS's (B) following treatment with GSK2141795.

SKOV3 monolayers and MTS's were treated with GSK2141795 for 48 hours. Analysis revealed expression levels of pPRAS40 in SKOV3 monolayers and MTS's exposed to GSK2141795 correlated closely with [^{3}H]FDG uptake, R^2 values = 0.910 and 0.979, respectively.

4.2.4 Effect of GSK2141795 on [^{18}F]FDG uptake in ovarian cancer tumour xenografts

The utility of [^{18}F]FDG uptake as a PD biomarker for GSK2141795 was assessed *in vivo* using SKOV3 xenografted mice. Mice bearing subcutaneous SKOV3 xenografts were fasted overnight and treated with 10mg/kg, 20mg/kg or 30mg/kg of GSK2141795 for 5 hours. Control animals were treated with vehicle (DMSO/PEG) for the same length of time. GSK2141795 treatment resulted in a dose-dependent decrease in tumour FDG uptake compared to control animals (Figures. 4.8 and 4.9). Analysis of the effect of GSK2141795 on [^{18}F]FDG uptake into SKOV3 xenografts indicated a reduced [^{18}F]FDG signal compared to vehicle reaching a maximum of 68% at the highest dose of 30 mg/kg of GSK2141795 (p<0.05) used in this study.

The relationship between the duration of the treatment and FDG uptake was also investigated. SKOV3 tumour bearing mice were treated with 30 mg/kg of GSK2141795 for time ranging from 1 to 72 hours prior to [^{18}F]FDG administration (one animal per time point). Treatment with GSK2141795 at a dose of 30 mg/kg resulted in a rapid decline of [^{18}F]FDG uptake in a time-dependent manner, reaching a maximum of 75% decrease at approximately 50 hours and continued up to 72 hours post-treatment (Figure 4.10).

A

B

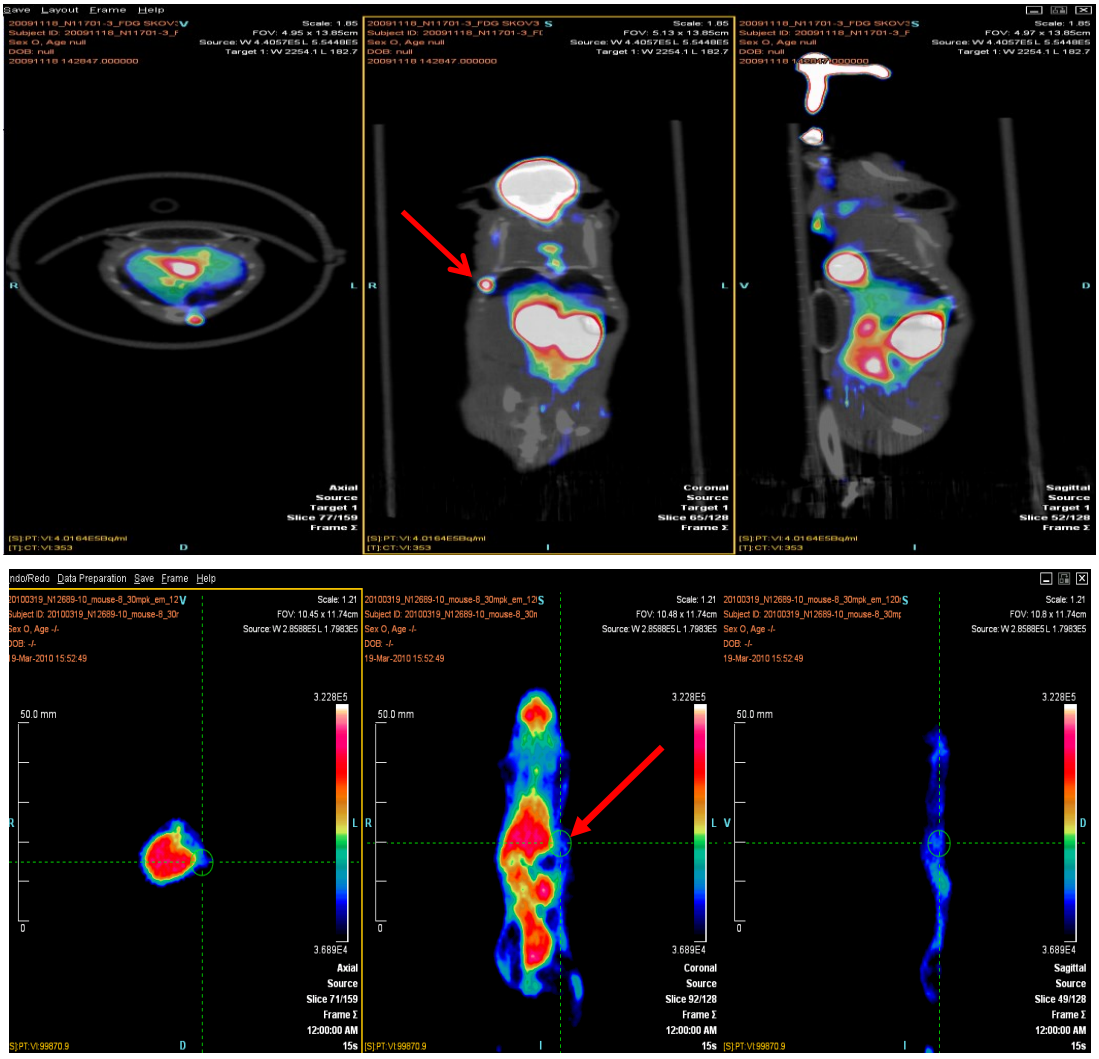


Figure 4-8 Positron emission tomography (PET) images of SKOV3 xenograft models treated with vehicle (a) or 30 mg/kg GSK2141795 (b) for 5 hours.

SKOV3 xenografts were treated with vehicle or 30 mg/kg GSK2141795 for 5 hours before being subjected to 60 min dynamic [^{18}F]FDG PET scan. A decrease in FDG uptake in tumours was observed in mice treated with GSK2141795 compared to the vehicle group Control PET images are co-registered with the CT images of the same animal to localise the PET signals. Images correspond to the same representative animal for each condition in three planes: axial, coronal and sagittal. The colour scale for all PET image data shows [^{18}F]FDG uptake in units of %ID/g (the percent injected dose per gram of tissue), with red corresponding to the highest activity and blue to the lowest activity. Arrows indicate the location of tumours.

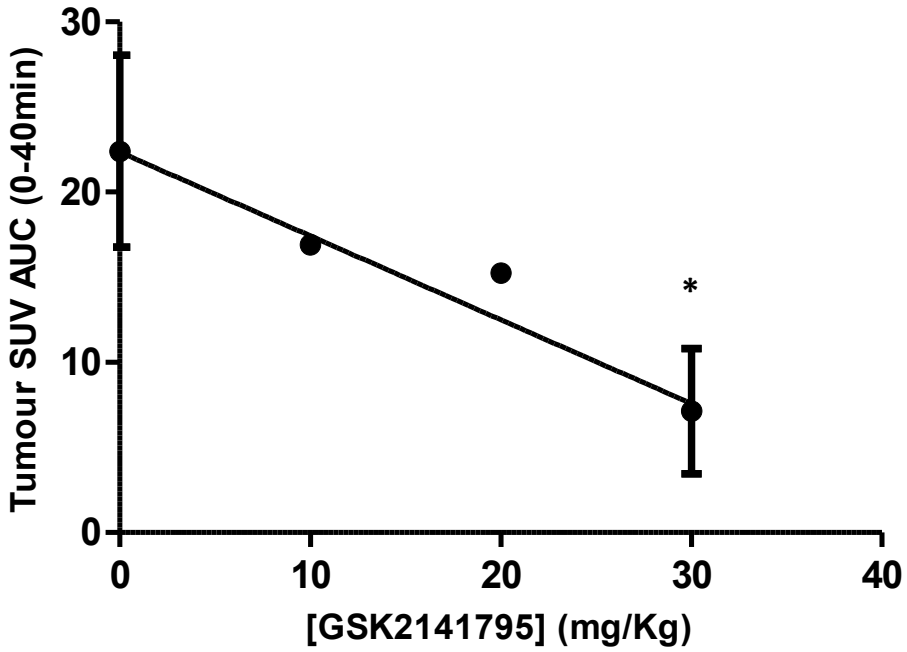


Figure 4-9Uptake of [^{18}F]FDG into tumours of SKOV3 xenografted mice following 5 hours drug treatment (10 - 30 mg/Kg).

SKOV3 xenograft-bearing mice were treated with either vehicle or GSK2141795. Standardized uptake value (SUV) were determined via [^{18}F]FDG imaging for each treatment group. Treatment of SKOV3 xenografts with GSK2141795 led to a dose-dependent decrease in mean [^{18}F]FDG SUV in each treatment group compared to vehicle controls . n=3 per dose except for 20 mg/Kg where n=1), *p<0.05 compared to vehicle treated animals. Data shown are mean ± SEM. SUV = standardised uptake values; AUC = area under the curve.

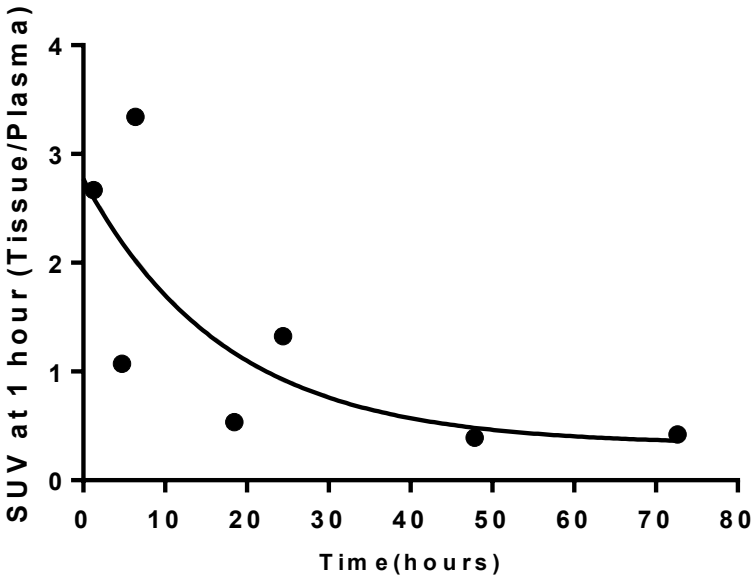


Figure 4-10 Time-course of tumour [^{18}F]FDG uptake in SKOV3 tumour xenografts in mice treated with GSK2141795.

SKOV3 xenograft bearing mice were treated with 30 mg/kg of GSK2141795 and tumours were imaged by small [^{18}F]FDG PET at different time points after treatment**.** Tumour time radioactivity curves were generated by calculating the ratio of the tumour to plasma uptake at each time point. GSK2141795 treatment resulted in a decrease in tumour FDG uptake compared to the control animals (t = 0) in a time-dependent manner (n = 1 animal / time point).

4.2.5 Pharmacokinetic analysis of GSK2141795 in the SKOV3 tumour xenograft model of ovarian cancer

To determine the level of GSK2141795 in blood and tumours a pharmacokinetic analysis was performed. Concentrations of GSK2141795 in tumour and blood taken from the *in vivo* SKOV3 xenograft time-course studies were measured. GSK2141795 accumulated in the SKOV3 xenograft tumours during the first 24 hours after treatment, with the t½ of the drug in the tumour determined to be ~12.5 hours (Figure 4.11 A). In comparison, levels of GSK2141795 were found to be maximised in the blood after 60 minutes following dosing (> 4000 ng/ml, decreasing rapidly to less than 1000 ng/ml within 4 hours (Figure 4.11 B). The ratio tumour to blood GSK2141795 concentrations showed a peak at 12 hours (ratio 22) declining to 9 by 24 hours and 2 by 48 hours (Figure 4.11 C).

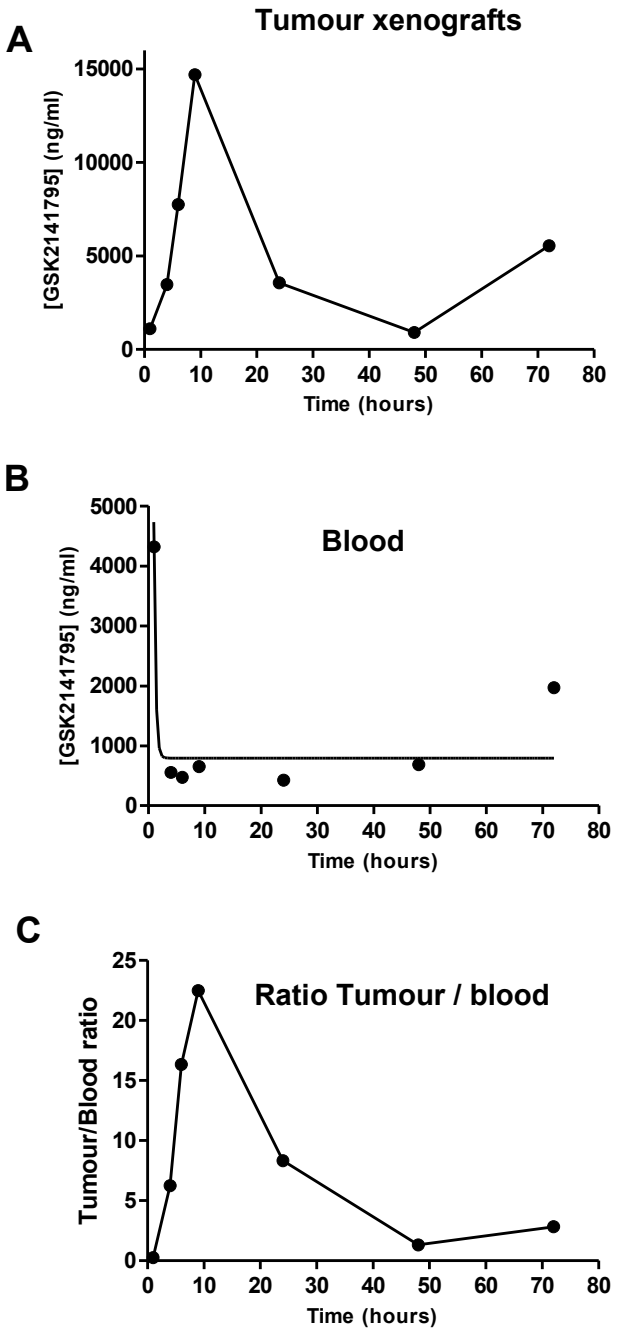


Figure 4-11 Pharmacokinetic data for GSK2141795 accumulating in xenograft tumours (A) and xenograft blood (B) up to 72 hours; with the subsequent ratio of tumour to blood (C).

SKOV3 tumour bearing mice were treated with 30 mg/kg GSK2141795 for 1, 4, 8, 12 ,24, 48 or 72 hours and tumour and plasma samples were collected. Tumour or blood samples collected at the same nominal times were pooled and GSK2141795 concentration in each sample was determined using mass spectrometry (MS). Blood concentration of GSK2141795 peaks within the first 60 minutes following the treatment and then decreases dramatically whereas the concentration of the compound in the tumour increases gradually following treatment.

4.2.6 Effect of inhibition of AKT inhibition by GSK2141795 on phosphorylation of the pharmacodynamic biomarker PRAS40 in human ovarian cancer xenografts

To assess the effect of inhibition of AKT using GSK2141795 on the phosphorylation of the downstream target PRAS40 *in vivo*, mice bearing SKOV3 xenografts were treated with 30 mg/kg GSK2141795, and after 5 hours animals were sacrificed, tumours harvested and the levels of total and pPRAS40 were determined in each sample by ELISA.

In addition mice bearing xenograft tumours were treated with combination of 1.5 mg/kg cisplatin and 30 mg/kg GSK2141795 or only cisplatin. When two drugs were combined, animals were treated with GSK2141795 two hour prior to the receiving of the cisplatin. Tissues were harvested 3 hours after the dosing with cisplatin and total protein was extracted from each tumour. Level of pPRAS40 in each sample was determined using ELISA.

Levels of phosphorylated PRAS40 were significantly reduced in SKOV3 xenografts following treatment with either GSK2141795 as a single agent ($p<0.01$) or in combination with cisplatin ($p<0.05$; Figure 4.12). This effect was not observed when cisplatin was used as a single agent in the xenografts, and therefore suggests that FDG-PET is a useful PD biomarker for AKT inhibition even in the context of combination cisplatin treatment.

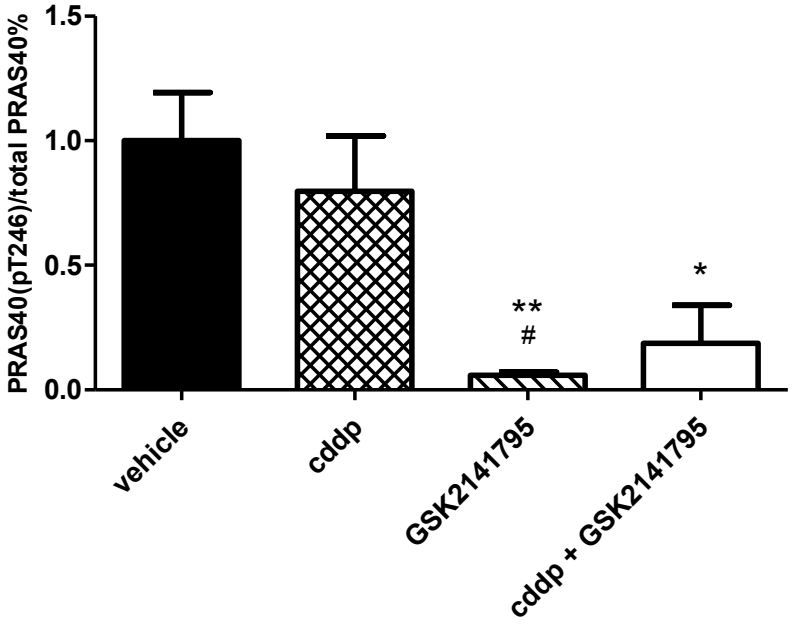


Figure 4-12 Concentration-dependent effect of AKT inhibition by GSK2141795 on phospho-PRAS40 (Thr246) level in SKOV3 xenografts

GSK2141795 (30 mg/Kg) abolished phosphorylation of PRAS40 at Thr246, both as single agent and in combination with cisplatin (cddp), in SKOV3 tumour xenografts, indicating the effective inhibition of AKT by GSK2141795 (mean of each treatment group is normalised to the mean of the vehicle group. n= 5 tumours). Data are presented as a phospho-PRAS40 / total PRAS40 decrease relative to untreated samples. Data are shown as the mean ± SEM for n = 5 animals (tumours) . *p<0.05 and **p<0.01, where the symbols * and # represent significant differences when compared to vehicle and cisplatin data, respectively.

4.3 Discussion

Findings described in chapter 3 suggested the potential ability of GSK2141795 to be used in combination with cisplatin to restore sensitivity to platinum in ovarian cancer patients. However, combining anti-cancer agents in the clinic is not always easy; doses of drugs used in combination often have to be reduced compared to when they are given as mono-therapies. Yet it is not always clear that the reduced dose will have the same effects on the target pathway, i.e. what is the minimal level of pathway inhibition required to maintain efficacy in a given situation. Ideally, quantification of pathway inhibition would be done with serial biopsies of a patient's tumour before and after drug treatment, in order to evaluate levels of phosphorylated AKT substrates. However, clinical implementation of this approach is challenging due to the reluctance of many patients to undergo repeat biopsies, lack of biopsiable tumours in all patients and tumour heterogeneity amongst other reasons. Therefore having a way to measure pathway inhibition in a non-invasive fashion would be very helpful to this process.

Positron emission tomography (PET) with [^{18}F]fluorodeoxy glucose (FDG-PET) evaluates cancer cells glycolysis as a surrogate for tumour response to chemotherapy. Two independent studies in 2002 and 2003 showed that early changes in FDG-PET signal can be used to predict imatinib response in gastrointestinal stromal tumours (Van den Abbeele and Badawi 2002, Stroobants, Goeminne et al. 2003). These findings led to much interest in using FDG-PET imaging as a predictive marker of response in development of novel cancer drugs.

Mechanistically, AKT activation causes increased transcription and plasma membrane localisation of Glucose Transporter 1 (GLUT1), a protein which facilitates transport of

glucose across the plasma membrane of mammalian cells. Membrane bound GLUT1 is an important mediator of FDG uptake in most of cancer cells (Brown, Goodman et al. 2002, Plas and Thompson 2005). Immunohistochemical studies have revealed decreased GLU1 staining in both plasma membrane and cytosol following PI3K/AKT/mTOR pathway inhibition in pancreatic tumour cells indicating that AKT inhibition inactivated GLUT1 transcription (Ma, Jacene et al. 2009). In addition, immunohistochemical studies on pancreatic tumours from patients treated with the mTOR inhibitor, temsirolimus, indicated a higher cytosolic to plasma membrane fraction of GLUT1, suggesting that PI3K/AKT/mTOR pathway inhibition disrupts the translocation of GLUT1 to plasma membrane (Ma, Jacene et al. 2009). AKT also regulates the translocation of Glut4 to the plasma membrane. Given the role of AKT in the regulation of glucose metabolism, [^{18}F]FDG-PET could serve as a powerful tool in assessing the effectiveness of AKT inhibitors in tumour cells.

The preclinical FDG-PET studies described in this chapter provide the justification for using FDG-PET to evaluate the effects of GSK2141795 on tumour glucose metabolism in the clinic, as well as the confidence that inhibition of glucose metabolism correlates strongly with AKT inhibition. Identical FDG-PET studies were performed using SKOV3 cells as (1) monolayers, (2) MTS and (3) *in vivo* xenografts. This allowed for comparison of the data obtained with the three models in order to determine the inter-predictability of each model and the relationship between changes in phospho-PRAS40 and FDG uptake in various models. Data described in this chapter shows that although phospho-PRAS40 levels were decreased by >50% in all 3 model systems, only MTS and *in vivo* xenografts showed >50% decrease in FDG uptake, suggesting that MTS may be a better *in vitro* representation of *in vivo* physiology than monolayer cultures. The decreases in FDG uptake and phospho-PRAS40/total PRAS40 were strongly positively correlated. Given that PRAS40 phosphorylation is a validated PD biomarker of AKT inhibition by GSK2141795, this finding

suggests that FDG uptake can also be used as a PD biomarker for GSK2141795 mediated target inhibition.

Although the decrease in FDG uptake by GSK2141795 was observed in both 2D and 3D *in vitro* models used in this study, the effectiveness of the compound was shown to be greater in the 3D model and more closely resembled the effects observed *in vivo*. This observation suggests that 3D models may be more suitable, efficient and predictive models than 2D models in determining dose and schedule optimisation prior to *in vivo* experiments. This may be important in minimising the cost of the studies and the use of animals in translational approaches.

The MTS studies also demonstrated a concentration-dependent decrease in FDG imaging signal following GSK2141795 administration, which was reproduced in xenograft studies. These data, coupled with the PK data obtained from xenograft studies, predict that concentrations ≥1 μM GSK2141795 would be required to determine the maximal decrease in phospho-PRAS40 and FDG uptake.

In summary, these results demonstrate that the pan-AKT inhibitor, GSK2141795, inhibits AKT signalling in platinum-resistant ovarian cancer cells lines *in vitro* and *in vivo*, and that [^{18}F]FDG uptake could be used as a non-invasive pharmacodynamic marker for guiding dose selection in ovarian cancer patients in future studies. These results found the rational for a phase I clinical trial of GSK2141795, an open label study to investigate the pharmacokinetics and pharmacodynamics of repeat escalating doses of the oral AKT inhibitor GSK2141795 by 18F FDG PET analysis in subjects with ovarian cancer (www.clinicaltrials.gov).

5 Comparison of the effect of AKT, PI3K and DNA-PK inhibition, in combination with cisplatin on cell proliferation and apoptosis

5.1 Introduction

Chapter 3 demonstrated that AKT inhibition with the small molecule GSK2141795 restored sensitivity to cisplatin in ovarian cancer platinum resistant cells both *in vitro* and in xenograft models. It has previously been shown that treatment of platinum resistant cells with csiplatin, results in AKT translocation into the nucleus where it is phosphorylated at S473 by catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). This DNA-PK mediated pro-survival mechanism does not appear to occur in platinum sensitive cells, PEO1 and PEA1. DNA-PK inhibition was shown in this study to prevent cisplatin-induced phosphorylation of AKT at S473, but not at T308. Moreover it was found that cisplatin-mediated AKT activation is mTORC2 independent (Stronach, Chen et al. 2011).

DNA-PK is a nuclear serine/threonine kinase. It is composed of two DNA binding proteins, Ku70 and Ku80 and a 470-kDa catalytic subunit, DNA-PKcs. DNA-PK plays a crucial role in nonhomologous end-joining (NHEJ) DNA repair. Ionizing radiation and radiomimic drugs induce double-strand breaks (DSB) that are mainly repaired by non-homologous end joining (NHEJ). In this process the two ends of DNA strand break are connected and resealed without the need for sequence homology between the ends (Mahaney, Meek et al. 2009). NHEJ double-strand DNA (dsDNA) break repair process is in contrast with the homologous recombination (HR) dsDNA break repair process in which DNA sequence from an intact DNA molecule, often the newly synthesised sister chromatid, is used to repair replication lesions (Arnaudeau, Lundin et al. 2001). In NHEJ DNA repair process each broken DNA end is first bound by one Ku 70/80 heterodimer and two heterodimer must come together to bridge matching ends to ensure efficient ligation. This complex is bound by the DNA-PK catalytic subunit which stabilises the protein/DNA binding therefore enabling the NHEJ DNA-repair process to carry on. The ligation of the DNA ends is accomplished by the XRCC4/ligase IV complex subsequently attracting the DNA-PKcs subunit. Together with

ataxia-telangiectasia and Rad3 related (ATR) and ataxia-telangiectasia mutant (ATM), DNA-PKcs forms a critical early component of the DNA damage response (Anderson and Lees-Miller 1992, Collis, DeWeese et al. 2005, Chen, Uematsu et al. 2007). DNA-PKcs, ATM and ATR are members of the PI3K-related protein kinases family (PIKKs). Members of the PIKKs family phosphorylate target proteins on serine or threonine residues using a C-terminal region related to the catalytic domain of PI3K. Other members of this family include mammalian target of rapamycin (mTOR), the catalytically inactive transformation/transcription domain-associated protein (TRRAP) and suppressor with morphological effect on genitalia 1 (SMG1) (Cimprich and Cortez 2008).

DNA-PK activation is regulated by phosphorylation. To date several phosphorylation sites of DNA-PK are identified which are differently phosphorylated dependent on the stimuli. For example, phosphorylation at Thr-2638/Thr-2647 is essential for radio-resistance conferred by DNA-PKcs (Soubeyrand, Pope et al. 2003).

In the classical model of AKT activation, PI3K acts upstream of AKT by phosphorylating membrane bound phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP_3), a second messenger that binds to the plectstrin homology (PH) domain of AKT, recruiting AKT to the plasma membrane. AKT is then phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at Thr308. Phosphorylated AKT in turn regulates a wide range of target proteins that control cell growth, proliferation, survival, and glucose metabolism (Bellacosa, Kumar et al. 2005) . In this model it is believed that phosphorylation of AKT at T308 by PDK1 is required for activation of AKT.

In this chapter it was investigated if nuclear DNA-PK mediated activation of AKT in response to cisplatin in platinum resistant cells is dependent or independent of PI3K activation. In this chapter, firstly the effect of DNA-PK or PI3K inhibitions with small molecular inhibitors of DNA-PK (NU7441) and PI3K (GSK2126485) on cisplatin sensitivity in ovarian cancer cells was assessed *in vitro* and in xenograft models. Furthermore, *PIK3CA* and *PIK3R1* depleted cells were used to investigate whether the nuclear DNA-PK mediated activation of AKT, in response to cisplatin, is dependent on activation of PI3K.

NU7441 (8-dibenzothiophen-4-yl-2-morpholin-4-yl-chromen-4-one) is a highly potent and selective DNA-PK inhibitor (IC_{50} = 14 nM), exhibiting ATP-competitive inhibition kinetics (Leahy, Golding et al. 2004, Zhao, Thomas et al. 2006). GSK2126458 is a highly potent inhibitor of PI3K which displays great selectivity for PI3K compared with other members of the PI3K-related protein kinase family. PI3K acts up-stream of AKT in the PI3K/AKT signalling cascade (Wang, Harris et al. 2008, Leung, Kim et al. 2011).

5.2 Results

5.2.1 Effect of DNA-PK inhibition on sensitivity to cisplatin *in vitro*

To assess the effect of DNA-PK inhibition on restoration of sensitivity to cisplatin *in vitro*, cisplatin resistant SKOV3 and PEO4, were treated with a range of concentrations of a potent DNA-PK inhibitor, NU7441 as a single agent or in combination with cisplatin for 24 hours. Effect of the treatment with NU7441 on apoptosis was assessed by measuring the activity of caspase 3/7. Inhibition of DNA-PK by NU7441 resulted in sensitisation to cisplatin in a concentration dependent manner in both cell lines. In addition, single agent NU7441 was also found to induce apoptosis at higher concentrations (Figure. 5.1).

5.2.2 Effect of DNA-PK inhibition of DNA-PK on tumour growth *in vivo*

To assess the effect of DNA-PK inhibition by NU7441 as a single agent or in combination with cisplatin *in vivo*, mice bearing SKOV3 tumour xenografts were treated with vehicle, cisplatin (1mg/kg twice weekly) or two different doses of NU7441 (7.5 mg/kg or 10 mg/kg daily) as a single agent or in combination with cisplatin for 14 days. Treatment of xenografts with single agent NU7441 did not have any effect on tumour growth and tumours grew at the same rate as the vehicle control groups (Figure. 5.2). In addition, treatment of tumours with 1 mg/kg cisplatin twice weekly did not slow tumour growth either. In contrast, DNA-PK inhibition in combination with cisplatin decreased tumour growth at 14 days by 89% (7.5mg/kg NU7441) ($p < 0.05$) and 90% (10mg/kg NU7441) ($p < 0.05$), relative to platinum only (Figure. 5.2).

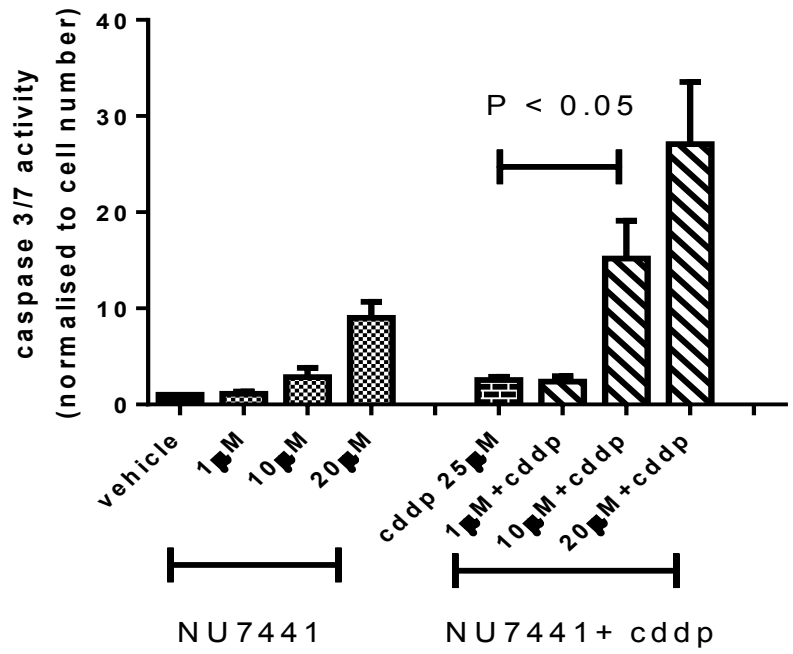


Figure 5-1 Caspase 3/7 activity in SKOV3 cells treated with the DNA-PK inhibitor NU7441 as single agent or in combination with cisplatin

Cells were pre-treated with NU7441 for 1 hour prior to exposure to cisplatin for 24 hours. Treatment with NU7441 resulted in a significant increase in caspase 3/7 activity in response to cisplatin compare to cells which were treated with cisplatin only. Data shown are the means ± SEM of 3 experiments performed in triplicate.

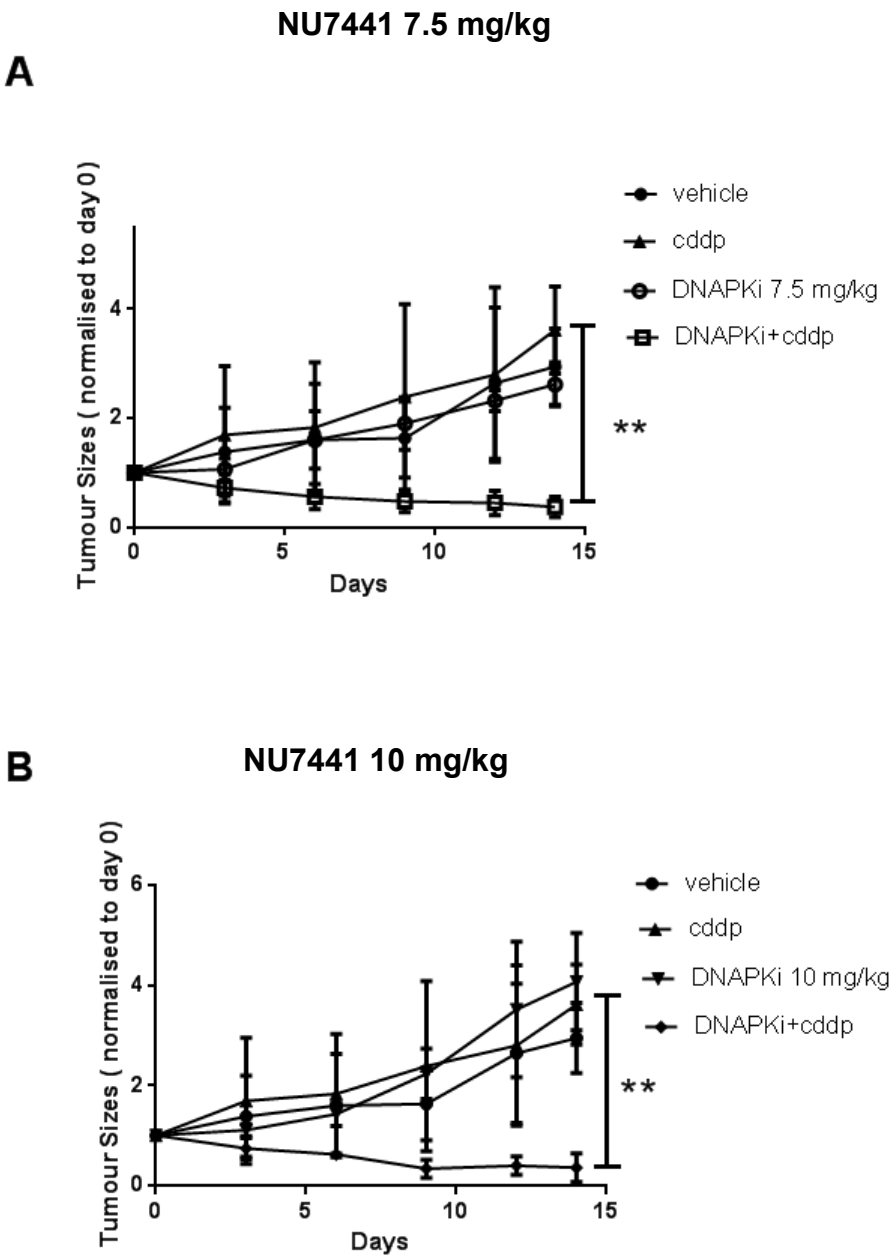


Figure 5-2 inhibition of DNA-PK restored response to cisplatin in chemo-resistant ovarian cancer cells *in vivo*

SKOV3 tumour cells were injected subcutaneously into the flanks of nude mice and treated with A) 7.5 mg/kg or B) 10 mg/kg DNA-PKcs inhibitor, NU7441 daily (5 days on/2 days off) for 2 weeks alone or in combination with 1 mg/kg cisplatin twice weekly. DNA-PK inhibition or cisplatin alone were ineffective however in combination decreased tumour growth at 14 days by 89% (7.5 mg/kg NU7441) and 90% (10 mg/kg NU7441), relative to platinum only treatment. Data shown are the mean ± SEM for n = 5 animals (tumours) / treatment. ***p*<0.01,

5.2.3 Effect of PI3K inhibition using GSK2126458 on sensitivity to cisplatin

In order to examine if inhibition of PI3K modulates cisplatin resistance in ovarian cancer cells, cisplatin resistant SKOV3 and PEO4 cell lines were treated with a range of concentrations of PI3K inhibitor GSK2126458 (0.1 nM - 40 nM) alone or in combination with 25 μM cddp for 24 hours. The dose range for GSK2126458 was selected based on the IC50 value of this compound in those two cell lines which was between 10 - 20 nM in both cell lines (Figure. 5.3). Treatment with GSK2126458 as a single agent resulted in either no increase, or a very slight increase in the induction of caspase 3/7 activity in both cell lines after 24 hours (Figure. 5.4a and 5.4b). This was similar to what was observed in the same cell lines where treated with the AKT inhibitor GSK2141795 for the same length of time (Figure. 5.4 a and 5.4.b).

In contrast with the AKT inhibitor GSK2141795, combination treatment of GSK2126458 with 25 μM cddp did not increase caspase-3/7 activity above levels measured in cells treated with cddp alone in either SKOV3 or PEO4 cells (Figure. 5.4c and 5.4d). Similar results were found when the treatment time was increased to 72 hours (Figure. 5.5). The growth-inhibitory effect of GSK2126458 was also assessed. However, as a single agent GSK2126458 was found to reduce cell viability in a dose dependent manner after 72 hours (Figure 5.6.a). Inhibition of the PI3K pathway by GSK2126458 was confirmed by measuring changes in the level of phosphorylation of the PI3K/AKT pathway PD biomarker PRAS40, in response to GSK2126458 treatment, which decreased in a dose dependent manner (Figure 5.6.b).

The effect of the inhibition of PI3K by GSK2126458 on the cisplatin IC_{50} curve in platinum resistant SKOV3 and PEO4 cells was also assessed. Cells were pre-treated with

GSK2126458 one hour prior to the treatment with a range of concentration of cisplatin (0.01 – 100 μM). Pre-treatment with GSK2126458 did not result in any significant change to the cisplatin IC50 curve in PEO4 and SKOV3 cells (Figure. 5.7).

In addition, reverse sequencing of the PI3K inhibitor and cisplatin treatments and increasing the treatment time also did not result in any shift in the cisplatin IC50 curves in PEO4 and SKOV3 cells (Figure 5.8) (PEO4 data not shown).

In conclusion neither post-treatment nor pre-treatment with GSK2126458, affected the sensitivity to cisplatin in SKOV3 and PEO4 cells.

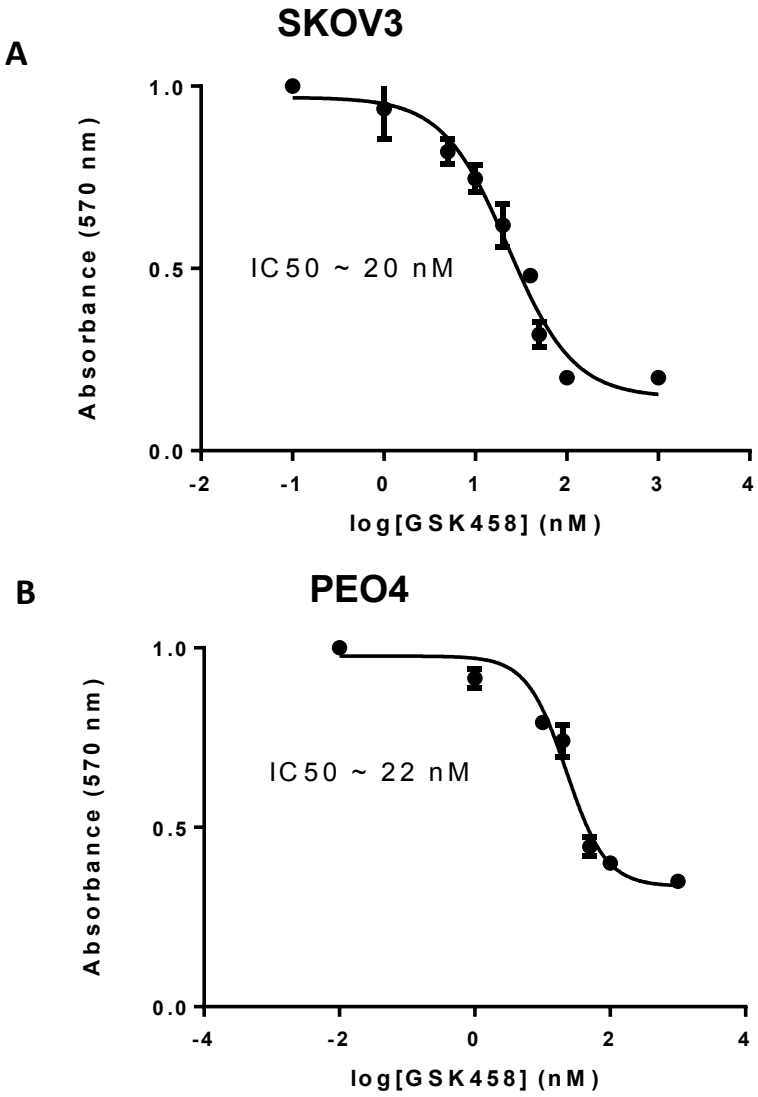


Figure 5-3 The half maximum inhibitory concentration of (IC50) of GSK2126458 in SKOV3 (A) and PEO4 (B)

Cells were treated with a range of concentration of GSK2126458 for 72 hours (approximately three doubling time). Cell viability was assessed by MTT assay after the completion of the treatment time. Data shown are the means ± SEM of n = 3 experiments performed in triplicate.

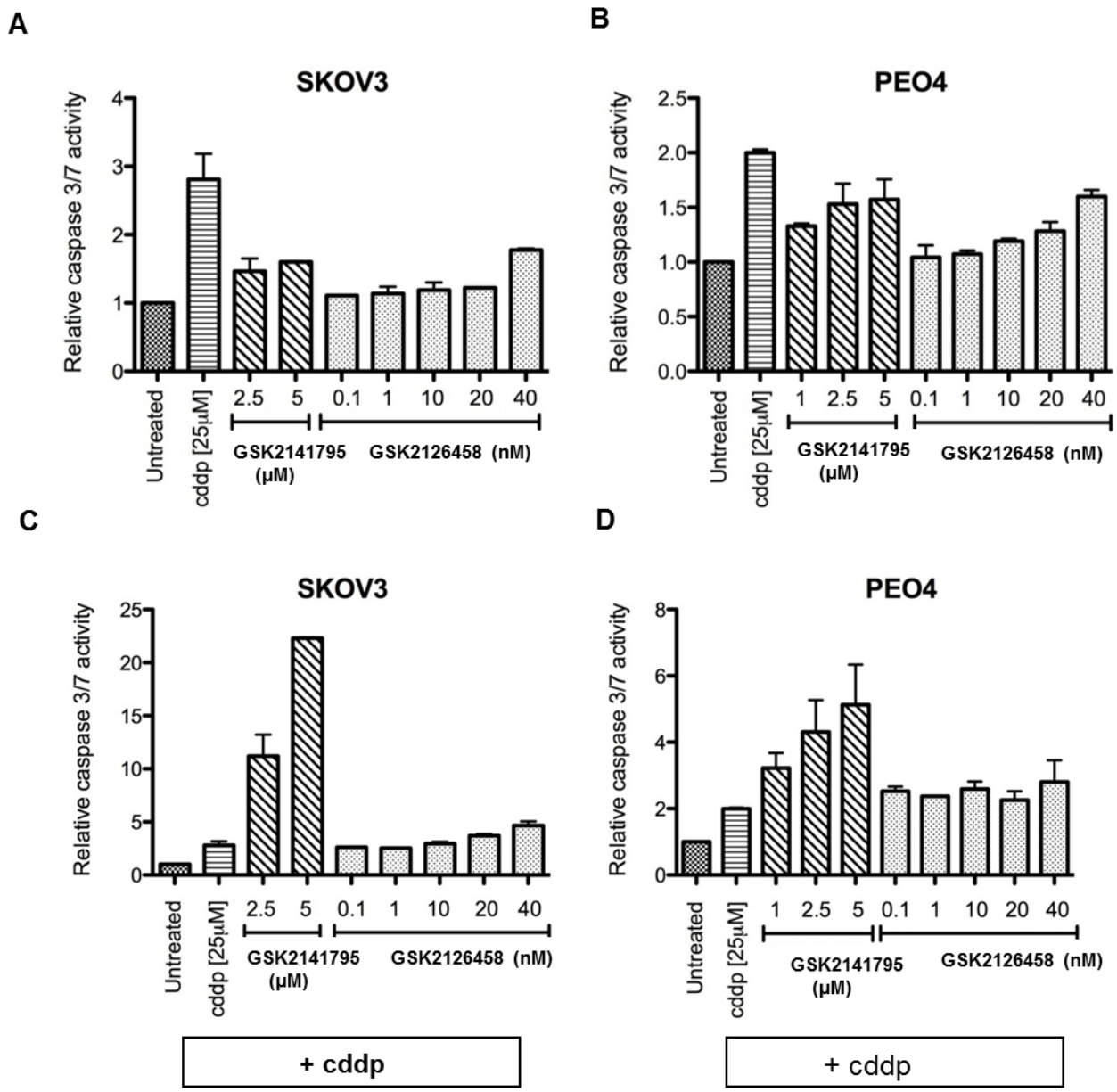


Figure 5-4 Caspase 3/7 activity (normalised to cell number) in SKOV3 and PEO4 monolayers treated with GSK2126458 and GSK2141795 – A and B: single agent treatments. C and D: combination with cddp

SKOV3 and PEO4 monolayers were treated with a range of concentrations of GSK2126458 for 24 hours as a single agent or in combination with cddp. Caspase 3/7 activity in cells treated with GSK2126458 was compared to caspase activity in cells treated with GSK2141795. Treatment with GSK2126458 did not affect induction of caspase 3/7 as a single agent or in combination with cddp whereas treatment with GSK2141795 re-sensitised cells to cddp. Data shown are the means ± SEM of n = 3 experiments performed in triplicate.

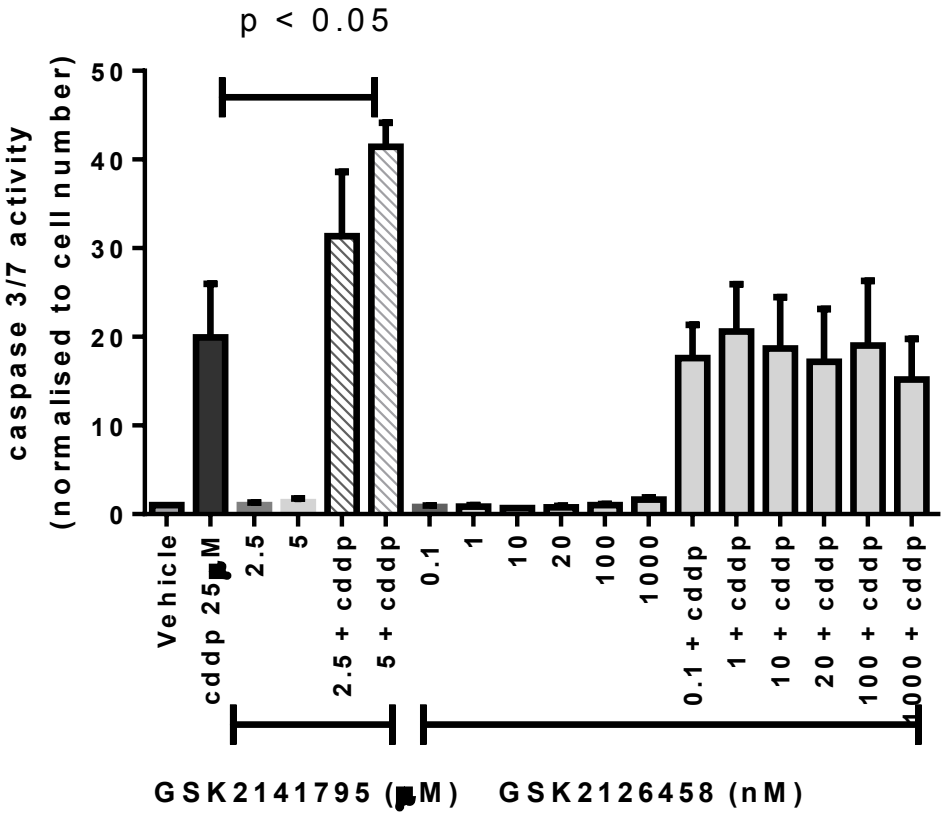


Figure 5-5 Comparison of the effect of 72 hours exposure to GSK2141795 and GSK2126458 on sensitisation to cisplatin in SKOV3 cells

SKOV3 cells were exposed to a range of concentration of the AKT inhibitor GSK2141795 or the PI3K inhibitor GSK2126458 as single agents or in combination with cisplatin for 72 hours. GSK2141795 significantly increased caspase 3/7 activity in response to cisplatin in SKOV3 cells but treatment with GSK2126458 did not have any effect on sensitivity to cisplatin in the same cell line. Data shown are the means ± SEM of n = 3 experiments performed in triplicate.

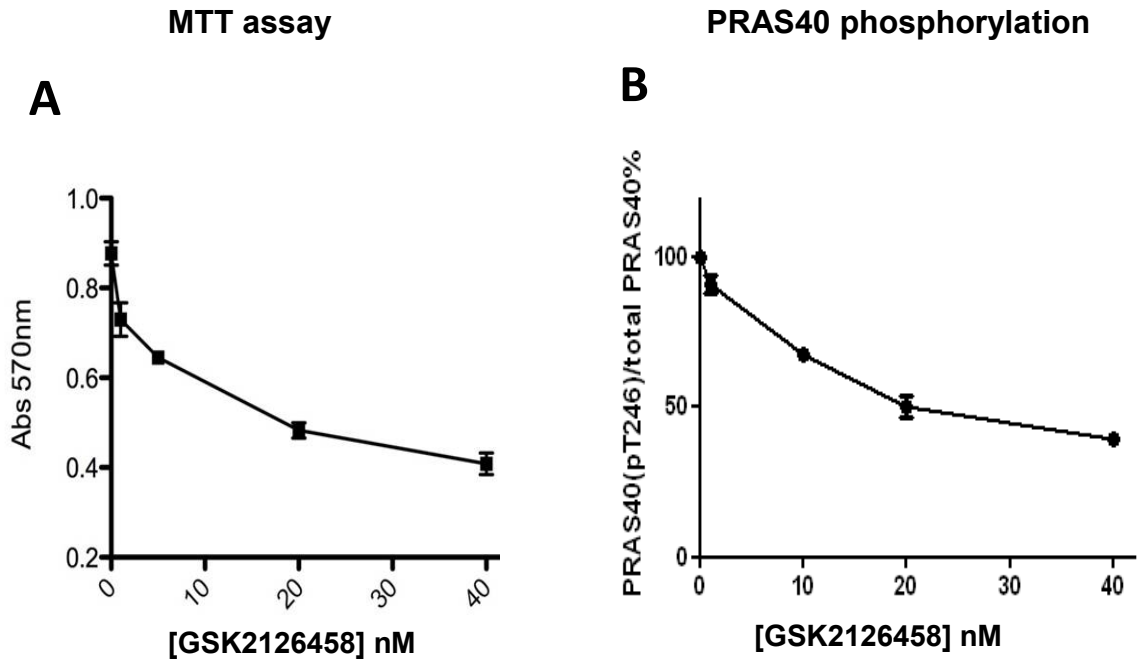


Figure 5-6 Effect of the treatment with GSK2126458 on the cell viability (A) and the phosphorylation levels of PRAS40 (B) in SKOV3 cells

SKOV3 monolayers were treated with a range of concentrations of GSK2126458 for 72 hours. Effect of the compound on the cell viability was assessed by MTT assay. GSK2126458 inhibited cell growth in a dose dependent manner (A, n = 3).Treatment with GSK2126458 also resulted in a dose dependent reduction in pPRAS40 level (B, n = 2). Data shown are the means ± SEM of n = 2/3 experiments

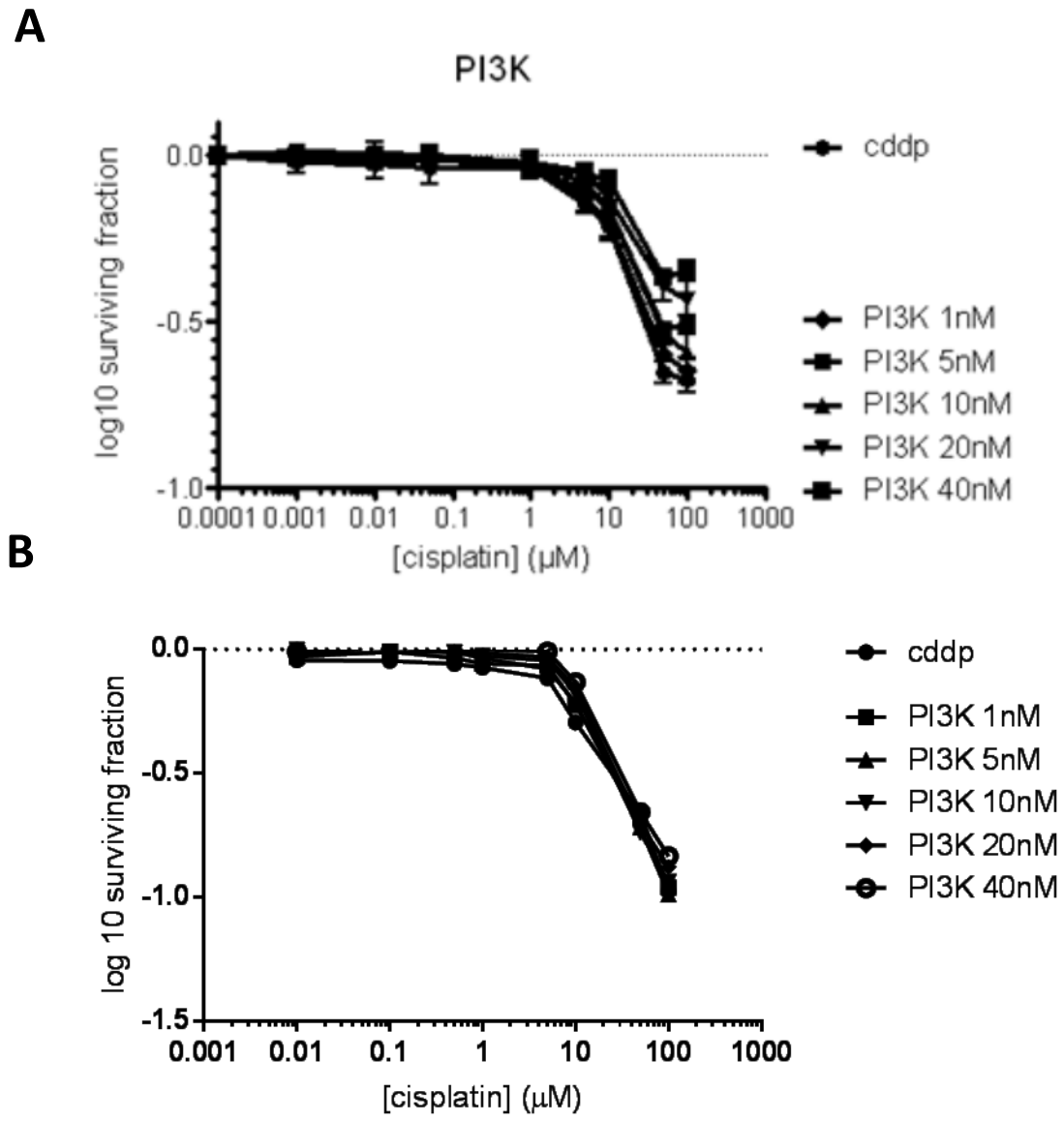
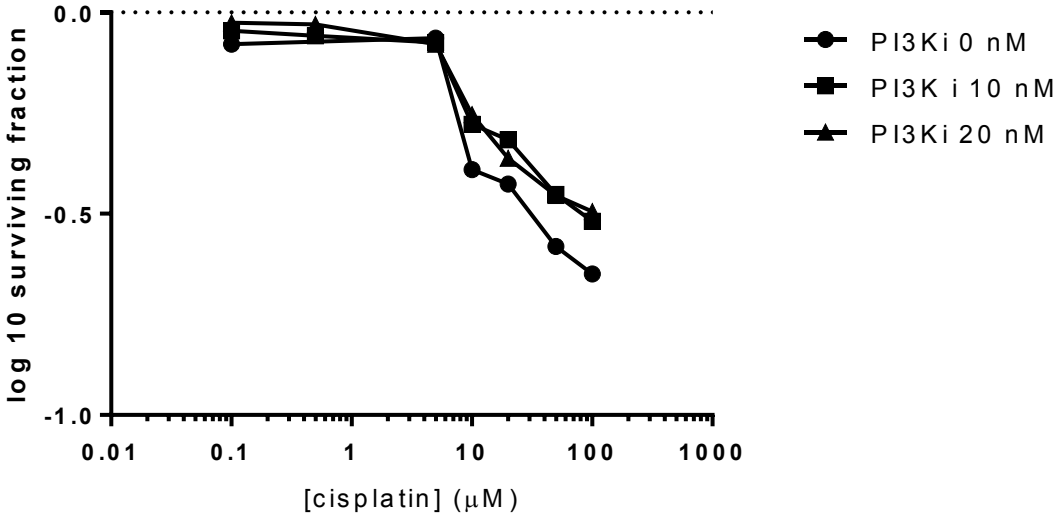


Figure 5-7 Effect of the exposure to GSK2126458 on IC50 curve of cisplatin in A) SKOV3 cells and B) PEO4 cells

Cells were pre-treated with different concentrations of the PI3K inhibitor GSK2126458 prior to exposure to a range of concentration of cisplatin. Treatment with GSK2126458 did not result in any significant change to the cisplatin IC50 curve in neither of the examined cell lines. Data shown are the means ± SEM of n = 3 experiments.

A



B

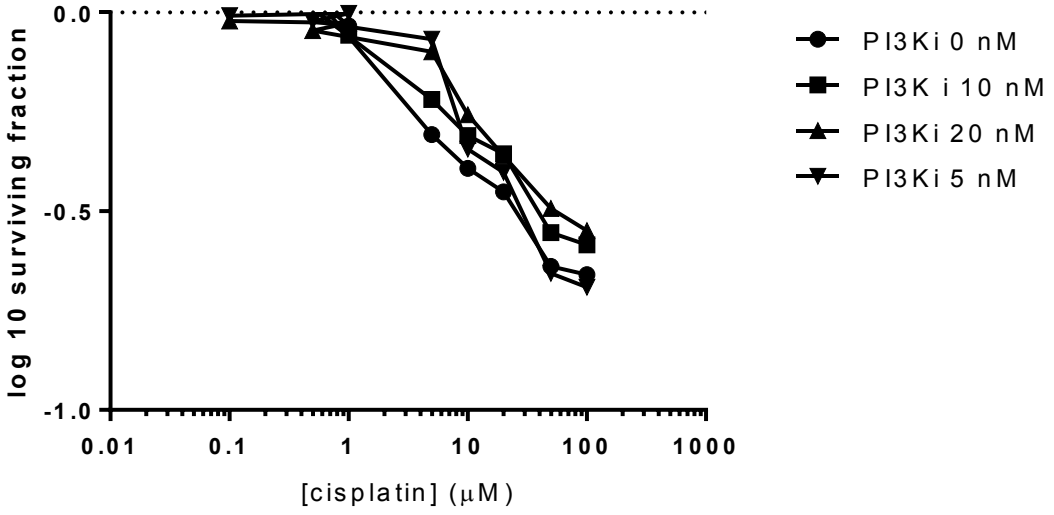


Figure 5-8 Effect of the post treatment with GSK2126458 on cddp IC50 curve A) 24 hours and B) 48 in SKOV3 cells

Cells were treated with different concentrations of the pI3K inhibitor GSK2126458 one hour after exposure to a range of concentration of cisplatin. Post-treatment with GSK2126458 for 24 or 48 hours did not result in any significant change to the IC50 curve of cisplatin in SKOV3 cells. Data shown are the means ± SEM of n = 3 experiments.

5.2.4 Effect of siRNA mediated PI3K knockdown on sensitivity to cisplatin *in vitro*

To confirm that the inhibition of PI3K has no effect on sensitisation to cisplatin in the assessed cell models as observed with GSK2126458 , siRNA knockdown of genes encoding for the regulatory subunit (p85,*PIK3R1*) and catalytic subunit (p110α, *PIK3CA*) of PI3K was performed and the effect of knockdown on sensitisation to cisplatin examined.

Knockdown of *PIK3CA* or *PIK3R1* did not enhance apoptotic responses to cisplatin in the either platinum resistant SKOV3 and PEO4 cells compared with transfection controls (Figure 5.9). SIRNA knockdown of *PIK3CA* and *PIK3R1* was confirmed by western blotting (Figure 5.10).

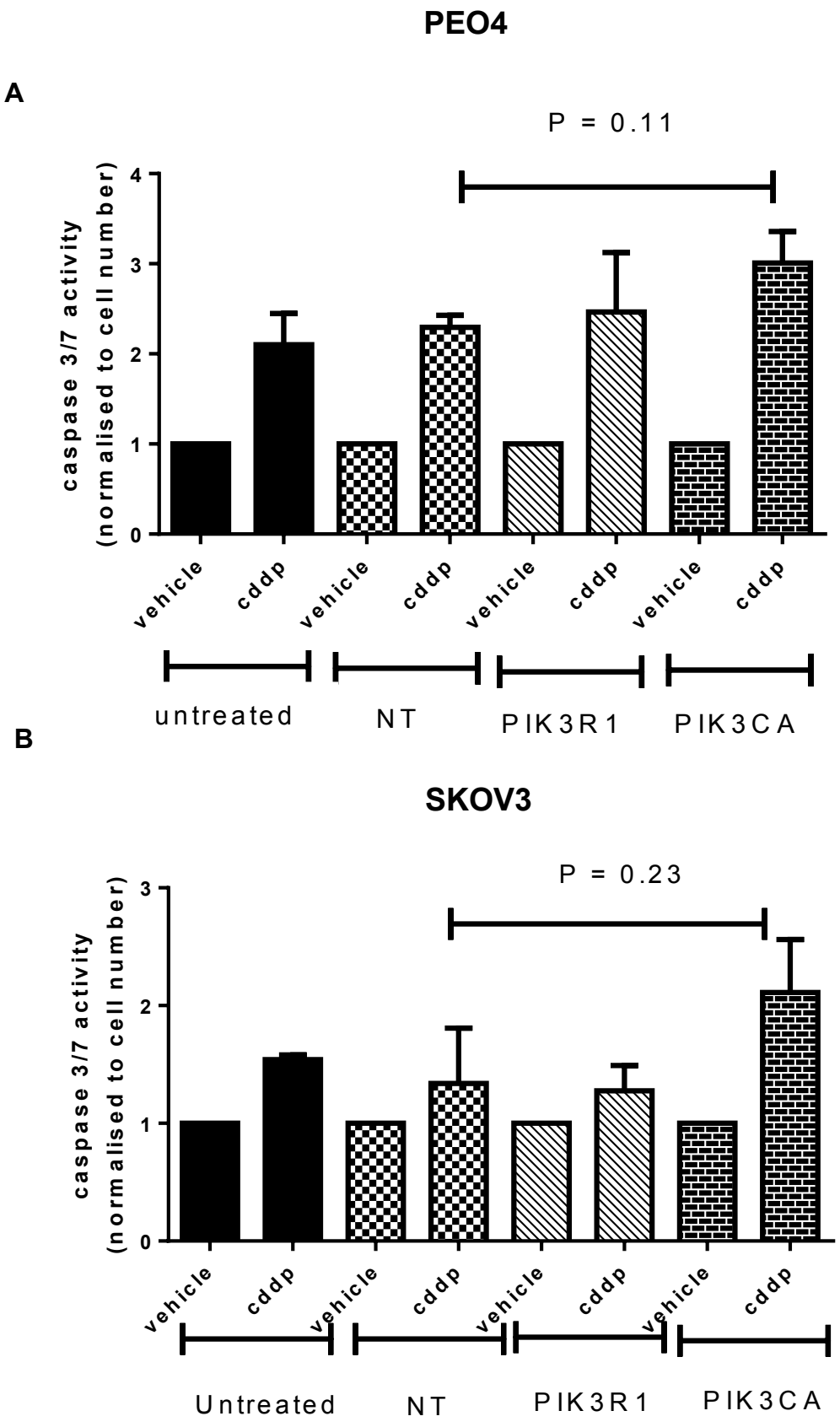


Figure 5-9 Effect of *PIK3CA* and *PIK3R1* knockdown on apoptotic response to cisplatin in A) PEO4 and B) SKOV3 cells

After knockdown of *PIK3R1* and *PIK3CA* in platinum resistant cells PEO4 A) and SKOV3 B), cells were treated with cisplatin for 24 hours and caspase 3/7 activity was assayed. *PIK3CA* and *PIK3R1* knockdown did not result in any significant change to apoptotic response to cisplatin in SKOV3 and PEO4 cell lines. Data shown are the means ± SEM of n = 3 experiments.

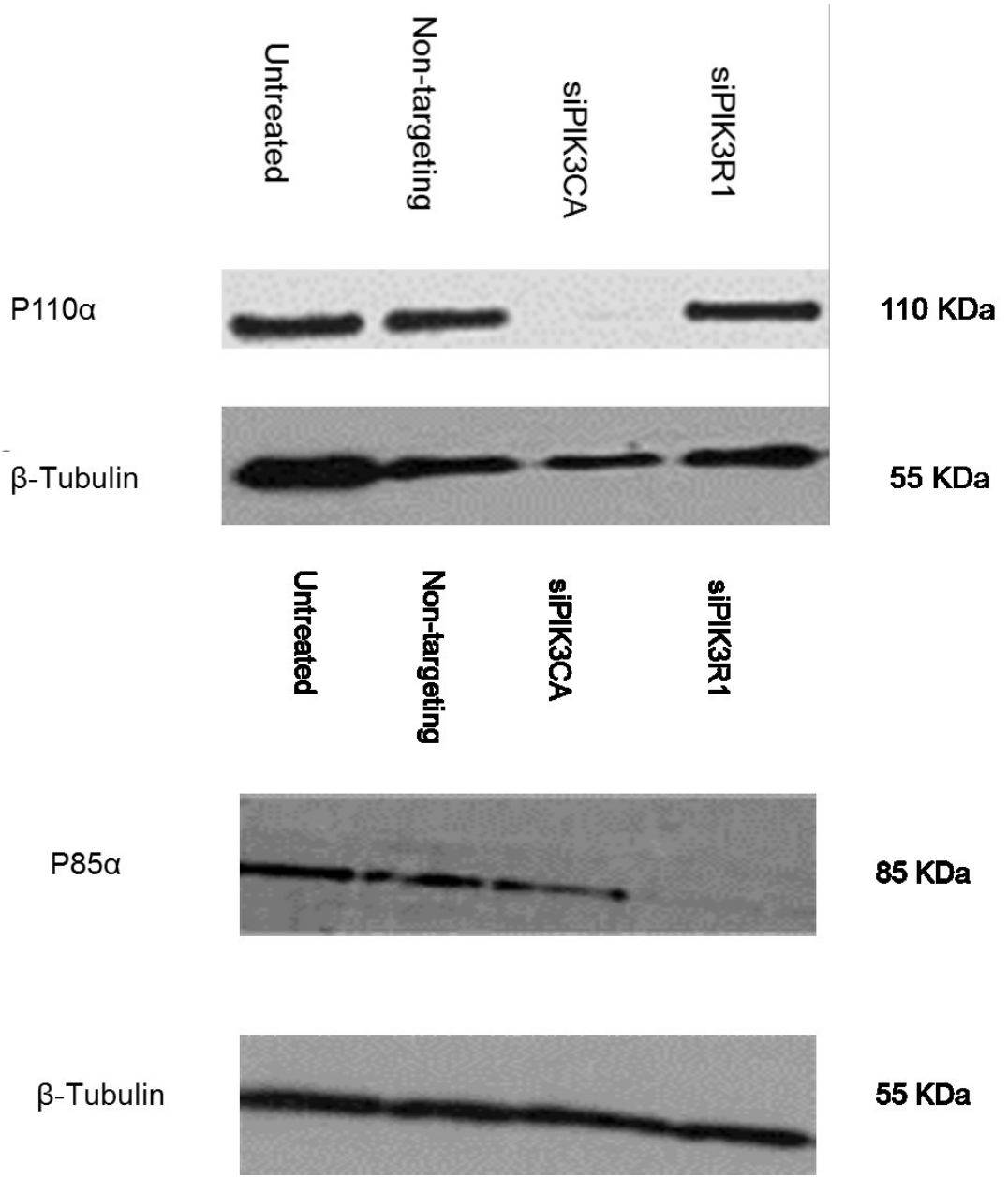


Figure 5-10 Confirmation of *PIK3CA* and *PIK3R1* knockdown in SKOV3 cells *in vitro* by western blotting

SKOV3 cells were transfected with siRNA directed to *PIK3CA*, *PIK3R1* or non-targeting control. Protein lysates were collected after 48 hours and expression of p110α (encoded by *PIK3CA*) and p85α (encoded by *PIK3R1*) were assessed by western blotting analysis. Western blotting analysis confirmed knockdown of *PIK3CA* and *PIKR1* genes. Beta-tubulin was used as loading control. Representative images from n=3 experiments.

5.2.5 DNA-PK modulates response to cisplatin independent of PI3K in cisplatin resistant ovarian cancer cells

To examine if PI3K is involved in modulation of response to cisplatin by DNA-PK, expression of both p85α and p110α subunits of PI3K were down-regulated by siRNA transfection of platinum resistant cells PEO4 and SKOV3 cells. Treatment of the transfected cells with the DNA-PK inhibitor NU7441 resulted in an similarly enhanced response to cisplatin as was seen in the non-transfected control cells, indicating that the DNA-PK dependent sensitisation to cisplatin in platinum resistant cells is not dependent on the expression of PI3K (Figure. 5.11).

In addition, *PIK3CA* and *PIK3R1* depleted SKOV3 cells were treated with cisplatin for 2 hours and the induction of pAKT S473 in response to cisplatin compared with control SKOV3 cells. Phosphorylation of pAKT at S473 was increased in response to cisplatin regardless of the siRNA treatment. Moreover, inhibition of DNA-PK by NU7441 abolished the phosphorylation at S473 in response to cisplatin in the PI3K wild type cells as well as in *PIK3CA* and *PIK3R1* siRNA transfected cells indicating that knockdown of either subunits of PI3K does not affect phosphorylation of AKT at S473 by DNA-PK. Treatment with cisplatin, NU7441 or combination of cisplatin and NU7441 did not affect the level of expression of total AKT in SKOV3 cells (Figure. 5.12).

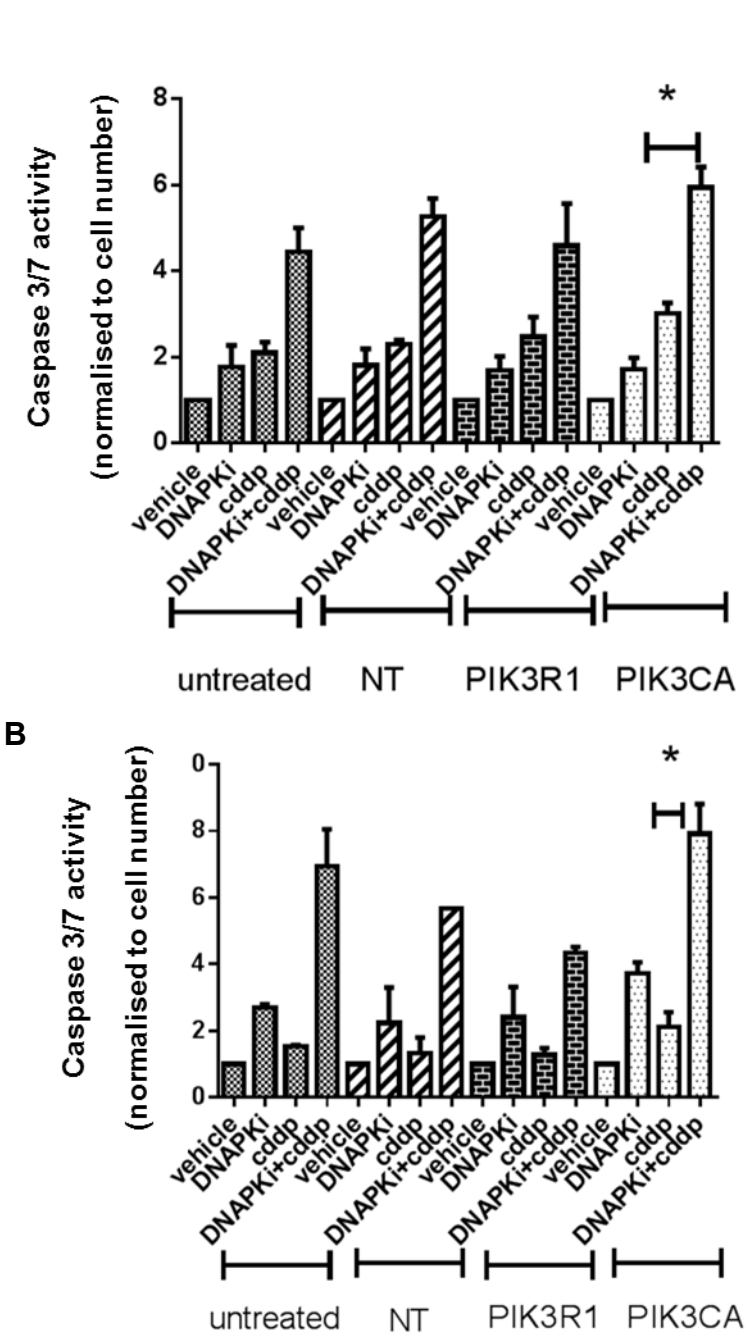


Figure 5-11 Inhibition of DNA-PK with NU7441 in combination with *PIK3CA* and *PIK3R1* knockdown in platinum treated and untreated A) PEO4 and B) SKOV3 cells.

SKOV3 cells were transfected with siRNA directed *to PIK3CA*, *PIK3R1* or non-targeting control for 48 hours. SiRNA transfected cells were treated with NU7441 or cisplatin or combination of NU7441 and cisplatin for 24 hours followed by caspase 3/7 activity assessment. siRNA knockdown of *PIK3CA* and *PIK3R1* genes did not affect the enhanced response to cisplatin in cells treated with NU7441 in combination with cisplatin compared with transfection controls. Data shown are the means ± SEM of 3–4 experiments performed in triplicate. *p<0.05

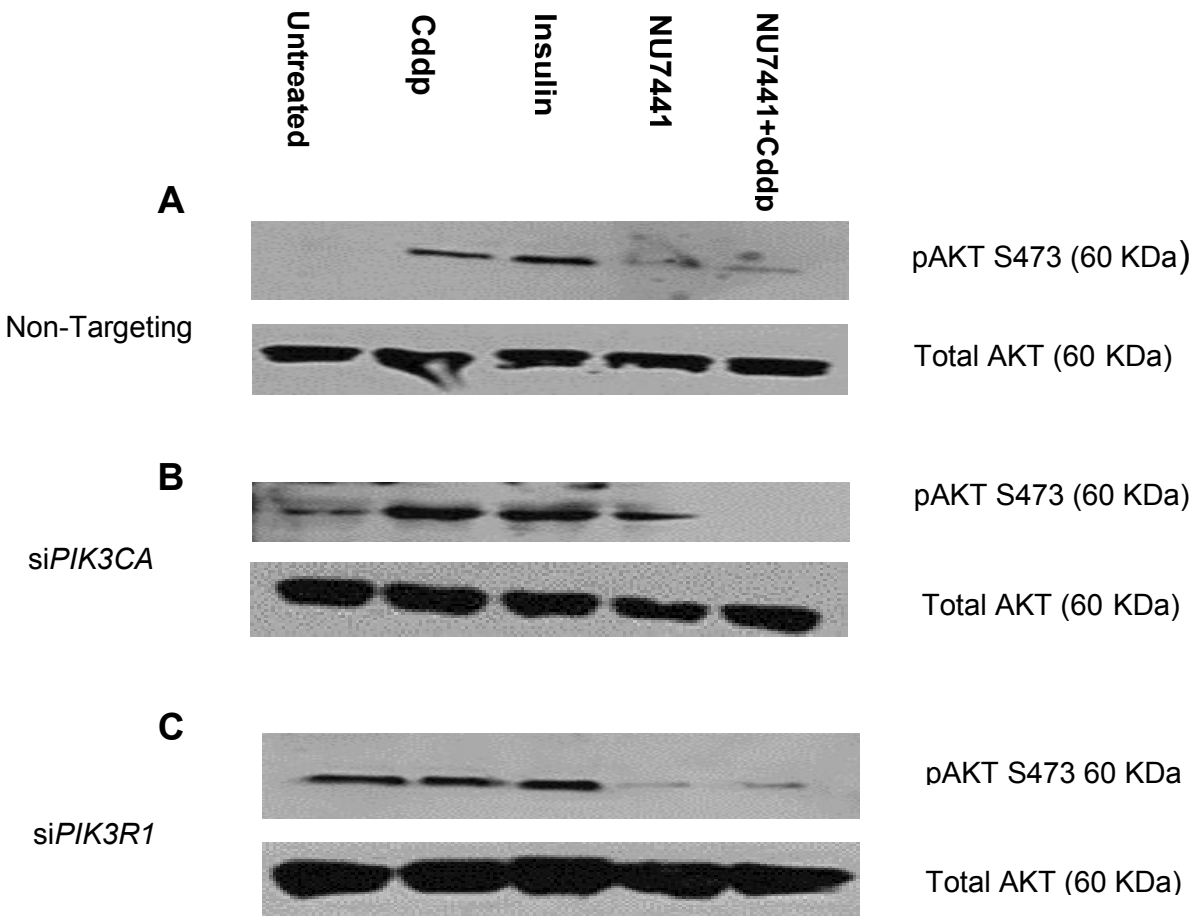


Figure 5-12 Effect of knocking down the *PIK3CA* and *PIK3R1* subunits of PI3K on phosphorylation of AKT at S473 by DNA-PK

SKOV3 cells were transfected with siRNA directed to *PIK3CA*, *PIK3R1* or non-targeting control for 48 hours followed by exposure to NU7441 or insulin or cisplatin or combination of cisplatin and NU7441 for 2 hours. Whole cell lysates were subjected to western blotting using pAKT (S473) and total AKT antibodies. Inhibition of DNA-PK with NU7441 abolished phosphorylation of AKT at S473 in non-transfected cells (A) as well as in *PIK3CA* (B) and PIK3R1 (C) siRNA transfected cells. Representative images from n=2 experiments.

5.3 Discussion

In chapter 3 AKT inhibition with GSK2141795 was shown to effectively sensitise platinum-resistant cells to cisplatin in both *in vitro* and *in vivo* models. In this chapter it was further investigated whether targeting PI3K or DNA-PK can also sensitise tumour cells to platinum treatment *in vitro* and *in vivo*. It was demonstrated that inhibition of DNA-PK with a potent DNA-PK inhibitor, NU7441, sensitised platinum resistant ovarian cancer cells to cisplatin both *in vitro* and in ovarian cancer xenograft models. In contrast, inhibition of PI3K with a potent and selective small molecule inhibitor GSK2126458 did not affect sensitivity to cisplatin in the same cell model *in vitro*. It was further demonstrated that the effects of DNA-PK activation on modulation of tumour cell resistant to cisplatin was independent of activation of PI3K.

Wallin et al. reported an increase in phosphorylation of AKT at S473 in the nucleus of breast cancer cell lines, and also in a breast cancer xenograft model, following doxorubicin treatment, and demonstrated that this phosphorylation was mediated by DNA-PK (Wallin, Guan et al. 2010). This is in agreement with recent findings in our laboratory showing that in response to cisplatin, AKT is translocated into the nucleus where it is phosphorylated at S473 by DNA-PK. (Stronach, Chen et al. 2011). This translocation was observed only in ovarian cancer platinum-resistant cells and not in sensitive cells (Stronach, Chen et al. 2011). Moreover, DNA-PK inhibition was shown to prevent cisplatin-induced phosphorylation of AKT at S473, but not at T308 (Stronach, Chen et al. 2011). Studies using DNA-PKcs-/- mice have demonstrated that DNA-PKcs is required for γIR induced DNA damage mediated activation of AKT but not insulin or growth factor induced activation (Surucu, Bozulic et al. 2008). No increase in blood glucose was detected in DNA-PKcs deficient mice compared

with wild-type controls when treated with either glucose or insulin suggesting that DNA-PKcs is not involved in glucose or insulin induced activation of AKT.

In this chapter it was further investigated if modulation of platinum resistant by DNA-PK and AKT was dependent on activation of PI3K, up-stream of AKT.

In chapter 3 it was also demonstrated that GSK2141795 combined with cisplatin increased apoptotic response to cisplatin in platinum-resistant 2D and 3D ovarian cancer cell models, and also in a platinum-resistant xenograft model. In the cell models examined, the enhanced apoptotic effect of cisplatin and GSK2141795 in combination with cisplatin was found to be synergistic. It was demonstrated in this chapter that inhibition of DNA-PK with NU7441 also restored sensitivity to cisplatin *in vitro* and *in vivo*. The importance of the role of DNA-PK in phosphorylation of AKT at S473 and activation of AKT in response to cisplatin has been already shown by our group and other groups. (Stronach, Chen et al. 2011). In the classical models of activation of AKT, it is believed that activation of PI3K up-stream of AKT is required for activation of AKT through phosphorylation at T308. In this chapter it was investigated if activation of PI3K is required for activation of AKT and DNA-PK in response to cisplatin. Interestingly, it was demonstrated in this chapter that re-sensitisation to cisplatin which was observed by inhibition of AKT or DNA-PK, was not observed when PI3K was inhibited using a potent selective PI3K inhibitor, GSK2126458, in the same cell models. However, inhibition of PI3K using GSK2126458 resulted in reduction in cell proliferation in a dose dependent manner. Inhibition of the AKT pathway by this PI3K inhibitor was confirmed by assessing the level of phosphorylation of the down-stream target, PRAS40. This finding is not in agreement with those of Westfall et al. who reported that inhibition of PI3K/AKT pathway by a potent PI3K inhibitor, LY294002, can sensitise ovarian cancer cells to the toxic effect of carboplatin (Westfall and Skinner 2005). However, it is important to bear in mind that LY294002 doesn't display a great selectivity for PI3K against other members of the

PI3K-related protein kinases and inhibits DNA-PK as well as PI3K (Muller, Blackburn et al. 2007) due to the great similarity between the sequence and structure of the catalytic subunit of PI3K and members of PI3K-related protein kinases.

In addition, siRNA knockdown of *PIK3CA* and *PIK3R1* genes, encoding for catalytic and regulatory subunits of PI3Kα, p110α and p85α respectively, did not sensitise platinum resistant cells to cisplatin. Although PI3K family is a large family of enzymes categorised into three classes and several subunits of PI3K are identified within each class, PI3Kα in particular has emerged as an attractive target for cancer therapeutic due to the high frequency of mutation and over-expression of this enzyme in many types of human cancers. Many PI3K inhibitors including GSK2126458 target the ATP binding site within the catalytic subunit of PI3Kα (McNamara and Degterev 2011). Therefore, in this study catalytic and regulatory subunits of PI3Kα were targeted by siRNA as they seemed to be mostly compatible with the effect of GSK2126458.

In this study it was demonstrated that PI3K knockdown, did not affect the phosphorylation of AKT at S473 in response to cisplatin in platinum resistant cell lines. Moreover, Inhibition of DNA-PK by NU7441 abolished phosphorylation of AKT at S473 in response to cisplatin in PI3K knockdown cells and controls alike, indicating that this phosphorylation is mediated by DNA-PK independently of PI3K.

Together, the findings in this chapter suggest PI3K independence of the role of AKT in the pro-survival response to DNA damage in platinum resistant tumour cells and that PI3K may not be involved in DNA-PK mediated activation of AKT in response to cisplatin treatment in platinum resistant ovarian cancer cells. It was also suggested that inhibition of PI3K may be less effective than inhibition of AKT or DNA-PK on reversal of platinum resistance in ovarian cancer. Previous studies in our group demonstrated that DNA-PK is not involved in

activation of AKT in response to insulin, separating DNA damage mediated effects from other physiological roles of AKT(Stronach, Chen et al. 2011).

Collectively, it can therefore now be hypothesised that AKT becomes activated through different protein signalling cascades dependent on the stimuli. It appears that AKT activation in response to DNA damage is mediated by DNA-PK, in a PI3K independent manner, whereas AKT activation in response to insulin and growth factors is distinct from this mechanism.

6 The effect of AKT inhibition on downstream pathway activation alone or in response to platinum-induced DNA damage

6.1 Introduction

The high rate of failure of drug development in oncology has led to an increased emphasis on establishing a pharmacological audit trail (PHAT) (Yap, Sandhu et al.).

.The emphasis of the PHAT is on testing all steps necessary for drug success systematically and sequentially starting *in vitro* and progressing eventually to the clinic. The purpose of these studies is to validate the proposed mechanism of action of drugs, and in addition to identify PD biomarkers to enable drug-target interaction to be measured, and also to develop predictive markers of activity. The PHAT thereby enables early data driven decisions on the continuation of development of a drug to minimise failures during the clinical phase.

In Chapter 4, AKT and PRAS40 phosphorylation were validated as PD biomarkers of AKT inhibition by GSK2141795. However, these are immediate endpoints of AKT inhibition. Since the downstream AKT pathway and feedback loops are complex and varied, identification of the dominant effecter pathways associated with GSK2141795 associated growth arrest and apoptosis in combination with cisplatin, has the potential to represent response associated PD and predictive biomarkers.

The purpose of studies discussed in this chapter was to identify PD biomarkers that measure the optimal inhibitory effects of GSK2141795 in cell lines, xenograft models and clinical tumour samples. Also to identify predictive biomarkers to be used for selection of patients who are most likely to respond to GSK2141795 treatment. For this purpose, tumour biopsies from ovarian cancer patients participated in a GSK2141795 dose-escalation phase I clinical trial were used. Identification of PD biomarkers would be beneficial for selection of

biologically active doses of GSK2141795 to support moving forward into phase I/II combination studies.

In addition, this chapter is aimed to understand effects of inhibition of AKT alone or in combination with cisplatin on cellular signalling cascades in ovarian cancer cells. This information would contribute towards better understanding of mechanism of platinum resistance in ovarian cancer and signalling cascades leading to reversal of platinum resistance.

In this chapter, the effect of the AKT inhibitor GSK2141795 on protein expression, and signalling cascades was therefore assessed, using Reverse Phase Protein Arrays (RPPA) (Tibes, Qiu et al. 2006). Similarly, the effect of GSK2141795 in combination with cisplatin was also assessed *in vitro* and *in vivo* to validate PD biomarkers identified for GSK2141795 in chapter 4, and also to identify other canonical and non-canonical pathway PD biomarkers.

6.2 Results

6.2.1 Effect of GSK2141795 on signalling cascades in ovarian cancer *in vitro*

SKOV3 monolayers were treated with GSK2141795 (5 μM) for 30 minutes and 8 hours. Thirty minutes and 8 hour time points were chosen to identify early and late response PD biomarkers for GSK2141795 respectively. Protein extracts from cells was analysed for alterations in protein expression using RPPA. Proteins showing less than 1.3 -fold difference between groups were discarded on the basis of unlikely biological relevance (Figure. 6.1 and 6.2).

In SKOV3 monolayers treated with GSK2141795 for 0.5 hour or 8 hours, phosphorylation of AKT at S473 and T308 was increased by 2.1 and 2.9-fold, and PRAS40 decreased by 1.3 and 1.8-fold respectively, consistent with previous data (Figure. 6.1). In addition to AKT, RPPA also revealed a dominant effect on the AKT-mTOR-p70S6K pathway with a consistent reduction(2-fold) in mTOR phosphorylation at pS2448, and the downstream pathway proteins p70S6K at T389 (2.6-fold in 0.5 hours; 2.5-fold in 8hours), S6 phosphorylation at pS235/S236 (4.1-fold in 0.5 hours and 14-fold by 8 hours), S240/S244 (3.7-fold in 0.5 hours and 7.8-fold by 8 hours) and 4EBP1 pS65 (1.3-fold in 0.5 and 8 hours) (Figure. 6.1).

In addition, in SKOV3 samples treated with GSK2141795 for 8 hours down-regulation was also seen in the phosphorylation of GSK-alpha/beta at S21 (4.2-fold) (Figure. 6.1). Interestingly, increase in phosphorylation of AMPK at T172 by 1.4-fold was seen in both groups, suggesting a feedback loop.

Unexpected changes in levels of pYAP(S127), pYB-1 (pS102), pACC (S79), were observed suggesting novel AKT associated pathways or off-target effects of GSK2141795.

To validate findings in SKOV3 cells in another platinum resistant ovarian cancer cell line, PEO4 monolayers were also treated with GSK2141795 for 0.5 hour and 8 hours. AKT-p308 showed the greatest level of up-regulation in PEO4 monolayers treated with GSK2141795 for 8 hours, as seen in SKOV3 monolayers (Figure. 6.2, b). In samples treated with the compound for 0.5 hour, the greatest decreases were seen in phosphorylation of S6 at S235/S236 (3.1-fold) and S240/S244 (3.1-fold), whereas the greatest increase were seen in phosphorylation of AMPK at T172 (1.66-fold) (Figure. 6.2, a) consistent with what seen in SKOV3 monolayers. In PEO4 monolayers treated with GSK2141795 for 8 hours phosphorylation of down-stream target of AKT, GSK-alpha/beta at S21 and S9 was decreased by 2.5 and 2.1-fold respectively. A two-fold reduction in phosphorylation of S6 at S235, down-stream of AKT, was also found in those samples. Findings in PEO4 cells suggest that changes in protein expression observed in SKOV3 monolayers in response to GSK2141795 were not cell line specific events.

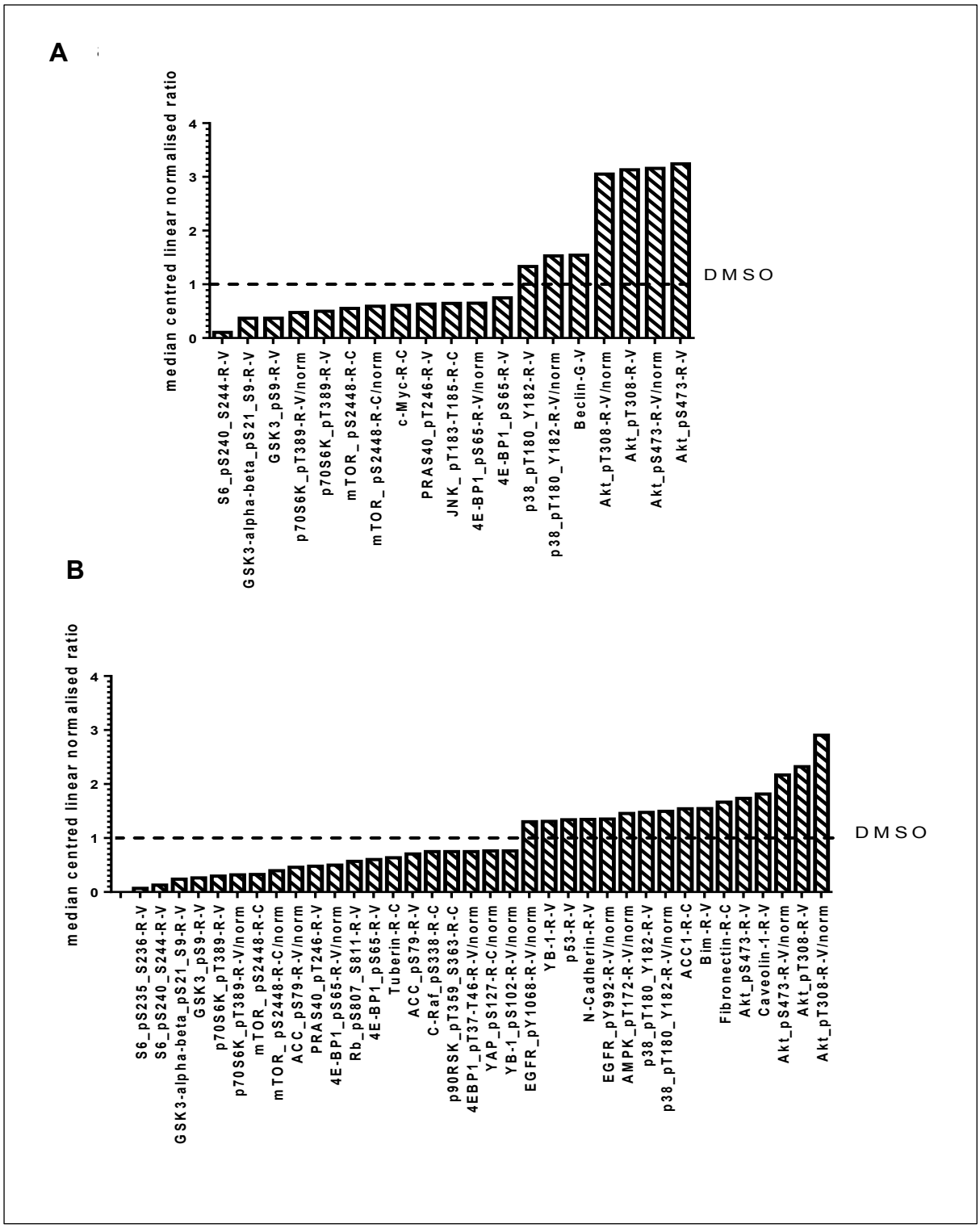


Figure 6-1 Protein expression levels in SKOV3 monolayers treated with GSK2141795 in comparison with vehicle treated samples.

SKOV3 monolayers were treated with 5 μM GSK2141795 for A) 0.5 hour and B) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and GSK2141795 (n=1).

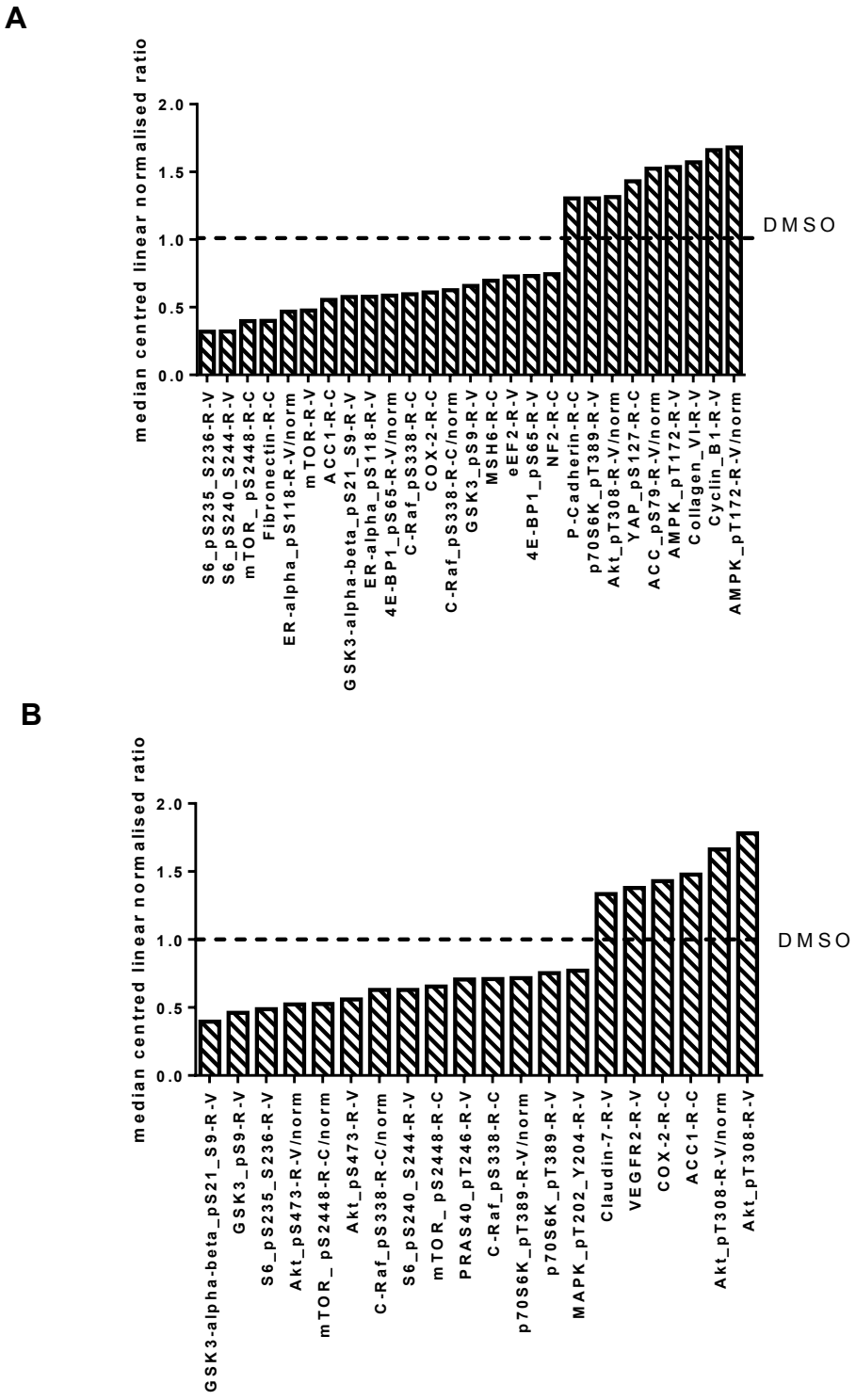


Figure 6-2 Protein expression levels in PEO4 monolayers treated with GSK2141795 in comparison with vehicle samples

PEO4 monolayers were treated with 5 µM GSK2141795 for A) 0.5 hour and B) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and GSK2141795 (n=1).

6.2.2 Effect of treatment with GSK2141795 on protein expression *in vivo*

SKOV3 xenograft tumours treated with GSK2141795 (30 mg/kg) were harvested 5 hours after oral administration of GSK2141795, and protein expression measured by RPPA, was compared following DMSO and GSK2141795 treatments (Figure 6.3). Overall 18 proteins were up-regulated (1.3-fold, t-test $p<0.05$; Table 6.1) and 24 proteins were down-regulated (1.3-fold, t-test $p<0.05$; Table 6.2).

Phospho-AKT308 and pAKTS473 with 5.8 and 4.8-fold increases in expression ($p = 5.3 \times 10^{-5}$ and 6.3×10^{-5} respectively) showed the greatest level of increase in expression in response to single agent GSK2141795, while no significant change in total AKT expression was seen. These findings were consistent with previous *in vitro* data in SKOV3 and PEO4 cells by ELISA and RPPA.

S6-pS235/S236, S6-pS240/S244 and Cyclin-B1 showed the greatest reduction (4-5 fold; p= 0.01, 0.015 and 0.0001 respectively) in expression in tumours treated with GSK2141795 compared to DMSO only. In addition, a 1.3-fold increase in expression of AMPK was seen (Figure 6.3). A 2-fold increase in expression of both p-EGFR, and p38 at T180 were also seen in xenograft samples treated with GSK2141795, suggesting feedback loops. In addition, alteration in expression of extracellular matrix proteins P-cadherin and fibronectin were observed in response to inhibition of AKT, potentially consistent with the role of AKT in cell migration and invasion (Fig 6.3).

GSK2141795 also affected expression of cell cycle proteins *in vivo*. In addition to cyclin B1, a 2-fold decrease in phosphorylation RB (retinoblastoma protein) was observed (Fig 6.3, Table 6.2). Interestingly, a 4-fold decrease in expression of cleaved caspase-7 and 1.3-fold

decrease in expression of cleaved caspase-9, was observed in xenograft samples treated with single agent GSK2141795, indicating a decrease in induction of apoptosis in response to GSK2141795. This may be due to the growth inhibitory effects of this compound as shown by reduction in expression of cell cycle progression proteins in this chapter and also demonstrated by MTT assay and FACS analysis in chapter 3 (Figure 6.3, Table 6.2).

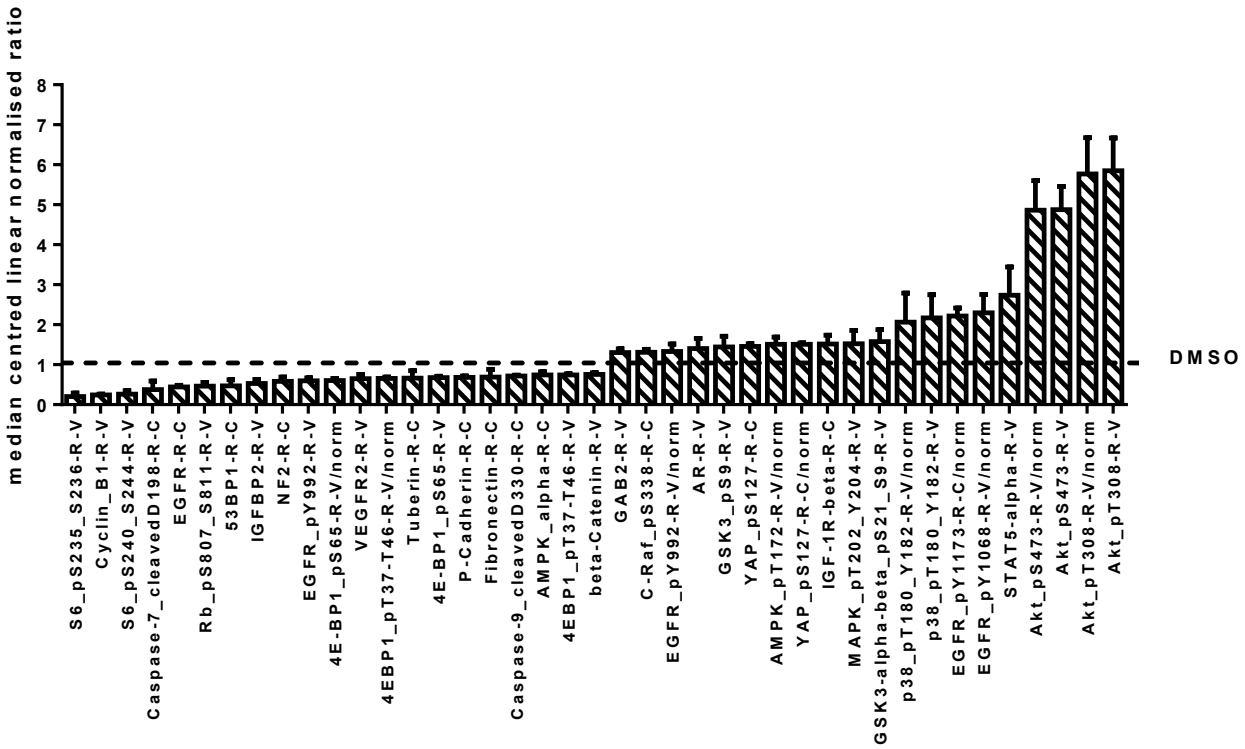


Figure 6-3 Proteins with significantly different levels (1.3-fold, t-test; p<0.05) between DMSO (n=5) and GSK2141795 (n=5) treatment in SKOV3 xenografts

Mice were treated with 30 mg/kg GSK2141795 for 5 hours and differences in level of protein expression between two groups were measured by RPPA analysis. Data shown are the means ± SD of replicates.

Table 6-1 Proteins showing more than 1.3-fold (t-test P<0.05) increase in expression in SKOV3 xenografts following treatment with GSK2141795 compared to DMSO (GSK795/DMSO ratio) treated animals

Up-regulated proteins	GSK795/DMSO Ratio	p-value
Akt_pT308	5.8	0.00002
Akt_pT308/norm	5.7	0.00005
Akt_pS473	4.8	0.00001
Akt_pS473/norm	4.8	0.00006
STAT5-alpha	2.7	0.003
EGFR_pY1068/norm	2.2	0.004
EGFR_pY1173/norm	2.2	0.0003
p38_pT180_Y182	2.1	0.007
p38_pT180_Y182/norm	2.0	0.03
GSK3-alpha-beta_pS21_S9	1.5	0.009
MAPK_pT202_Y204	1.5	0.02
IGF-1R-beta	1.5	0.005
YAP_pS127/norm	1.5	0.00007
AMPK_pT172/norm	1.5	0.001
YAP_pS127	1.4	0.0004
GSK3_pS9	1.4	0.01
AR	1.4	0.02
EGFR_pY992/norm	1.3	0.02
C-Raf_pS338	1.3	0.0003
GAB2	1.3	0.03

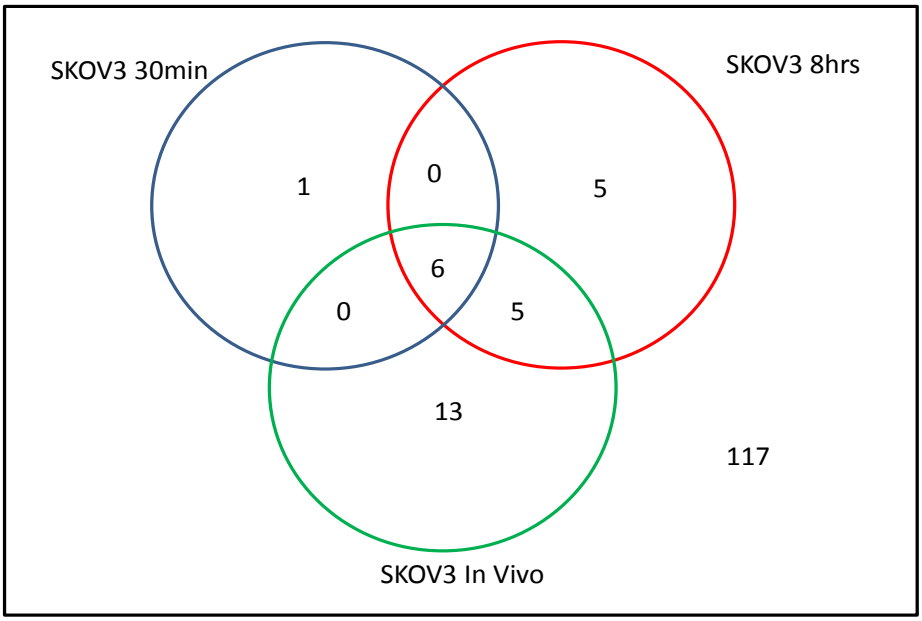
Table 6-2 Proteins showing more than 1.3-fold decrease (t-test P<0.05) in expression in SKOV3 xenografts following treatment with GSK2141795 compared to DMSO treated animals (GSK795/DMSO ratio)

Protein	GSK795/DMSO Ratio	p- value
S6_pS235_S236	0.20	0.01
Cyclin_B1	0.24	0.0001
S6_pS240_S244	0.26	0.01
Caspase-7_cleavedD198	0.37	0.007
EGFR	0.44	0.008
Rb_pS807_S811	0.46	0.002
53BP1	0.47	0.01
IGFBP2	0.52	0.006
NF2	0.58	0.008
EGFR_pY992	0.59	0.02
4E-BP1_pS65/norm	0.60	0.001
VEGFR2	0.65	0.02
4EBP1_pT37-T46/norm	0.65	0.03
Tuberin	0.66	0.03
4E-BP1_pS65	0.67	0.001
P-Cadherin	0.68	0.002
COX-2	0.69	0.1
Fibronectin	0.69	0.05
Caspase-9_cleavedD330	0.71	0.0001
AMPK_alpha	0.74	0.008
4EBP1_pT37-T46	0.74	0.009
beta-Catenin	0.75	0.08

To confirm the identity of the proteins where expression altered in response to GSK2141795 in both *in vitro* and *in vivo* model systems, changes in protein expression in SKOV3 monolayers (both 0.5 and 8 hours) and in xenografts were compared. Six proteins were identified which were up-regulated and 4 proteins which were down-regulated in both time-points in monolayer samples as well as *in vivo* (Figure. 6.4 and Table 6.3). Five proteins were up-regulated and 4 proteins were down-regulated in common in SKOV3 monolayer at 8 hours and in SKOV3 xenografts but not in monolayer samples exposed to GSK2141795 for only 30 minutes (Figure. 6.4 and Table 6.3). Increased phosphorylation of AKT at S473 and T308 and increased phosphorylation of p38 at T180-Y182 was found to be common between *in vivo* SKOV3 xenografts and SKOV3 monolayers regardless of the duration of exposure to GSK2141795.

To identify proteins changing expression in response to treatment with GSK2141795 at early time-points (early response) compared to those affected by the compound at later time points (late response), protein samples extracted at 30 minutes and 8 hours following exposure to GSK2141795 were compared in both SKOV3 and PEO4 cells. Three proteins were up-regulated and 10 proteins were down-regulated in common in both SKOV3 and PEO4 samples exposed to GSK2141795 for 8 hours. In contrast, only 1 protein was found to be up-regulated and 7 to be down-regulated in common in both PEO4 and SKOV3 samples exposed to GSK2141795 only for 30 minute (Figure 6.5, Table 6.4). The comparison of effects in PEO4 cells alone at both 30 min and 8 hour time-points is shown in Figure 6.6 and Table 6.5.

A



B

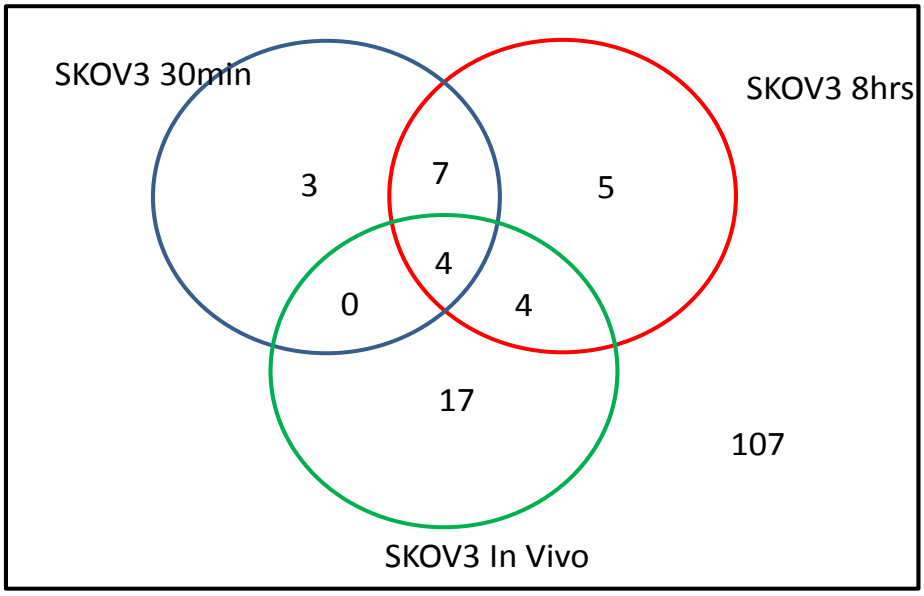


Figure 6-4 Comparison of A) up-regulation and B) down-regulation of 147 proteins in SKOV3 *in vitro* and *in vivo* models

Protein expression was compared in SKOV3 monolayers treated with GSK2141795 for 30 minutes and 8 hours and with SKOV3 xenografts.

Table 6-3 Proteins expression in SKOV3 xenografts in response to treatment with GSK2141795 compared with the same treatment in SKOV3 monolayers in vitro (0.5 and 8 hours) relative to DMSO

Up-regulated proteins	*in vitro* 0.5 hours	*in vitro* 8hours	*in vivo*	Venn group
Akt_pS473	Yes	Yes	Yes	7
Akt_pS473/norm	Yes	Yes	Yes	7
Akt_pT308	Yes	Yes	Yes	7
Akt_pT308/norm	Yes	Yes	Yes	7
p38_pTYes80_Y182	Yes	Yes	Yes	7
p38_pT180_Y182/norm	Yes	Yes	Yes	7
AMPK_pT172/norm	No	Yes	Yes	5
Bim	No	Yes	Yes	5
EGFR_pY1068/norm	No	Yes	Yes	5
EGFR_pY992/norm	No	Yes	Yes	5
YB-1	No	Yes	Yes	5

Down-regulated proteins	*in vitro* 0.5 hours	*in vitro* 8hours	*in vivo*	Venn group
4E-BP1_pS65	Yes	Yes	Yes	7
4E-BP1_pS65/norm	Yes	Yes	Yes	7
S6_pS235_S236	Yes	Yes	Yes	7
S6_pS240_S244	Yes	Yes	Yes	7
GSK3_pS9	Yes	Yes	No	4
GSK3-alpha-beta_pS21_S9	Yes	Yes	No	4
mTOR_ pS2448	Yes	Yes	No	4
mTOR_ pS2448/norm	Yes	Yes	No	4
p70S6K_pT389	Yes	Yes	No	4
p70S6K_pT389/norm	Yes	Yes	No	4
PRAS40_pT246	Yes	Yes	No	4
4EBP1_pT37-T46/norm	No	Yes	Yes	5
ACC_pS79	No	Yes	Yes	5
Rb_pS807_S811	No	Yes	Yes	5
Tuberin	No	Yes	Yes	5

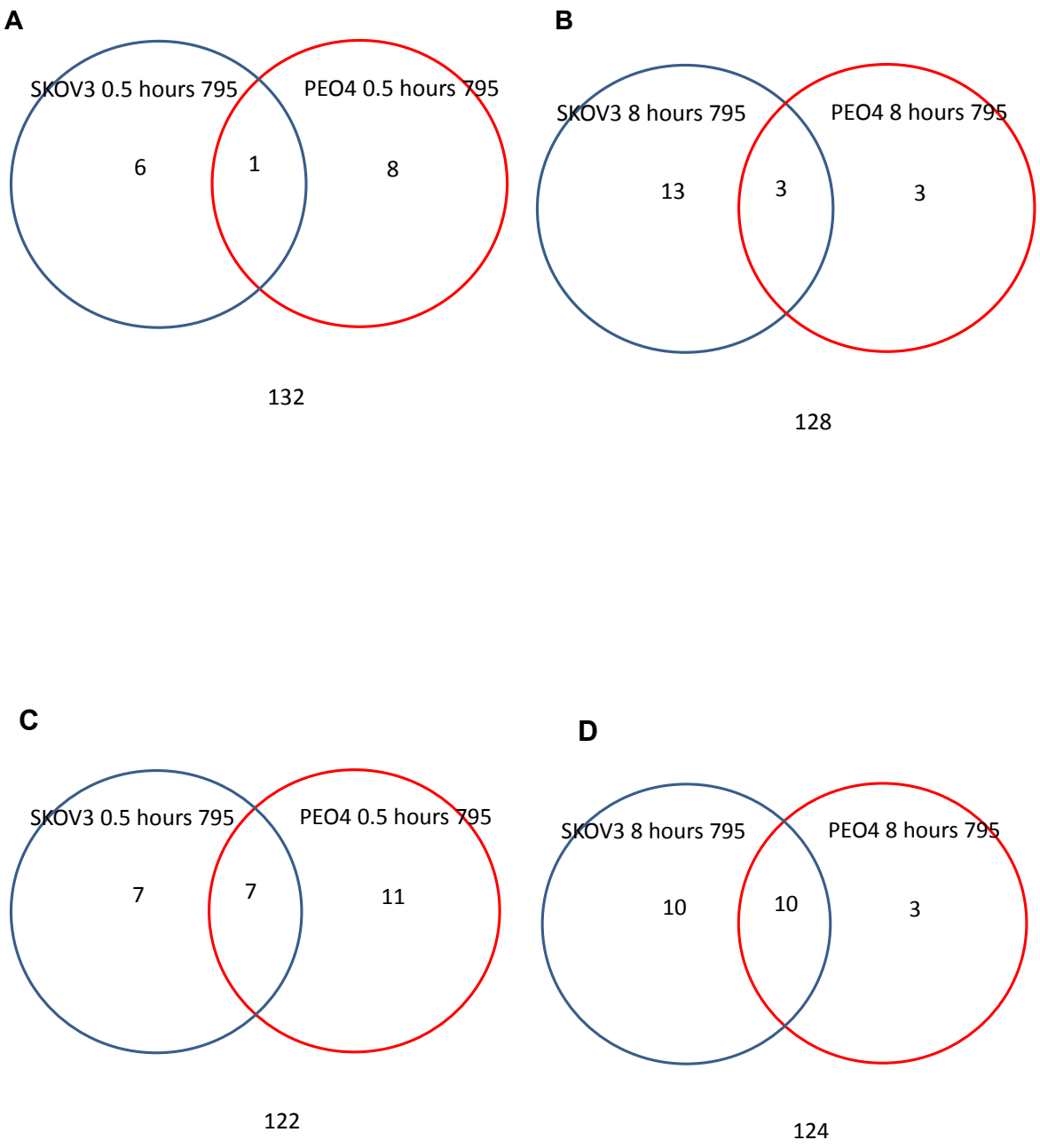


Figure 6-5 Comparison of protein expression in SKOV3 and PEO4 monolyers in response to treatment with GSK2141795

One protein was up-regulated A) and 7 down-regulated C) in both SkOV3 and PEO4 treated with GSK2141795 for 30 minutes. Three proteins were upregulated B) and 10 proteins were down-regulated in both SKOV3 and PEO4 treated with GSK2141795 for 8 hours.

Table 6-4 Proteins which showed alternation in expression in both SKOV3 and PEO4 monolayers treated with GSK2141795.

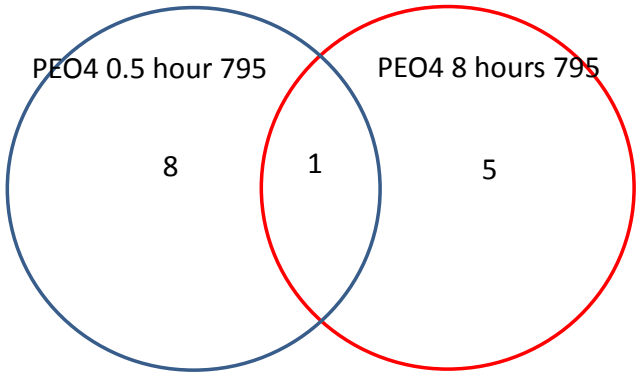
Up-regulated proteins	SKOV3 0.5h	PEO4 0.5h	Venn group
Akt_pT308/norm	Yes	Yes	3

Down-regulated proteins	SKOV3 0.5h	PEO4 0.5h	Venn group
4E-BP1_pS65	Yes	Yes	3
4E-BP1_pS65/norm	Yes	Yes	3
GSK3_pS9-R-V	Yes	Yes	3
GSK3-alpha-beta_pS21_S9	Yes	Yes	3
mTOR_ pS2448	Yes	Yes	3
S6_pS235_S236	Yes	Yes	3
S6_pS240_S244	Yes	Yes	3

Up-regulated proteins	SKOV3 8h	PEO4 8h	Venn group
ACC1-R	Yes	Yes	3
Akt_pT308	Yes	Yes	3
Akt_pT308/norm	Yes	Yes	3

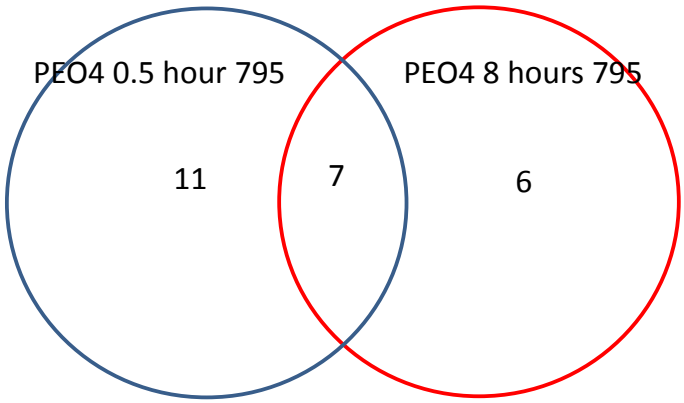
Down-regulated proteins	SKOV3 8h	PEO4 8h	Venn group
C-Raf_pS338	Yes	Yes	3
GSK3_pS9	Yes	Yes	3
GSK3-alpha-beta_pS21_S9	Yes	Yes	3
mTOR_ pS2448	Yes	Yes	3
mTOR_ pS2448/norm	Yes	Yes	3
p70S6K_pT389	Yes	Yes	3
p70S6K_pT389/norm	Yes	Yes	3
PRAS40_pT246	Yes	Yes	3
S6_pS235_S236	Yes	Yes	3
S6_pS240_S244	Yes	Yes	3

A



133

B



123

Figure 6-6 Comparison of protein expression alternation in PEO4 monolayers treated with GSK2141795 for 30 minutes or 8 hours

One protein was up-regulated A) and 7 proteins were down-regulated B) in both time-points.

Table 6-5 Proteins which their expressions altered in response to GSK2141795 in PEO4 monolayers at both 0.5 and 8 hours

Up-regulated proteins	0.5 hours	8 hours	Venn group
Akt_pT308/norm	Yes	Yes	3

Down-regulated proteins	0.5 hours	8 hours	Venn group
C-Raf_pS338	Yes	Yes	3
C-Raf_pS338/norm	Yes	Yes	3
GSK3_pS9	Yes	Yes	3
GSK3-alpha-beta_pS21_S9	Yes	Yes	3
mTOR_ pS2448	Yes	Yes	3
S6_pS235_S236	Yes	Yes	3
S6_pS240_S244	Yes	Yes	3

6.2.3 Effect of treatment with GSK2141795 on protein expression in ovarian cancer patients

In a phase I clinical trial of single agent GSK2141795 in ovarian cancer, 12 patients were treated with GSK2141795 for 4 weeks at a dose of 25-75 mg once daily orally. Tumour biopsies were taken prior to the start of treatment from all patients, and after 4 weeks on GSK2141795 from 10. Due to disease progression and death, week 4 biopsies could not be obtained from 2 patients. Biopsies were examined for differences in level of protein expression by RPPA.

PD biomarkers

Protein expression at week 4 was compared to protein expression in biopsies taken from the same patient prior to the start of treatment (week 0), in the ten evaluable patients, to identify PD biomarkers for GSK2141795. Proteins showing less than 1.3-fold alterations in protein expression in post-treatment samples compared with the pre-treatment samples were excluded. Phosphorylation of AKT at S473 and T308 was significantly increased in all samples treated with GSK2141795 regardless of tumour sub-type and the dose of the drug (6.4 and 4.5-fold respectively) (Table 6.6). This finding was consistent with findings in *in vitro* and *in vivo* ovarian cancer models suggesting that p-AKT is a robust PD biomarker for GSK2141795. In addition, a slight decrease (1.3-fold) in expression of total AKT was also observed (Table 6.6).

In addition, increase in expression of p38-T180 and p70S6K-pT389, in response to GSK2141795, was observed in clinical samples, consistent with observations *in vitro* and *in vivo*. However, these alterations were not significant in clinical samples despite significant

changes for the same proteins observed *in vitro* and *in vivo*. This could be due to the small sample size and inter-tumour heterogeneity in tumour biopsies.

In addition, tumour biopsies from the 10 patients treated with GSK2141795 for 4 weeks were classified into three different sub-groups based on their clinical diagnosis; serous (6 samples), clear cell (2 samples) and endometrioid (2 samples), and protein expression compared. Proteins showing less than 1.3-fold alternation in protein expression in post-treatment samples compared with the pre-treatment samples were excluded for further analysis (see Appendix Figures A.1, A.2 and A.3 and Table A.1).

Phosphorylation of AKT at both phosphorylation sites S473 and T308 was greatly increased in all three tumour sub-types as was seen *in vitro* and *in vivo.*

Expression of the epithelial tight junction protein, Claudin 7 and the extracellular matrix protein E-cadherin in serous samples (1.4-fold), expression of VEGFR , up-stream of AKT and the down-stream target of AKT, phospho-PRAS40 in clear cell samples (1.4-fold) and expression of the extracellular matrix protein cadherin-P in endometrioid samples (3-fold) showed the most down-regulations (Figures A.1, A.2. A.3). Altogether, AKT-pS473, AKT-pT308, Caveolin-1,HER2-pY1248 and p38-pT180/Y182 were found to be up-regulated in all three tumour types. Up-regulation of AKT-pT08, AKT-S473 and p38-pT180/Y182 was consistent with what was observed earlier in *in vitro* monolayers and *in vivo* xenografts. However, except alterations in expression of AKT and pAKT ($p<0.05$), other alterations in protein expression were not found to be significant due to the low number of samples and tumour heterogeneity.

Response biomarkers

Ovarian cancer patients were treated with daily GSK2141795 for 4 weeks and pre and post-treatment tumour biopsies were used for determination of response biomarkers. Response to the treatment was evaluated by measuring of the CA125 levels in the pre and post-treatment blood samples. CA125 is a biomarker of ovarian cancer. Elevation of CA125 level correlates with poor response to treatment whereas a fall in CA125 level has been associated with response to treatment. Prior to the analysis, tumour biopsies were divided into two groups, according to the changes in the level of expression of CA125 in response to treatment with GSK2141795. Patients showing more than 30% drop in the level of expression of CA125 were grouped as responders and the rest as non-responders. Protein expression profiles were determined using RPPA. Protein expression profiles in pre-treatment and post-treatment samples taken from each patient were compared to determine expression alterations in response to the treatment in each patient. Alterations in protein expression 4 weeks after the initiation of the daily treatment with GSK2141795 were relative to levels prior to the start of treatment, were compared between responders (n=5) and non-responders (n=5). Expression of RAF-C pS338, ER-alpha pS118 and EGFR pY1173 were significantly increased, and the expression levels of Cyclin B1 and SRC pY416 significantly decreased in responders compared to non-responders (Table 6.7). Increase in AKT phosphorylation in response to GSK2141795 was observed in both responders and non-responders groups (Figure 6.7).

Table 6-6 PD Biomarkers: Proteins showed more than 1.3-fold alteration in expression in response to treatment with GSK2141795 in ovarian cancer tumours

.

Protein	Ratio wk4/wk0	p-value
AKT_pS473/norm	**6.4**	**0.02**
AKT_pS473	**4.7**	**0.01**
AKT_pT308/norm	**4.5**	**0.01**
AKT_pT308	**3.2**	**0.007**
Rad50	2.2	0.2
HER2_pY1248	1.9	0.1
Caveolin-1	1.9	0.2
Era	1.7	0.4
CHK2_pT68/norm	1.6	0.3
p38_T180_182/norm	1.6	0.4
Tau	1.5	0.2
HER2_pY1248	1.5	0.2
p70S6K_pT389/norm	1.5	0.4
p70S6K_pT389/norm	1.4	0.6
Raf-C_pS338/norm	1.3	0.4
ATM_	1.3	0.8
p70S6K_pT389/norm	1.3	0.7
Raf-C__pS338/norm	1.3	0.3
p27_pT198/norm	1.3	0.3
PR	1.3	0.7
MAPK_pT202_	1.3	0.3
EGFR	1.3	0.1
FAK	1.3	0.4
p27_pT198	1.3	0.3
Cox-2	1.3	0.1
Notch-1	1.3	0.2
MAPK__pT202_204	1.3	0.2
EGFR	1.3	0.1
Xiap	1.3	0.2
eEF2K	1.3	0.3
AKT	**0.7**	**0.01**
Cadherin	0.7	0.2

Table 6-7 Response-biomarkers: significant response-dependent protein-change (more than 1.3-fold) as a result of treatment with GSK2141795

Protein	Ratio	p-value
Raf.C_.pS338./norm	1.7	0.04
ERa_pS118.V/norm	1.6	0.03
ERa_pS118/norm	1.6	0.04
EGFR.C.	1.4	0.05
EGFR_pY1173	1.3	0.02
TAZ_pS89	1.3	0.007
YB1.V	1.3	0.03
Snail.C	1.3	0.01
PKca	0.7	0.04
P27	0.7	0.04
YB1_pS102./norm	0.7	0.03
ChK2	0.7	0.04
Src_pY416/norm	0.7	0.03
Src_pY416	0.7	0
Cyclin.B1	0.7	0.001
Rb-pS809	0.6	0.05
YAP-pS127	0.5	0.04

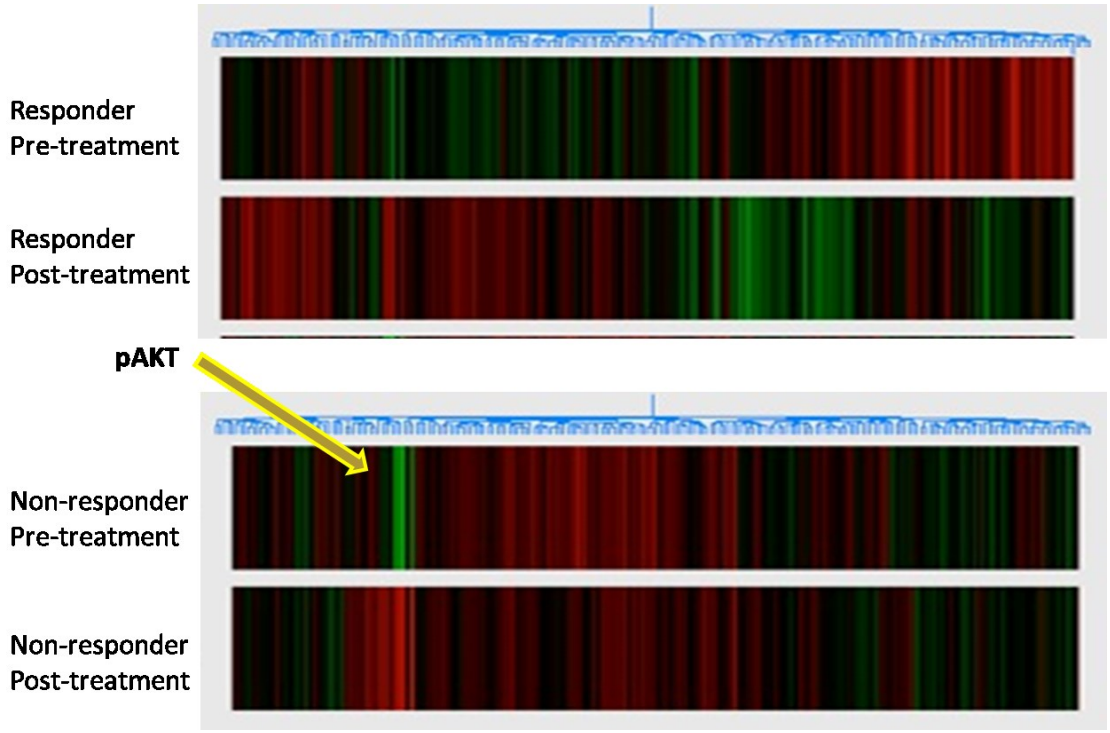


Figure 6-7 Phosphorylation of AKT (S473 and T308) was increased in response to treatment with GSK2141795 in both responder and not-responder groups

Ovarian cancer patients were treated with GSK2141795 daily for 4 weeks. Patients were divided into two groups according to the changes in the level of expression of CA125 in response to treatment with GSK2141795. Patients showing more than 30% drop in the level of expression of CA125 were grouped as responders and the rest as non-responders. Protein expression levels in pre-treatment and post-treatment tumour biopsies taken from patients were analysed using RPPA. AKT phosphorylation level was increased in both groups in response to the treatment. Down-regulation is indicated by green whereas up-regulation is indicated by red.

Predictive biomarkers

To find biomarkers which can be used to predict response to AKT inhibition by GSK2141795, protein expression in tumour biopsies taken just before the start of the treatment with GSK2141795 was compared between responders and non-responder groups. Expression of pMAPK (T202) was found to be significantly lower in responders compared to non-responders, suggesting that pMAPK (T202) may represent a predictive biomarker for GSK2141795 activity (Figure 6.8). Additional data for all assessed proteins are shown in Table A.15.

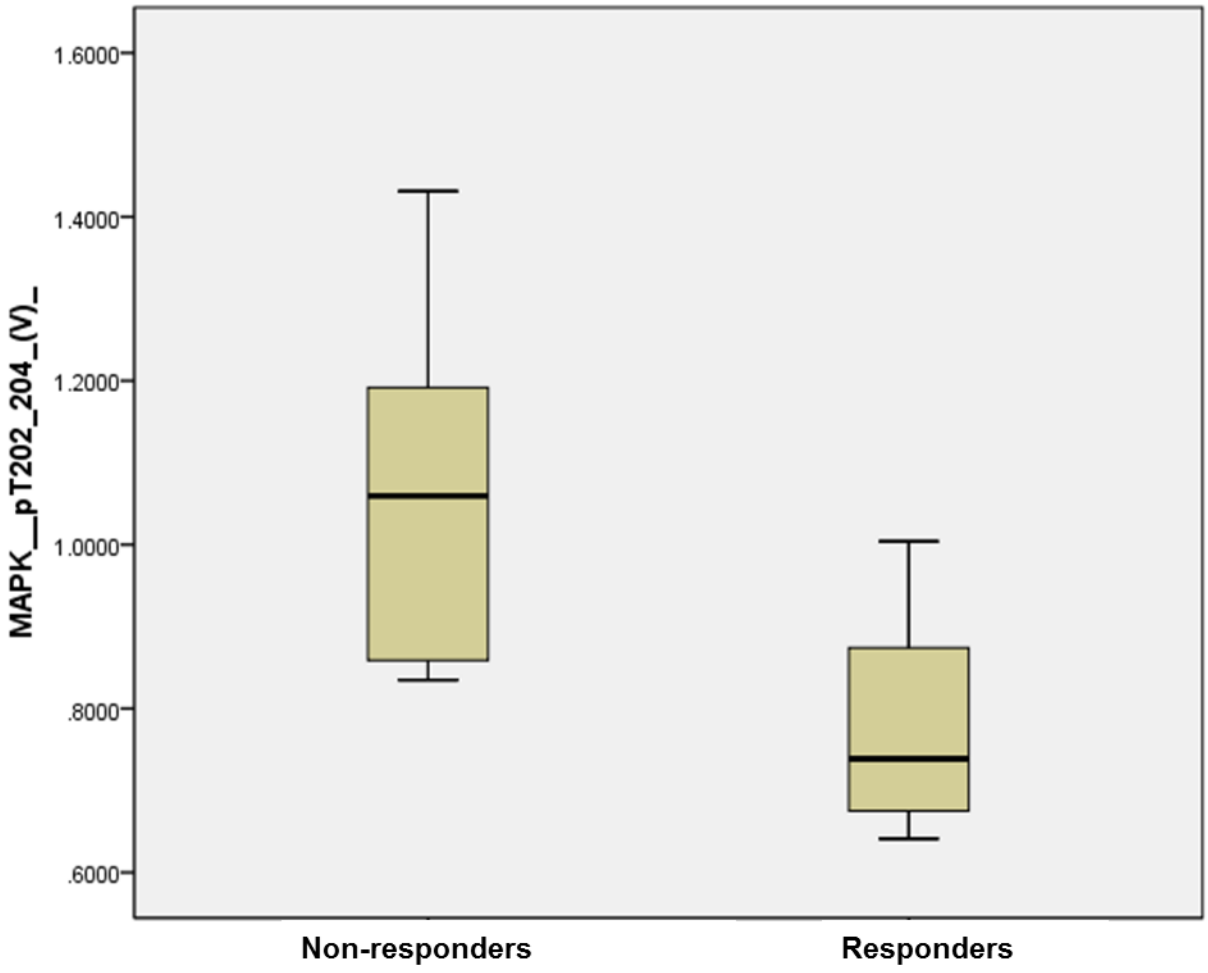


Figure 6-8 Expression of pMAPK (T202) in responders and non-responders

Protein expression profiles of tumour biopsies taken from patients prior to the treatment with GSK2141795 were compared between responders and non-responders. Expression of pMAPK (pT202) was found to be significantly lower (1.4-fold) in responders compared to non-responders before the initiation of treatment with GSK2141795 suggesting pMAPK (T202) can be used as a predictive biomarker for response to GSK2141795. The data shows mean of n=5 samples ± SD, p = 0.02

6.2.4 Effect of treatment with cisplatin on signalling cascades

In Vitro

The effect of treatment with cisplatin on protein signalling cascades in SKOV3 and PEO4 monolayers for 30 minutes or 8 hours was examined (see Appendix). Only 3 proteins were up-regulated and 2 proteins were down-regulated in SKOV3 cells treated with cisplatin for 30 minutes (Figure A.4A). After 8 hours 5 proteins were up-regulated and 3 proteins were down-regulated in SKOV3 monolayers (Figure A.4B). More proteins showed alternation in expression in response to cisplatin in PEO4 monolayers. Eight proteins were up-regulated and 7 down-regulated in PEO4 monolayers treated with cisplatin for 30 minutes (Figure. A.5.A). After 8 hours 5 proteins were up-regulated and 12 proteins down-regulated in PEO4 cells (Figure. A.5B).

In Vivo

To validate these effects *in vivo*, protein expression was analysed in platinum-resistant SKOV3 tumour xenografts treated with 1.5 mg/kg cisplatin and compared to DMSO vehicle treatment, by RPPA (Figure 6.9). Overall, 29 proteins were down-regulated (Table 6.9), and 25 proteins up-regulated (Table 6.8).

Up-regulation of ER-alpha-pS118 was common between PEO4 monolayers and SKOV3 monolayers and xenografts (Figure A.4, A.5. 6.10). AMPK-pT172 was up-regulated in both SKOV3 monolayers and xenografts, but not PEO4 cells (Figure A.4. 6.10). ER-alpha(total) and AMPK(total) were found to be down-regulated in SKOV3 xenografts (Figure 6.9).

Phosphorylation of EGFR at Y1173 (2.3-fold) and MEK1 at pS217 were increased (2.6 and 1.3-fold respectively) suggesting activation of EGFR and MEK survival pathways in response to cisplatin in SKOV3 xenografts. The anti-apoptotic protein Bcl-2 was up-regulated, and consistent with this, a 2-fold decrease in expression of cleaved caspase-7 was observed. Down-regulation of p27 phosphorylation at T157 (1.3-fold) was found in cisplatin treated SKOV3 xenografts, suggesting increase in cell cycle progression at G1 but on the other hand, reduced expression of cyclin B1 in the same samples, suggested decrease in the proportion of cells in S-phase (Figure 6.9, Table 6.8, 6.9).

Importantly, a four-fold decrease was found in expression of S6, down-stream of mTOR, in SKOV3 xenografts, suggesting down-regulation of AKT-mTOR signalling and decline in protein synthesis in response to cisplatin (Figure 6.8, Table 6.9).

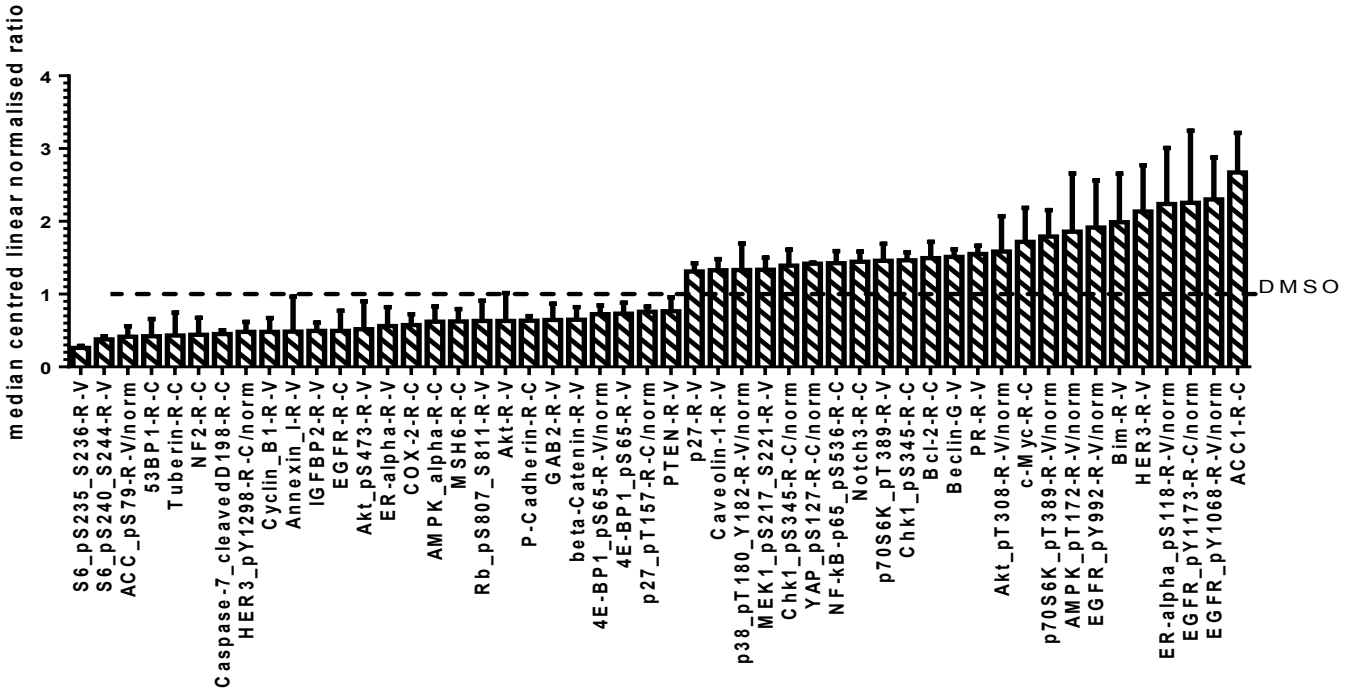


Figure 6-9 Proteins with significantly different levels (t-test; p<0.05) between DMSO and cisplatin treatment in SKOV3 xenografts

SKOV3 xenografts were treated with 1.5 mg/kg cisplatin for 3 hours and differences in level of protein expression between two groups were measured by RPPA analysis. The graphs indicates proteins showing changes more than 1.3-fold in expression. The data shows mean of n=4 samples ± SD.

Table 6-8 Proteins showing more than 1.3-fold increase in expression in SKOV3 xenografts following treatment with cisplatin (CDDP) compared with DMSO treated animals (CDDP/DMSO ratio)

Up-regulated Proteins	CDDP/DMSO Ratio	p-value
ACC1	2.6	0.02
EGFR_pY1068/norm	2.3	0.01
EGFR_pY1173/norm	2.2	0.05
ER-alpha_pS118/norm	2.2	0.02
HER3	2.1	0.02
Bim	1.9	0.05
EGFR_pY992/norm	1.9	0.03
AMPK_pT172/norm	1.8	0.07
p70S6K_pT389/norm	1.7	0.01
c-Myc	1.7	0.02
Akt_pT308/norm	1.5	0.09
PR	1.5	0.003
Beclin	1.5	0.001
Bcl-2	1.4	0.01
Chk1_pS345	1.4	0.001
STAT5-alpha	1.4	0.1
p70S6K_pT389	1.4	0.02
Notch3	1.4	0.001
NF-kB-p65_pS536	1.4	0.009
YAP_pS127/norm	1.4	0.0009
Chk1_pS345/norm	1.3	0.02
p38_pT180_Y182	1.3	0.19
MEK1_pS217_S221	1.3	0.01
p38_pT180_Y182/norm	1.3	0.17
Caveolin-1	1.3	0.06
p27	1.3	0.01

Table 6-9 Proteins showing more than 1.3-fold decrease in expression in SKOV3 xenografts following treatment with cisplatin compared with DMSO treated animals.

Down-regulated proteins	CDDP/DMSO Ratio	p-value
S6_pS235_S236	0.26	0.03
S6_pS240_S244	0.37	0.06
ACC_pS79/norm	0.41	0.03
53BP1	0.42	0.03
Tuberin	0.43	0.02
NF2	0.44	0.01
Caspase-7_cleavedD198	0.45	0.01
HER3_pY1298/norm	0.48	0.02
Cyclin_B1	0.48	0.01
Annexin_I	0.48	0.08
IGFBP2	0.49	0.01
EGFR	0.49	0.06
p21	0.50	0.17
Akt_pS473	0.51	0.07
ER-alpha	0.56	0.03
COX-2	0.57	0.09
AMPK_alpha	0.62	0.02
MSH6	0.62	0.01
Rb_pS807_S811	0.63	0.08
Akt	0.63	0.15
P-Cadherin	0.63	0.005
GAB2	0.64	0.08
beta-Catenin	0.64	0.07
VEGFR2	0.68	0.11
eEF2	0.69	0.26
4E-BP1_pS65/norm	0.72	0.03
4E-BP1_pS65	0.73	0.04
p27_pT157/norm	0.75	0.03
PTEN	0.76	0.11
Akt_pS473/norm	0.77	0.25

6.2.5 Effect of combination of GSK2141795 and cisplatin on protein signalling cascades in SKOV3 and PEO4 monolayers and xenograft models

SKOV3 xenografts were treated with a combination of 30 mg/kg GSK2141795 and 1.5 mg/kg cisplatin and protein expression data measured by RPPA (Figure 6.10). The highest increase in phosphorylation was seen for AKT at both phosphorylation sites T308 and S473 (4.9 and 4.3-fold respectively) (Table 6.10). In contrast, the greatest decrease in phosphorylation was seen for S6 at both phosphorylation sites S236 and S244 (4 and 3-fold decrease respectively) (Table. 6.11) as was seen in xenografts treated with single agent GSK2141795.

In addition SKOV3 and PEO4 monolayers were treated with combination of 5 μM GSK2141795 and 25 μM cisplatin for 30 minutes or 8 hours (Figures A.8 and A9).

In SKOV3 monolayers, phosphorylation of AKT at both phosphorylation sites T308 and S473 was dramatically increased at both 30 minutes and 8 hours time points as it was seen in SKOV3 xenografts. GSK3 and P70S6K showed great decreases in both time points whereas decrease in phosphorylation of S6 was only observed in cells treated with the combination treatment for 8 hours. These findings showed down-regulation in AKT signalling pathway as a result of the combination treatment. In general, increase in phosphorylation of AKT at both S473 and T308 and decrease in expression of down-stream effectors of AKT including pS6, pGSK3, pmTOR, PRAS40 and 4EBP1 was observed in PEO4 monolayers and SKOV3 monolayers and xenografts, treated with the combination of GSK2141795 and cisplatin, showing the efficacy of GSK2141795 in the combination treatment. (Figures. 6.10, A.8, A9).

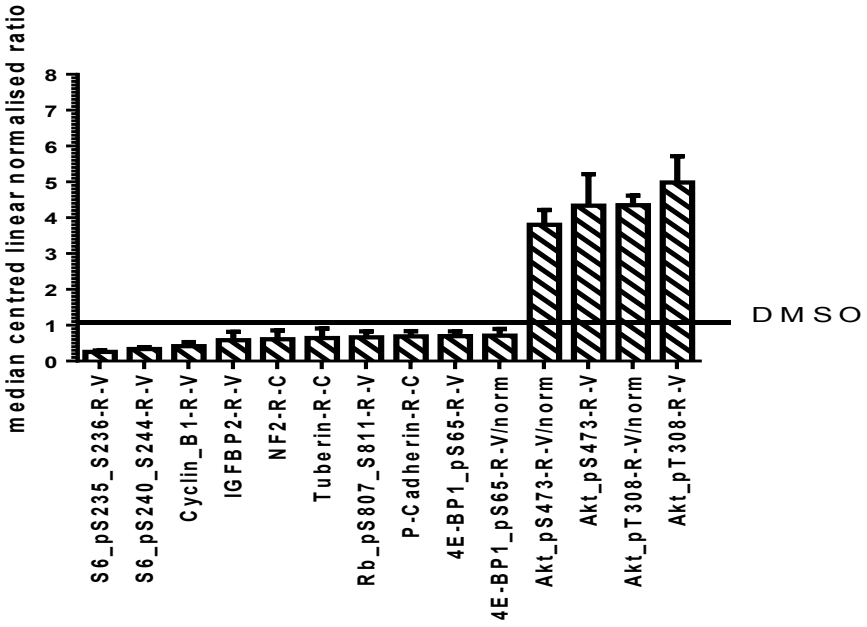


Figure 6-10 Proteins with significantly different levels (t-test; p<0.05) between DMSO and combination of GSK2141795 and cisplatin treatment in SKOV3 xenografts

SKOV3 xenografts were treated with combination of 1.5 mg/kg cisplatin and 30 mg/kg GSK2141795. Differences in level of protein expression between two groups were measured by RPPA analysis. The graphs indicates proteins showing significant changes in expression between DMSO and combination groups (n=4).

Table 6-10 Proteins showing more than 1.3-fold increase in expression in SKOV3 xenografts following treatment with combination of cisplatin and GSK2141795 compared with DMSO treated animals.

Up-regulated Proteins	Ratio	p-value
Akt_pT308	4.9	0.00003
Akt_pT308/norm	4.3	0.000002
Akt_pS473	4.3	0.0003
Akt_pS473/norm	3.8	0.00002
Bim-R-V	2.0	0.22
EGFR_pY1173/norm	1.8	0.24
STAT5-alpha	1.7	0.1
EGFR_pY1068/norm	1.7	0.08
AMPK_pT172/norm	1.7	0.2
ER-alpha_pS118/norm	1.6	0.3
EGFR_pY992/norm	1.4	0.2
Caveolin-1	1.4	0.2
Caspase-7_cleavedD198	1.3	0.05
Collagen_VI	1.3	0.1
Beclin	1.3	0.06
YAP_pS127/norm	1.3	0.06

Table 6-11 Proteins showing more than 1.3-fold decrease in expression in SKOV3 xenografts following treatment with combination of cisplatin and GSK2141795 compared with DMSO treated animals.

Down-regulated Proteins	Ratio	p-value
S6_pS235_S236	0.25	0.01
S6_pS240_S244	0.33	0.02
Cyclin_B1	0.41	0.001
IGFBP2	0.58	0.03
NF2	0.61	0.04
Tuberin	0.64	0.05
53BP1	0.64	0.1
EGFR	0.65	0.1
Rb_pS807_S811	0.66	0.03
COX-2	0.67	0.1
P-Cadherin	0.69	0.01
4E-BP1_pS65	0.69	0.01
4E-BP1_pS65/norm	0.71	0.04
Fibronectin	0.74	0.1
beta-Catenin	0.76	0.2
eEF2	0.76	0.3
ER-alpha	0.76	0.2
MSH6	0.76	0.1

Majority of proteins expression alterations found in samples treated with combination of GSK2141795 and cisplatin were common with the ones found in samples treated with either single agents. Therefore, to identify proteins alterations only specific to the combination treatment, up-regulation and down-regulation of protein expression levels in response to cisplatin and GSK2141795 and combination of two were compared in SKOV3 xenografts and SKOV3 and PEO4 monolayers. The purpose of this analysis was to identify proteins involved in the mechanism leading to sensitisation to cisplatin, as shown in chapter 3 as phenotypic effect of the combination treatment *in vitro* and *in vivo* and also to identify biomarkers for the combination treatment.

Cleaved caspase-7 which was found to be down-regulated In SKOV3 xenografts treated with cisplatin only, but up-regulated in xenografts treated with the combination of cisplatin and GSK2141795 (1.3-fold, p=0.05), consistent with the increase in apoptosis in response to the combination treatment (Table 6.12). In addition, MSH6 was found to be down-regulated in xenografts treated with the combination treatment, suggesting decrease in DNA mismatch repair when cisplatin is combined with GSK2141795. However, down-regulation of MSH6 was not found to be statistically significant (Table 6.13). Up-regulation of cellular membrane protein, caveolin-1 (1.4-fold) and collagen-VI (1.3-fold) were found to be up-regulated in response to the combination treatment in xenografts, suggesting involvement of the extra-cellular matrix in cell senstitisation to cisplatin. However, these up-regulations were not statistically significant. In addition, four proteins were found to be up-regulated and 5 to be down-regulated only in response to cisplatin not in combination with GSK2141795 (Figure 6.11, Table 6.13, 6.14) and 11 proteins to be up-regulated and 7 to be down-regulated only in response to GSK2141795 not in combination with cisplatin (Figure 6.11, Table 6.12, 6.13).

In SKOV3 monolayers treated with the combination treatment for 30 minutes, six proteins were found to be up-regulated and 5 proteins were found to be down-regulated only in response to the combination treatment but not in response to cisplatin or GSK2141795 alone .Three proteins were identified to be up-regulated and 1 protein to be down-regulated only in cisplatin treated cells and 1 protein was found to be up-regulated and two proteins were down-regulated only in GSK2141795 treated cells not in the combination group (Figure. A13, Table. A.9).

In SKOV3 monolayers treated with the combination of GSK2141795 and cisplatin for 8 hours, 4 proteins were found to be up-regulated and 8 proteins to be down-regulated only in response to the combination treatment but not in GSK2141795 and cisplatin only treated cells. No protein was found to be up-regulated and only one to be down-regulated only in cisplatin treated cells and five to be up-regulated and two proteins to be down-regulated only in cells treated with GSK2141795 (Figure. A.14, Table A.10).

Interestingly, the Yes Associated Protein (YAP) was found to be down-regulated in SKOV3 monolayers treated with combination of GSK2141795 and cisplatin but not in samples treated with single agent GSK2141795 or cisplatin suggesting the Hippo signalling pathway may be involved in sensitisation of SKOV3 cells to platinum (Table A.11).

In PEO4 monolayers treated with GSK2141795 or cisplatin or combination of both for 30 minutes, 8 proteins were found to be up-regulated and 6 to be down-regulated only in response to the combination treatment and two proteins to be up-regulated and two proteins to be down-regulated in response to cisplatin alone and one protein to be up-regulated and

three proteins to be down-regulated only in response to GSK2141795 not in the combination treatment (Figure. A.15, Tables.A.12, A.13).

In PEO4 monolayers treated with GSK2141795 or cisplatin or combination of both for 8 hours, five proteins were found to be up-regulated and seven proteins to be down-regulated only in response to the combination treatment not in the GSK2141795 and cisplatin only treated cells (Figure. A.16, Table A.14).

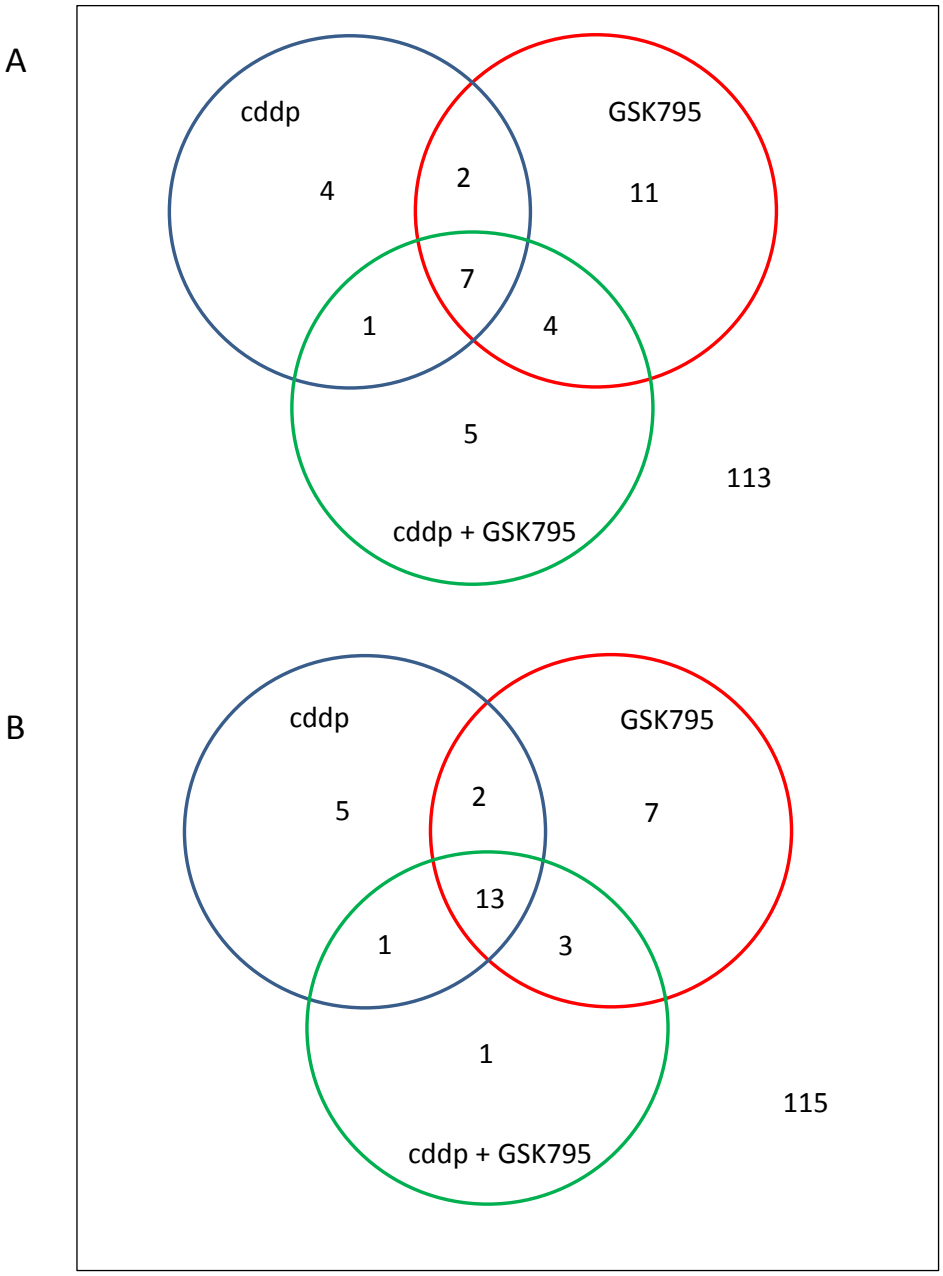


Figure 6-11 Comparison of changes in protein expression in response to cddp or GSK2141795 as single agents or in combination in SKOV3 xenografts

SKOV3 xenografts were treated with cddp (3 hours) or GSK2141795 (5 hours) or combination of two drugs (2 hours pre-treatment with GSK2141795 followed by 3 hours treatment with cddp). Up-regulations (A) or down-regulations (B) of protein expression in response to the three treatments were compared in Venn diagrams.

Table 6-12 Comparison of up-regulations in protein expression in response to cddp and GSK2141795 as single agents or in combination in SKOV3 xenografts

Protein	skov_vivo_cddp	skov_vivo_795	skov_vivo_cddp_795	Venn group
AMPK_pT172/norm	Yes	Yes	Yes	7
Bim	Yes	Yes	Yes	7
EGFR_pYYes068	Yes	Yes	Yes	7
EGFR_pY1068/norm	Yes	Yes	Yes	7
HER3	Yes	Yes	Yes	7
STAT5-alpha	Yes	Yes	Yes	7
YAP_pS127/norm	Yes	Yes	Yes	7
EGFR_pY1173/norm	Yes	Yes	No	4
EGFR_pY992/norm	Yes	Yes	No	4
Beclin	Yes	No	Yes	6
ACC1	Yes	No	No	1
c-Myc	Yes	No	No	1
ER-alpha_pS118/norm	Yes	No	No	1
p70S6K_pT389/norm	Yes	No	No	1
Akt_pS473	No	Yes	Yes	5
Akt_pS473/norm	No	Yes	Yes	5
Akt_pT308	No	Yes	Yes	5
Akt_pT308/norm	No	Yes	Yes	5
AR-R	No	Yes	No	2
C-Raf_pS338	No	Yes	No	2
GAB2	No	Yes	No	2
GSK3_pS9	No	Yes	No	2
GSK3-alpha-beta_pS21_S9	No	Yes	No	2
IGF-1R-beta	No	Yes	No	2
MAPK_pT202_Y204	No	Yes	No	2
p38_pT180_Y182	No	Yes	No	2
p38_pT180_Y182/norm	No	Yes	No	2
YAP_pS127	No	Yes	No	2
YB-1	No	Yes	No	2
Bcl-X	No	No	Yes	3
Caspase-7_cleavedD198	No	No	Yes	3
Caveolin-1	No	No	Yes	3
Collagen_VI	No	No	Yes	3
Src_pY527	No	No	Yes	3

Table 6-13 Comparison of down-regulations in protein expression in response to cddp and GSK2141795 as single agents or in combination in SKOV3 xenografts

Protein	skov_vivo_cddp	skov_vivo_795	skov_vivo_cddp+795	Venn group
53BP1	Yes	Yes	Yes	7
beta-Catenin	Yes	Yes	Yes	7
COX-2	Yes	Yes	Yes	7
Cyclin_B1	Yes	Yes	Yes	7
eEF2	Yes	Yes	Yes	7
EGFR	Yes	Yes	Yes	7
IGFBP2	Yes	Yes	Yes	7
NF2	Yes	Yes	Yes	7
P-Cadherin	Yes	Yes	Yes	7
Rb_pS807_S811	Yes	Yes	Yes	7
S6_pS235_S236	Yes	Yes	Yes	7
S6_pS240_S244	Yes	Yes	Yes	7
Tuberin	Yes	Yes	Yes	7
AMPK_alpha	Yes	Yes	No	4
Caspase-7_cleavedD198	Yes	Yes	No	4
ER-alpha	Yes	No	Yes	6
ACC_pS79/norm	Yes	No	No	1
Akt_pS473	Yes	No	No	1
Annexin_I	Yes	No	No	1
HER3_pY1298/norm	Yes	No	No	1
p21	Yes	No	No	1
4E-BPYes_pS65	No	Yes	Yes	5
4E-BP1_pS65/norm	No	Yes	Yes	5
Fibronectin	No	Yes	Yes	5
4EBP1_pT37-T46	No	Yes	No	2
4EBP1_pT37-T46/norm	No	Yes	No	2
ACC_pS79	No	Yes	No	2
ACC1	No	Yes	No	2
Caspase-9_cleavedD330	No	Yes	No	2
EGFR_pY992	No	Yes	No	2
VEGFR2	No	Yes	No	2
MSH6	No	No	Yes	3

6.2.6 Validation of RPPA method using ELISA

To validate the results of the RPPA analysis, showing increase in AKT phosphorylation at T308 and S473, the effect of treatment with GSK2141795 with or without cisplatin on the phosphorylation of AKT at S473 and T308 phosphorylation sites in xenograft tumours were assessed using ELISA. SKOV3 tumour bearing mice were treated with 30 mg/kg GSK2141795 as a single agent or in combination with 1.5 mg/kg cisplatin for 5 hours. Treatment with GSK2141795 resulted in an increase in phosphorylation of AKT at both phosphorylation sites, S473 and T308 compared to the untreated or only cisplatin treated animals (Figure 6.12), consistent with previous data using RPPA, validating the previous results and the RPPA method for PD discovery.

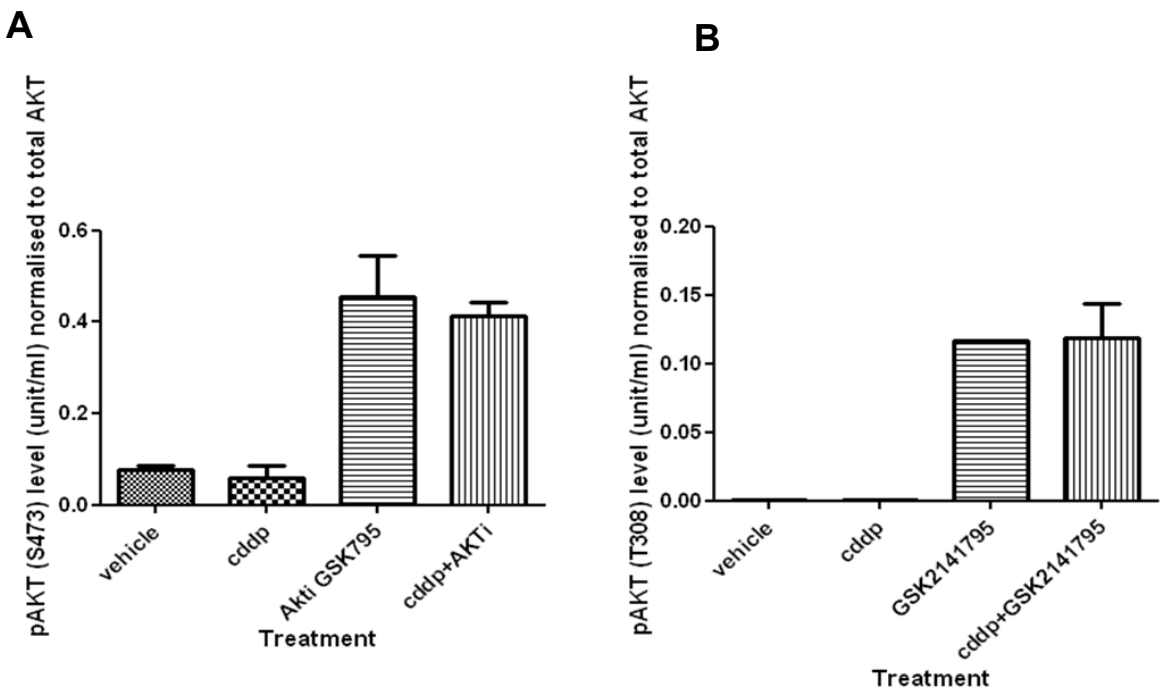


Figure 6-12 Effect of treatment with GSK2141795 as single agent or in combination with cddp on the level of phosphorylation of AKT at S473 (A) and T308 (B) in SKOV3 xenograft mouse models

SKOV3 tumour bearing mice were treated with vehicle, or cisplatin (1.5 mg.kg) or 30 mg/kg GSK2141795 or combination of GSK2141795 and cisplatin for 5 hours. Level of AKT phosphorylation at T308 and S473 in protein lysates extracted from tumours was determined using ELISA. Treatment with GSK2141795 resulted in an increase in the phosphorylation of AKT at both phosphorylation sites, S473 and T308. Data are the means ± SEM of n = 3

6.3 Discussion

One of the main goals in oncology drug development is to improve the specificity and efficacy of targeted therapy, through the identification and evaluation of predictive and PD response biomarkers for such agents. The aim of this chapter was to identify specific molecular biomarkers for efficacy of GSK2141795 as a single agent or in combination with cisplatin. To achieve this aim, *in vitro*, *in vivo* and ovarian cancer clinical samples treated with GSK2141795 as a single agent or in combination with cisplatin were screened with 170 validated antibodies against total and activated proteins involved in canonical and non-canonical pathways of AKT activation using RPPA.

Single agent GSK2141795 – PD biomarkers

In vivo PD biomarkers were investigated by comparison of tumour samples from untreated animals and samples from animals treated with GSK2141795. Clinical PD biomarkers for GSK2141795 were investigated by comparison of tumour biopsies taken from patients before and after treatment with GSK2141795. Analysis of ovarian cancer tumours from patients and *in vivo* tumour xenografts along with cell monolayers following treatment with GSK2141795 revealed several alterations within the AKT pathway. Phosphorylation of AKT at T308 and S473 was found to be increased following treatment with GSK2141795 in *in vitro* monolayer cell models, in tumour xenografts and also in tumour samples from ovarian cancer patients, regardless of the histology of tumour or the level of clinical response to AKT inhibition. The same result was achieved by ELISA using the same xenograft samples. These findings suggest that phosphorylation of AKT at T308 or S473 can be used as a potential PD biomarker for GSK2141795 *in vitro*, *in vivo* and in ovarian cancer patients, and validates RPPA's utility for PD biomarker discovery.

Consistent with the above, AKT hyper-phosphorylation also has been reported using other ATP competitive AKT inhibitors such as A-443654 (O'Reilly, Rojo et al. 2006, Han, Leverson et al. 2007). Two hypotheses prevail to explain this. Firstly inhibition of mTOR signalling downstream of AKT may alleviate a negative feedback loop exerted through p70-S6K/IRS-1 mediated inhibition of PI3K (O'Reilly, Rojo et al. 2006, Han, Leverson et al. 2007). Secondly, occupancy of the nucleotide binding pocket of AKT kinases by ATP competitive inhibitors enables intermolecular interactions that restrict phosphatase access, and sustain AKT phosphorylation (Chan, Zhang et al. 2011). In this study, increased phosphorylation of P70S6K at T389 was also observed in *in vitro*, *in vivo* and in ovarian cancer tumours suggesting increased activation of this protein, supporting the effect of the mTOR feedback loop. The increased phosphorylation of AKT in this study might be a result of the combination of both hypotheses as GSK2141795 is an ATP competitive inhibitor of AKT.

Decreased pPRAS40 (T246) expression was observed in both PEO4 and SKOV3 monolayers treated with GSK2141795 for 8 hours, but not in samples treated with GSK2141795 for 30 minutes, suggesting that alterations in expression of pPRAS40 is a late cellular events in response to GSK2141795. This finding was in agreement with results of ELISA experiment in chapter 4 showing down-regulation of pPRAS40 in response to treatment with GSK2141795 both *in vitro* and *in vivo*. PRAS40 is a negative regulator of mTORC1 (Sancak, Thoreen et al. 2007), and inhibits the activity of mTORC1 by preventing its binding to S6K1 and 4E-BP1. Phosphorylation of PRAS40 at T246 by AKT leads to the release of PRAS40 from the complex therefore stimulation of mTORC1 signalling (Wang, Harris et al. 2007, Wang, Harris et al. 2008). In keeping with this, a decrease in phosphorylation of mTOR and its down-stream targets 4E-BP1 and S6 was observed.

A decrease in phosphorylation of S6 and 4E-BP1 following treatment with GSK2141795 was found in both monolayer cell models and *in vivo* xenografts, and also in clinical samples. S6 is a ribosomal protein required for protein translation and is phosphorylated at S235 and S240 by p70S6-kinase in the mTOR branch of the AKT pathway. 4EBP1 is a translational repressor protein that interacts with and inhibits initiation factor eIF4e. Both S6 and 4E-BP1 are down-stream effectors of mTOR and mTOR controls cell growth and protein synthesis by mediating the regulation of these two proteins (Laplante and Sabatini 2012).

As demonstrated in chapter 3, inhibition of AKT by GSK2141795 resulted in inhibition of cell proliferation and tumour growth *in vitro* and *in vivo.* This observation may be result of inhibition of mTOR mediated cell growth and proliferation signalling following inhibition of AKT, with a decrease in level of phosphorylation and therefore activation of S6 and 4E-BP1 in response to treatment with GSK2141795, as seen here.

Moreover, decrease in phosphorylation of mTOR at S2448, GSK3 at S9 and GSK alpha/beta at S21, all down-stream targets of AKT, in response to GSK2141795 was found in all *in vitro* samples regardless of the length of the treatment. GSK-3 β phosphorylates cell cycle proteins such as cyclin D1 and c-Myc (Alt, Cleveland et al. 2000). Phosphorylation by GSK-3 β targets those proteins for destruction. On the other hand, AKT inactivates GSK-3 β by phosphorylation which promotes cell-cycle entry by stabilizing cell cycle proteins such as cyclin D1. Therefore, inhibition of AKT causes cell cycle arrest as was seen in chapter as a result of inhibition of AKT by GSK2141795 *in vitro* and *in vivo*.

Increase in phosphorylation of p38 in response to GSK2141795 was found in SKOV3 *in vitro* monolayers and *in vivo* xenografts and also in clinical samples regardless of tumour sub-

type. This may be due to cross talk between AKT/MAPK. Cross talk between AKT and MAPK pathways has been shown in various cell lines demonstrating that down-regulation of one pathway may result in up-regulation of the other pathway(Aksamitiene, Kiyatkin et al.). For example, Rane et al have demonstrated that down-regulation of AKT activation during hyperglycemia results in up-regulation of p38 MAPK activation. The same authors have also shown that inhibition of PI3K/AKT pathway with LY294002 or silencing AKT expression *in vitro* promoted p38 MAPK phosphorylation in the absence of hyperglycemia (Rane, Song et al. 2010). Therefore, in this study, up-regulation of p38 may be a direct result of inhibition of AKT by GSK2141795

Expression levels of YB-1, pEGFR (Y992) and Bim were increased and expression levels of Tuberin and pACC (S79) were decreased in SKOV3 xenografts and also SKOV3 monolayers treated with GSK2141795 for 8 hours but not at 30 minutes indicating that this is a late event. Overexpression of YB-1 and pEGFR (Y992) was found in the clinical samples in response to GSK2141795. YB-1 is a RNA-binding protein that is involved in regulation of mRNA transcription, splicing, translation, and stability. Evdokimova et al have demonstrated that AKT mediates phosphorylation of YB-1 (Evdokimova, Ruzanov et al. 2006) so down-regulation of YB-1 here, is expected as a result of AKT inhibition by GSK2141795.

EGFR is a receptor tyrosine kinase that acts upstream of AKT and controls survival, cell proliferation, migration and metabolism by activating the PI3K/AKT, ERK and RSK pathway. Up-regulation of EGFR in this analysis shows activation of compensatory survival pathways regulated by EGFR in the absence of AKT signalling. In addition, ER et al have demonstrated that AKT facilitates the endocytic trafficking of EGFR to promote its degradation. Inhibition of AKT signalling decreased both EGFR degradation and recycling within the cells resulting in increased activation of RSK and ERK pathways (Er, Mendoza et

al. 2013). This finding is in agreement with findings in this study showing overexpression of pEGFR in response to treatment with GSK2141795. Phosphorylation of HER2 at Y1248 was also increased in response to GSK2141795 in clinical samples suggesting activation of similar compensatory pathways in response to the AKT inhibition by GSK2141795.

Activation of compensatory survival pathways in response to AKT inhibition shows importance of considering cross-talking pathways in targeted therapy drug and development of rational combination therapies such as combination of AKT and MEK inhibitors.

Overall, it can be concluded that increased phosphorylation of AKT at T308 and decreased phosphorylation of ribosomal protein S6 phosphorylation at S235 and S240 and translational repressor protein 4EPB1 at S65 which were seen in *in vitro*, *in vivo* and clinical tumour samples following treatment with GSK2141795 may represent stable molecular biomarkers of AKT inhibition. These biomarkers, subject to further validation, can be eventually used in clinic to assess the modulation of AKT pathway by GSK2141795. In addition, alterations in phosphorylation of GSK3 and PRAS40, immediate down-stream targets of AKT, showed effective inhibition of AKT by GSK2141795 and efficacy of this compound *in vitro*, *in vivo* and in patients. Figure 6.13 illustrates interactions between proteins that altered expression in response to GSK2141795.

Single agent GSK2141795 'response biomarkers' and 'predictive biomarkers'

Tumour biopsies taken from patients who showed decline in CA125 level in response to 4 week treatment with GSK2141795 were used for investigation of response biomarkers. Response biomarkers were investigated by comparison of protein expression profiles between tumour samples taken from patients before and after 4 weeks treatment with GSK2141795. Although the same approach was used to investigate the PD biomarkers but

unlike PD biomarkers which were investigated by comparison of samples from all participant before and after treatment with GSK2141795, regardless of CA125 level, here only samples from patients who showed more that 30% decline in CA125 level were used for the analysis. Significant increase in expression of RAF-C and YB-1, and phosphorylation of EGFR, TAZ and ER-alpha, and significant decreases in expression of cyclin B1, PKCa, p27 and phosphorylation of YB-1 and Src were observed in ovarian cancer patients showing more than 30% decline in expression of CA125, in response to treatment with single agent GSK2141795. While the effect of AKT inhibition on cyclin B1, EGFR and p27 has been discussed above, the relationship between AKT inhibition and changes in some of the other proteins, and their relationship to response requires further study. Although these biomarkers can be used to assess the modulation of AKT pathway with GSK2141795 and effects of inhibition of AKT on down-stream signalling cascades in two different groups of patients (the ones who showed decline in CA125 level in response to the treatment and the ones who did not), but due to the short length of treatment with GSK2141795 (4 weeks) in this clinical trial, longer treatment with GSK2141795 and later biopsies may be more informative for assessment of response and resistance to GSK2141795.

Predictive biomarkers were investigated by comparison between single pre-treatment tumour biopsies taken from patients who showed more that 30% decline in CA125 levels and the ones who did not. Expression of pMAPK (T202) was found to be significantly lower in tumour biopsies taken from responder patient prior to the initiation of the treatment with GSK2141795 compared to biopsies taken from non-responders. This finding suggests that pMAPK (T202) may represent a predictive biomarker for GSK2141795 activity. This predictive biomarker, subject to further validation in the following clinical trials for GSK2141795, can be used in advance of therapy to estimate the response of a specific patient to GSK2141795 treatment. This is particularly important for selection of patients who are most likely to response to the treatment with GSK2141795. High level of pMAPK (T202)

expression also suggests the potential benefit of combining GSK2141795 with inhibitors of MAPK pathway to maximise the effect of AKT inhibition by GSK2141795 in clinic.

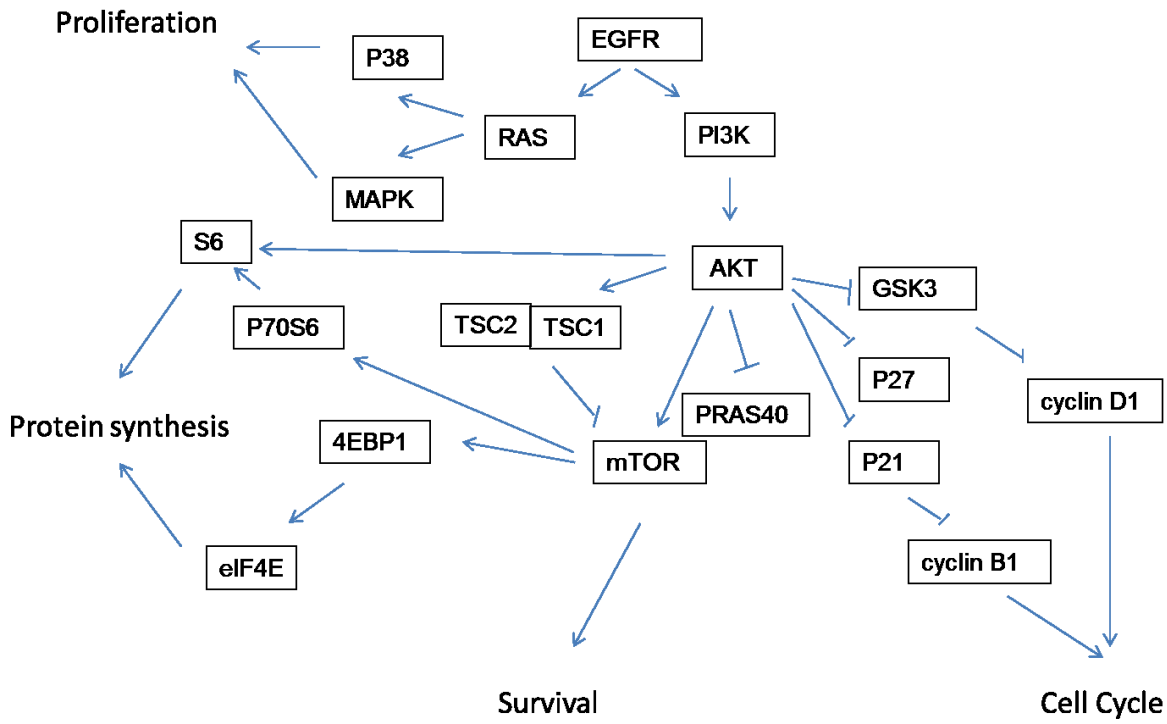


Figure 6-13 Interactions between proteins showed alteration in expression in response to GSK2151795.

In general, down-stream effectors of AKT down-regulated in response to GSK2141795 while proteins involved in compensatory survival signalling pathways such as RAS/MAPK pathway up-regulated. In addition, some proteins up-stream of AKT, such as EGFR, showed up-regulations suggesting a feedback loop.

Cisplatin – PD Biomarkers

More proteins were found showing altered expression in response to cisplatin *in vivo* compared with *in vitro* samples. Phosphorylation of ER-alpha was increased in SKOV3 *in vivo* xenografts and also in SKOV3 monolayers at both 30 minutes and 8 hour time points. Involvement of ER in apoptotic signalling in response to cisplatin has already been demonstrated. In 2003, Mandic et al reported a novel nucleus independent mechanism of action for cisplatin. They showed cisplatin induced apoptosis in the absence of DNA damage in enucleated cells and ER to be cisplatin's non-nuclear target (Mandic, Hansson et al. 2003).

Increase in phosphorylation of p70S6K (T389) was seen in SKOV3 *in vivo* xenografts and also in SKOV3 monolayers 30 minutes after treatment with cisplatin. Dhar et al have demonstrated constitutive activation of p70S6K to be associated with intrinsic resistance to cisplatin (Dhar and Basu 2008). They showed the levels of phosphorylated p70S6K, but not total p70S6K was elevated in cisplatin resistant cells following treatment with cisplatin (Dhar and Basu 2008). This is in agreement with finding in this study, as SKOV3 cells are also known to be platinum resistant. However, in this study, down-regulation of S6-pS235 was found in xenografts treated with cisplatin, suggesting down-regulation of mTOR signalling and protein synthesis in response to cisplatin. Decrease in protein synthesis might be due to the down-regulation of S-G1 cell cycle progression and reduced cell proliferation as was indicated by reduced expression of cyclin-B in response to cisplatin in xenograft samples.

Interestingly, the anti-apoptosis protein Bcl2 was found to be up-regulated in response to cisplatin in SKOV3 xenografts. Up-regulation of Bcl-2 has been previously reported in myeloma cells in response to exposure to other cytotoxic agents including etoposide and doxorubicin, and this up-regulation has been associated with resistance to the cytotoxic

agents (Tu, Xu et al. 1996). In addition, reduced expression of cleaved caspase-7 in the same samples suggested resistance to platinum-induced apoptosis in SKOV3 xenografts. This finding is in agreement with findings in chapter 3, showing resistance to cisplatin in SKOV3 xenografts, and also in SKOV3 monolayers demonstrated by caspase 3/7 apoptosis assay. In addition, up-regulation of phospho-MEK (S217) and phospho-EGFR (Y1173) was found in SKOV3 xenografts treated with cisplatin, suggesting up-regulation of survival signalling in response to cisplatin in SKOV3 xenografts. Benhar et al have also shown activation of EGFR in response to cisplatin in human glioma and breast cancer cells, and they suggest the cisplatin-dependent EGFR activation to be a survival response that reduces the efficacy of cisplatin treatment (Benhar, Engelberg et al. 2002).

Together, RPPA analysis of SKOV3 monolayers and xenografts treated with cisplatin, provided more evidence supporting resistance to cisplatin-induced apoptosis in this cell model both *in vitro* and *in vivo*, and identified several PD markers of cisplatin.

Combination of GSK2141795 and cisplatin- PD Biomarkers

Several proteins were found to change expression in the combination treatment with cisplatin and GSK2141795, but not following cisplatin or GSK2141795 as single agents. Identifying these proteins may help to understand mechanisms which led to increased induction of apoptosis *in vitro* and tumour size reduction *in vivo,* in response to the combination of cisplatin and GSK2141795, compared to treatment with single agent GSK2141795, as demonstrated in chapter 3.

The apoptosis related protein cleaved caspase 7, was found to be up-regulated in SKOV3 xenografts treated with combination of cisplatin and GSK2141795, but not in xenografts

treated with cisplatin or GSK2141795 only, indicating induction of apoptosis in xenograft tumours treated with the combination treatment. This finding suggest that reduction in tumour size observed in chapter 3 in the same xenografts in response to combination of GSK2141795 and cisplatin was a results of increased apoptosis.

The DNA mismatch repair protein MSH6 was found to be down-regulated in SKOV3 xenografts treated with the cisplatin and GSK2141795 combination treatment. This down-regulation was not found in xenografts treated with cisplatin or GSK2141795 only suggesting decrease in DNA mismatch repair and increase in platinum induced DNA damage when cisplatin is combined with GSK2141795 compared to single agent cisplatin treatment.

RAF/MEK/ERK pathway proteins were found to be down regulated in response to the combination of cisplatin and GSK2141795, in PEO4 and SKOV3 monolayers, suggesting decrease in cell proliferation and survival in samples treated with combination of GSK2141795 and cisplatin compared to single agent treatment.

Together, the findings in this study show up-regulation of apoptosis signalling and down-regulation of survival signalling via the mismatch repair and RAF/MEK/ERK pathways when cisplatin is combined with GSK2141795. This is in agreement with phenotypic observations in cell growth and apoptosis in both monolayers and 3D cell cultures and also in ovarian cancer xenografts, described in chapter 3.

Limitations of the study

Several proteins were found to similarly change expression in response to the GSK2141795, cisplatin or the combination treatment in cell monolayers, xenografts and tumour samples from ovarian cancer patients. However, there were also differences in expression pattern between the *in vitro*, *in vivo* and in clinical ovarian cancer samples in response to the same treatments. This observation can be explained in several ways.

Firstly, the *in vitro*, *in vivo* and clinical samples were collected on different time points and although care was taken to collect samples in relevant time-points but equivalent timing cannot be exactly estimated in the three models systems. Secondly, the number of samples collected was different for *in vitro*, *in vivo* and in clinical samples. The difference in the number of samples may result in statistical differences in calculation of significant changes.

Moreover, *in vitro* monolayers and subcutaneous xenograft models may not fully mirror changes the happen within the cells in response to anti-cancer drugs in patients. This could be due to the differences in tumour cell microenvironment in cell monolayers, subcutaneous tumour xenograft and within the body in ovarian cancer patients, and inter-patient tumour heterogeneity.

The advantage of using 3D microenvironment rather than 2D cell monolayers has been subject of many discussions in the last decades. *In vivo* cells form contacts and communicate with different cell types through the receptors distributed throughout the entire cell surface. However, in 2D monolayers, cells lose this three dimensional cell contact and only communicate along a small proportion of their membrane (Lin and Bissell 1993, Boudreau and Bissell 1998). It has been demonstrated that dissociation of non-malignant

mammary epithelial cells upon culture on plastic resulted in rapid loss of many features of the differentiated cells (Lin and Bissell 1993, Boudreau and Bissell 1998). In the case of malignant cells it has been reported that the effects of inactivation of tumour-suppressor genes or activation of oncogenes are different in cells cultured in different microenvironments (Debnath, Mills et al. 2002, Wrobel, Debnath et al. 2004). It has also been suggested that tumour cells grown in different microenvironments respond differently to chemotherapy and other factors (Teicher, Herman et al. 1990, Kobayashi, Man et al. 1993, Weaver, Lelievre et al. 2002, Neve, Chin et al. 2006). The importance of cell-cell interactions in other tumour characteristics such as metastasis has also been demonstrated (Thiery and Chopin 1999).

In addition, the novel PD biomarkers discovered in this study need to be verified with a follow up assay and the probability of false discovery has to be computed by multiple testing correction (Noble 2009). Response biomarkers investigated in this study are preliminary due to the short length of the phase I clinical trials (4 weeks) and has to be validated in the following longer clinical trials for GSK2141795. Repeat biopsies at longer time points than 4 weeks may be more informative for investigation of response and resistance biomarkers for GSK2141795.

Finally, findings in this chapter are another emphasis on tumour heterogeneity in ovarian cancer. The fact that the protein expression profile in response to the same treatment was different across ovarian cancer cells lines and tumour samples assessed in this study, shows that the same treatment may result in different responses in different ovarian cancer patients and even in different cells within the same tumour. Therefore, it is important to personalise treatment for each patient and even each tumour based on the unique and specific features of that tumour such as its protein or gene expression profile.

Conclusion

In conclusion, in this chapter PD biomarkers for GSK2141795 were identified *in vitro*, *in vivo* and in ovarian cancer patients by applying RPPA protein analysis. These PD markers, specifically increased phosphorylation of AKT at T308 which was common in all *in vitro*, *in vivo* and clinical samples, can be used to assess the effectiveness of GSK2141795 *in vitro*, *in vivo* and in ovarian cancer patients. In addition, predictive response biomarkers for GSK2141795 were identified in ovarian cancer patients, potentially could be used to predict patient response to GSK2141795. It was also demonstrated that combination of GSK2141795 with cisplatin increased apoptotic signalling and decreased survival signalling in cultured cells and also in tumour xenografts compared to samples treated with GSK2141795 or cisplatin only. These results support the further clinical development of GSK2141794 as a single agent and in combination with cisplatin in ovarian cancer, and the validation and use of the above PD, response and predictive biomarkers for treatment/trial optimisation.

7 The effect of DNA-PK inhibition, alone or in combination with cisplatin, on cellular signalling cascades

7.1 Introduction

It was demonstrated in chapter 5 that inhibition of DNA-PK with NU7441 resulted in sensitisation of platinum resistant ovarian cancer cells to cisplatin *in vitro* and *in vivo*, while single agent NU7441 showed no activity. This shows the potential of DNA-PK inhibitors to be used in clinic to overcome platinum resistance in ovarian cancer.

To facilitate development of DNA-PK inhibitors to be used in combination with cisplatin in clinic, it would be useful to identify PD biomarkers to monitor the activity of these agents in patients. The aim of this chapter was to identify PD biomarkers for NU7441 as single agent or in combination with cisplatin *in vitro* and *in vivo*, and key proteins involved in sensitisation of SKOV3 cells to platinum in response to combination of the DNA-PK inhibitor NU7441 and cisplatin.

To achieve this aim, RPPA analysis was used to assess the effect of DNA-PK inhibition with NU7441, alone or in combination with cisplatin, on cellular signalling cascades in SKOV3 monolayers and tumour xenografts.

7.2 Results

7.2.1 Effect of single agent NU7441 or in combination with cisplatin on cellular protein signalling cascades *in vitro*

To assess the effect of DNA-PK inhibition as single agent or in combination with cisplatin on cellular signalling cascades *in vitro*, SKOV3 monolayers were treated with 10 μM NU7441 alone for 8 hours, or in combination with 25 μM cisplatin (NU7441 was added an hour prior to cisplatin), and protein lysates were analysed by RPPA, and normalised to DMSO treated cells.

Expression levels of pMAPK (T202-Y182) and p38 (T180-Y182) increased (~1.5 and 1.4-fold respectively), whereas pS6 (S240 – S244) (S235 – S236) was down-regulated (~5-fold) in response to NU7441 (Figure 7.1).

Phosphorylation of ER-alpha at S118, and ChK1 at S45 were increased (1.7 and 1.5-fold respectively), whereas expression of PI3K-p110, ER-alpha and YB-1 were decreased about 2-fold in in response to single agent cisplatin, compared to DMSO treated cells (Figure 7.2).

Twenty one proteins were found to be up-regulated and 13 to be down-regulated in samples treated with the combination of NU7441 and cisplatin, compared with vehicle controls. Among these proteins beclin, phospho-ER-alpha (S118), cleaved caspase-3 and pChK1 (S345) showed the highest level of increase in expression (~2-fold), and pS6 (S235-S236) and pS6 (S240-S244) showed the decrease in expression (~4-fold) (Figure. 7.3).

To identify proteins which their expressions altered only in response to NU7441 and cisplatin combination treatment, but not in samples treated with each drug as single agent, protein expression levels were compared in samples treated with NU7441, or cisplatin, or combination of both (Figure 7.4; Table 7.1 and 7.2). Nine proteins were found to be up-regulated and 16 to be down-regulated in samples treated with combination of NU7441 and cisplatin, which did not alter in response to NU7441 or cisplatin as single agents. Up-regulation of beclin was seen in response to the combination treatment showing increase in cellular stress. Up-regulation of the pro-apoptotic protein, Bim and activated caspase-3 which was only seen in response to the combination treatment, shows increase in apoptosis in cells treated with combination of cisplatin and NU7441 compared to the ones treated with cisplatin or NU7995 only. This was consistent with findings using the caspase 3/7 apoptosis assay in chapter 5, that increase in induction of apoptosis was observed in cells treated with combination of NU7441 and cisplatin. Interestingly, down-regulation of pTAZ and pYAP was found in cells treated with the combination of NU7441 and cisplatin. Down-regulation of pYAP was also seen in SKOV3 monolayers treated with combination of GSK2141795 and cisplatin in chapter 6 (Figure. A.17, Table.A.11), suggesting a potential role for the Hippo pathway in mechanism of sensitisation to cisplatin in platinum resistant cells.

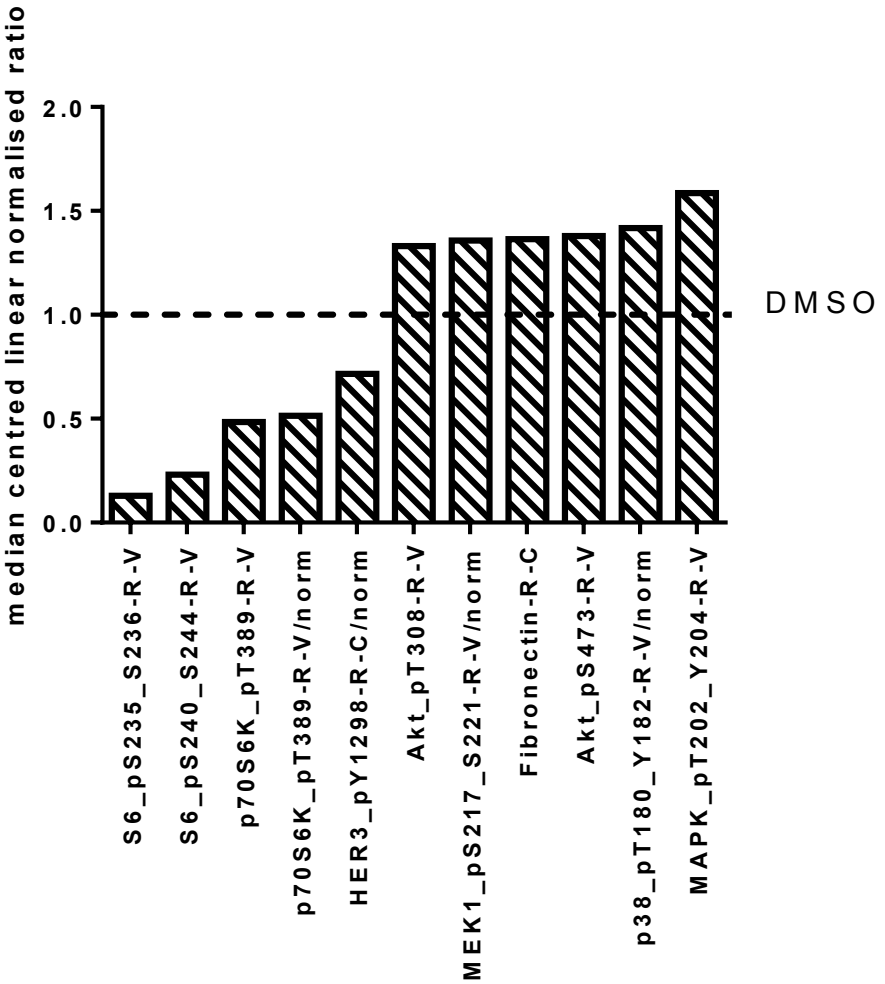


Figure 7-1 Proteins showing up-regulation or down-regulation in SKOV3 monolayers treated with NU7441 compared with vehicles

SKOV3 monolayers were treated with 10 μM NU7441 and level of protein expression was compared with DMSO treated samples. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated samples, n=1.

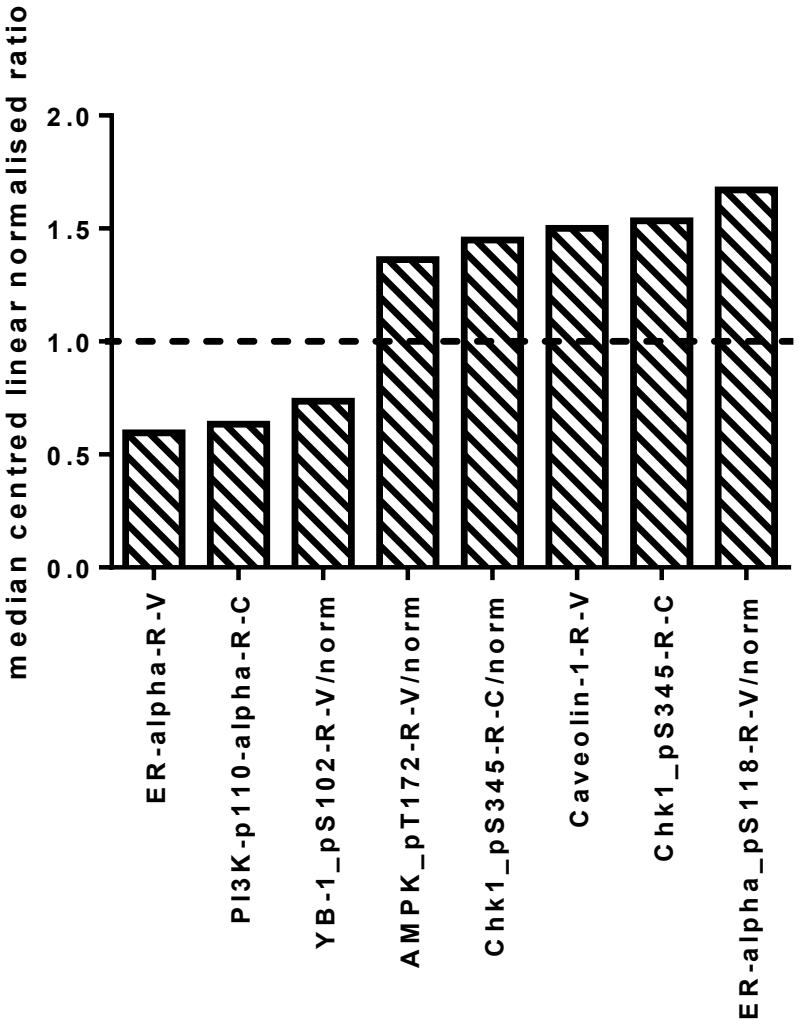


Figure 7-2 Proteins showing up-regulation or down-regulation in SKOV3 monolayers treated with cisplatin compared with vehicles

SKOV3 monolayers were treated with 25 μM cisplatin and level of protein expression was compared with DMSO treated samples. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated samples, n=1.

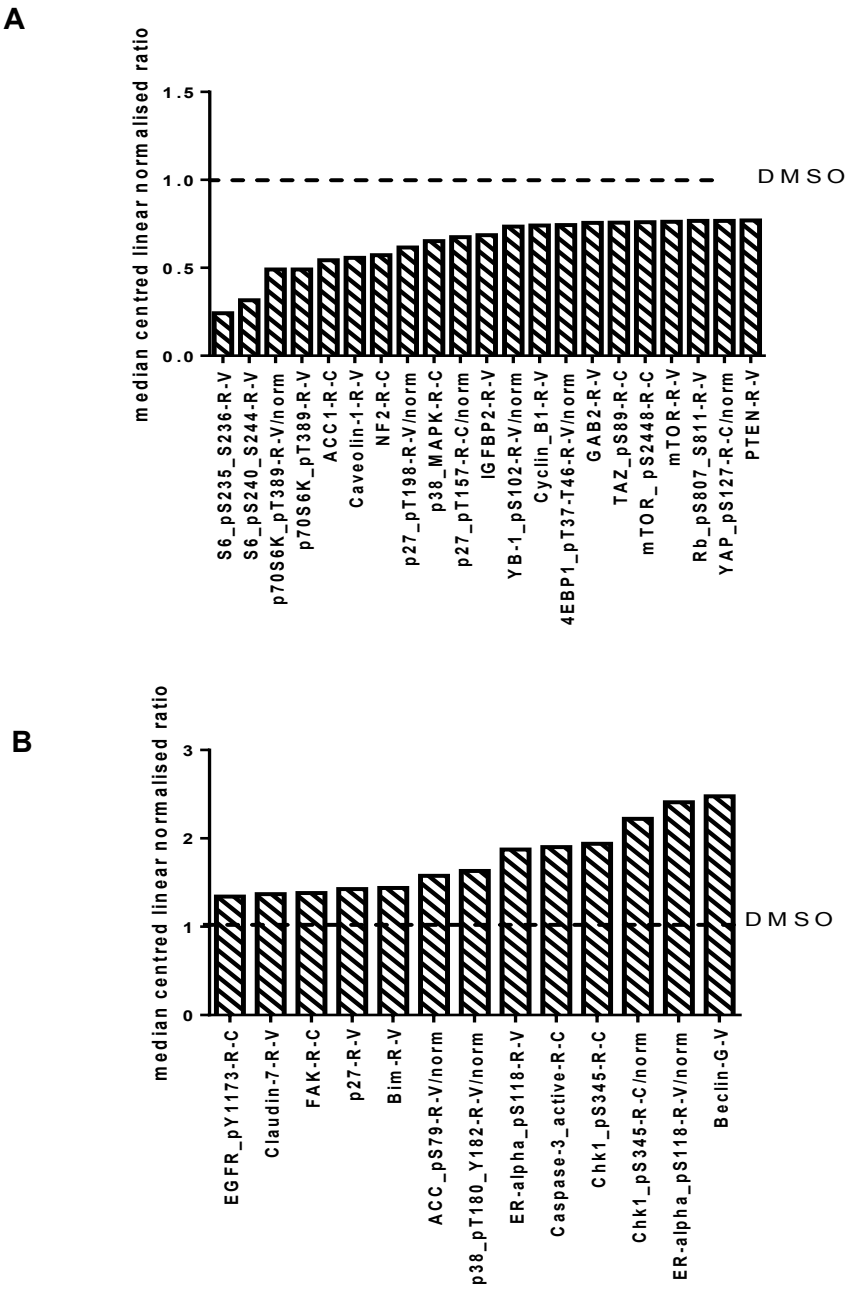


Figure 7-3 Proteins showing down-regulation A) or up-regulation B) in SKOV3 monolayers treated with combination of NU7441 and cisplatin compared with vehicles

SKOV3 monolayers were treated with combination of 10 μM NU7441 and 25 μM cisplatin and level of protein expression was compared with DMSO treated samples. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated samples, n=1.

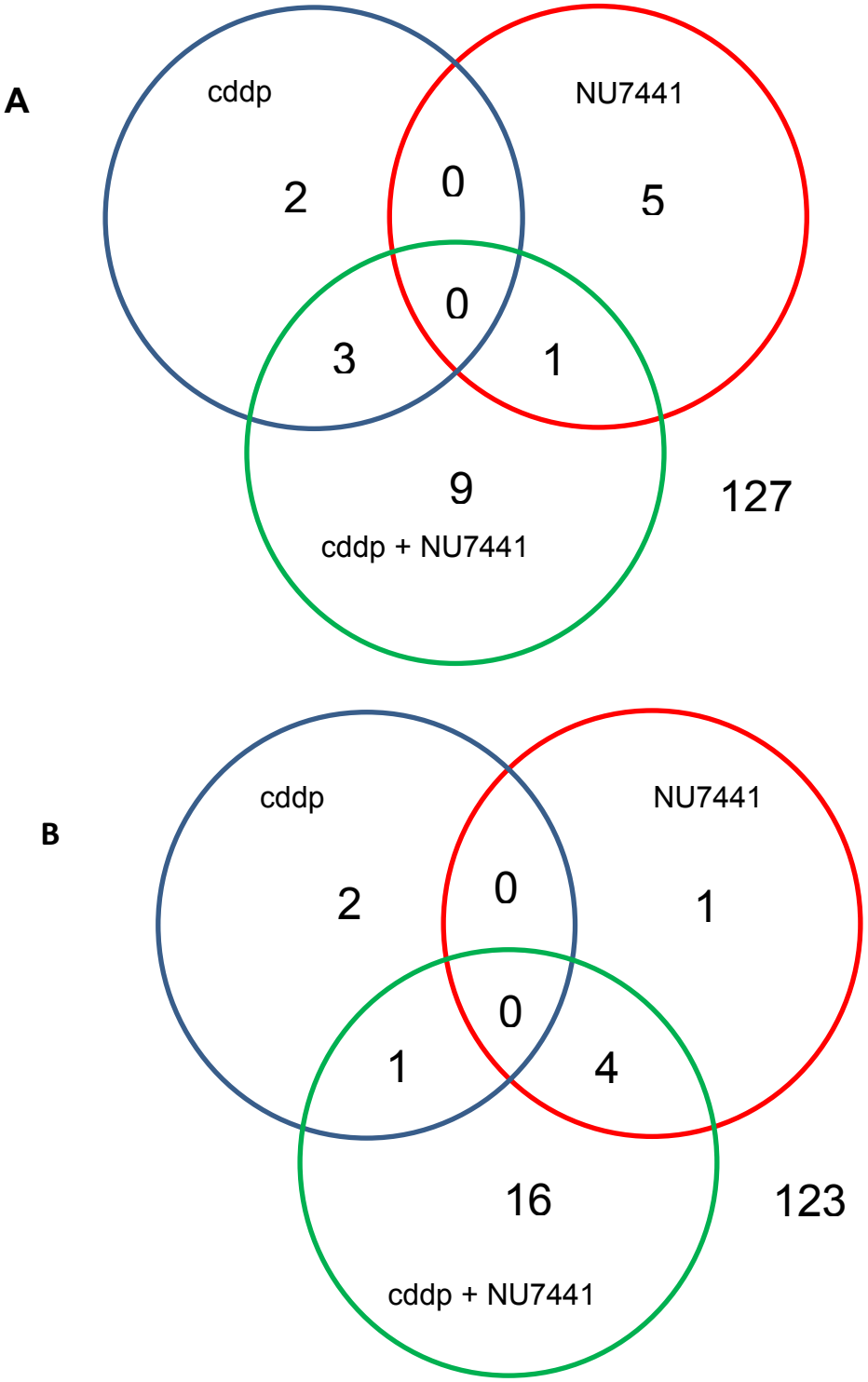


Figure 7-4 Comparison of up-regulations a) and down-regulations b) in protein expression in SKOV3 monolayers treated with NU7441 or cisplatin or combination of both.

Nine proteins were up-regulated and 1 protein was down-regulated only in combination treatments but not in cddp and NU7441 treated samples.

Table 7-1Comparison of protein up-regulations in SKOV3 monolayers treated with NU7441 or cisplatin or combination of NU7441 and cisplatin.

Up-regulated proteins	CDDP + NU7441	NU7441	CDDP
p38_pT180_Y182-/norm	Yes	Yes	No
Chk1_pS345	Yes	No	Yes
Chk1_pS345/norm	Yes	No	Yes
ER-alpha_pS118-/norm	Yes	No	Yes
ACC_pS79/norm	Yes	No	No
Beclin	Yes	No	No
Bim	Yes	No	No
Caspase-3_active	Yes	No	No
Claudin-7	Yes	No	No
EGFR_pY1173	Yes	No	No
ER-alpha_pS118	Yes	No	No
FAK	Yes	No	No
p27	Yes	No	No
Akt_pS473	No	Yes	No
Akt_pT308	No	Yes	No
Fibronectin	No	Yes	No
MAPK_pT202_Y204	No	Yes	No
MEK1_pS217_S221/norm	No	Yes	No
AMPK_pT172/norm	No	No	Yes
Caveolin-1	No	No	Yes

Table 7-2 Comparison of protein down-regulations in SKOV3 monolayers treated with NU7441 or cisplatin or combination of NU7441 and csiplatin.

Down-regulated proteins	CDDP + NU7441	NU7441	CDDP
p70S6K_pT389	Yes	Yes	No
p70S6K_pT389/norm	Yes	Yes	No
S6_pS235_S236	Yes	Yes	No
S6_pS240_S244	Yes	Yes	No
YB-1_pS102-/norm	Yes	No	Yes
4EBP1_pT37-T46-/norm	Yes	No	No
ACC1	Yes	No	No
Caveolin-1	Yes	No	No
Cyclin_B1	Yes	No	No
GAB2	Yes	No	No
IGFBP2	Yes	No	No
mTOR_ pS2448	Yes	No	No
mTOR	Yes	No	No
NF2	Yes	No	No
p27_pT157/norm	Yes	No	No
p27_pT198/norm	Yes	No	No
p38_MAPK	Yes	No	No
PTEN	Yes	No	No
Rb_pS807_S811	Yes	No	No
TAZ_pS89	Yes	No	No
YAP_pS127/norm	Yes	No	No
HER3_pY1298-/norm	No	Yes	No
ER-alpha	No	No	Yes
PI3K-p110-alpha	No	No	Yes

7.2.2 Effect of inhibition of DNA-PK with NU7441 as single agent or in combination with cisplatin on cellular protein signalling cascades *in vivo*

To investigate the effect of inhibition of DNA-PK with NU7441 as a single agent or in combination with cisplatin on protein signalling cascades *in vivo*, mice bearing SKOV3 xenografts were treated with 10 mg/kg NU7441, or 1 mg/kg cisplatin, or combination of both drugs. Protein samples extracted from xenografts were analysed for changes in expression in response to each treatment by RPPA.

Proteins with 1.3-fold increase or decrease in expression in xenografts treated with NU7441 as single agent compared to xenografts treated with DMSO only are shown in Figure 7.5 and Table 7.3. Phosphorylation of AKT at S473 significantly decreased showing the effective inhibition of DNA-PK, as AKT in an immediate down-stream target of AKT. However, phosphorylation of down-stream targets of AKT, PRAS40 and GSK3, increased in response to DNA-PK inhibition suggesting that phosphorylation of AKT at S473 may not be required for activation of the mTOR branch of AKT signalling. A 1.5-fold increase in expression of the DNA mismatch repair protein, MSH6 was also observed. EEF2K and YAP phosphorylated at S127 showed the highest increase in expression (~1.5-fold, $p< 0.05$) and fibronectin and EGFR showed the greatest decrease (~ 2-fold, $p < 0.05$).

In xenografts treated with 1 mg/kg cisplatin for 3 hours, very few proteins showed significant a change in expression (<1.3-fold, $p<0.05$) (Figure. 7.6 and Table 7.4). HER3 and p70S6K showed the highest increase, suggesting activation of cellular survival pathways in response to cisplatin and ER-alpha showed the highest decrease (~2-fold, $p < 0.05$) (Figure 7.2, Table 7.2).

In xenografts treated with the combination of cisplatin and NU7441, fibronectin expression decreased the most (2-fold, $p<0.05$) and eEF2K, MSH6 and cyclin_B1 showed the highest increase (1.5-fold). In addition, p38 and phospho-AKT(S473) were among the proteins showing significant down-regulation, and phospho-GSK3(S9), phospho-4EBP1, phospho-YAP(S127), phospho-PRAS40(T246) and MEK1 were among the proteins showing the highest significant up-regulation (Figure 7.7 and Table 7.5).

Alterations in protein expression in cisplatin or NU7441 single agent treated groups were identified and compared to alteration in protein expression in the combination group. This comparison was done to identify proteins which their level of expression changed in response to the combination treatment but remained unchanged in response to either cisplatin or NU7441 single agent treatment, to identify proteins involved in the mechanism of re-sensitisation to cisplatin (Figure 7.8; Tables 7.6 and 7.7). Up-regulation in expression of cyclin B1, MEK-1 and 4EBP1, and down-regulation of p21 and phospho-EGFR were only found in xenografts treated with combination treatment and not in xenografts treated with the single agent cisplatin or NU7441.

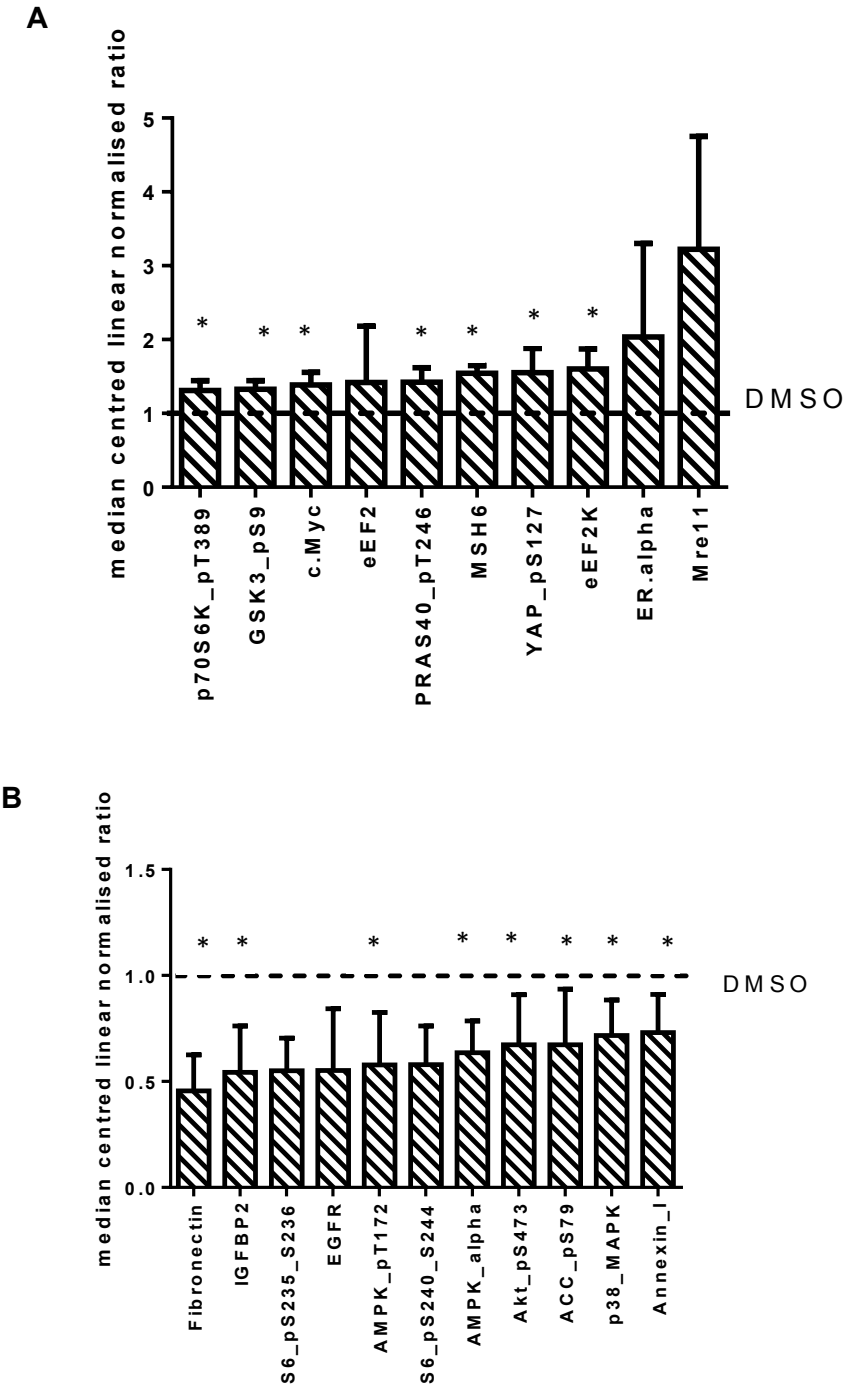


Figure 7-5 Proteins showing up-regulation A) or down-regulation B) in SKOV3 xenografts treated with NU7441

SKOV3 xenografts were treated with 10 mg/kg NU7441 and level of protein expression was compared with the DMSO treated xenografts. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated xenografts. * indicates t-test p values ≤ 0.05, n=9. Data shows mean of results for 9 samples ± SD.

Table 7-3 Proteins showing ≥ 1.3-fold increase or decrease in expression in SKOV3 xenografts treated with NU7441 compared with DMSO treated samples (n=9)

Protein	Ratio NU7441/DMSO	p-value
Mre11	3.2	0.1
ER.alpha	2.0	0.6
eEF2K	1.6	0.002
YAP_pS127	1.5	0.001
MSH6	1.5	0.000
PRAS40_pT246	1.4	0.001
eEF2	1.4	0.2
c.Myc	1.3	0.001
GSK3_pS9	1.3	0.003
p70S6K_pT389	1.3	0.006
Annexin_I	0.7	0.00
p38_MAPK	0.7	0.001
ACC_pS79	0.6	0.02
Akt_pS473	0.6	0.009
AMPK_alpha	0.6	0.004
S6_pS240_S244	0.5	0.09
AMPK_pT172	0.5	0.00
EGFR	0.5	0.08
S6_pS235_S236	0.5	0.1
IGFBP2	0.5	0.05
Fibronectin	0.4	0.01

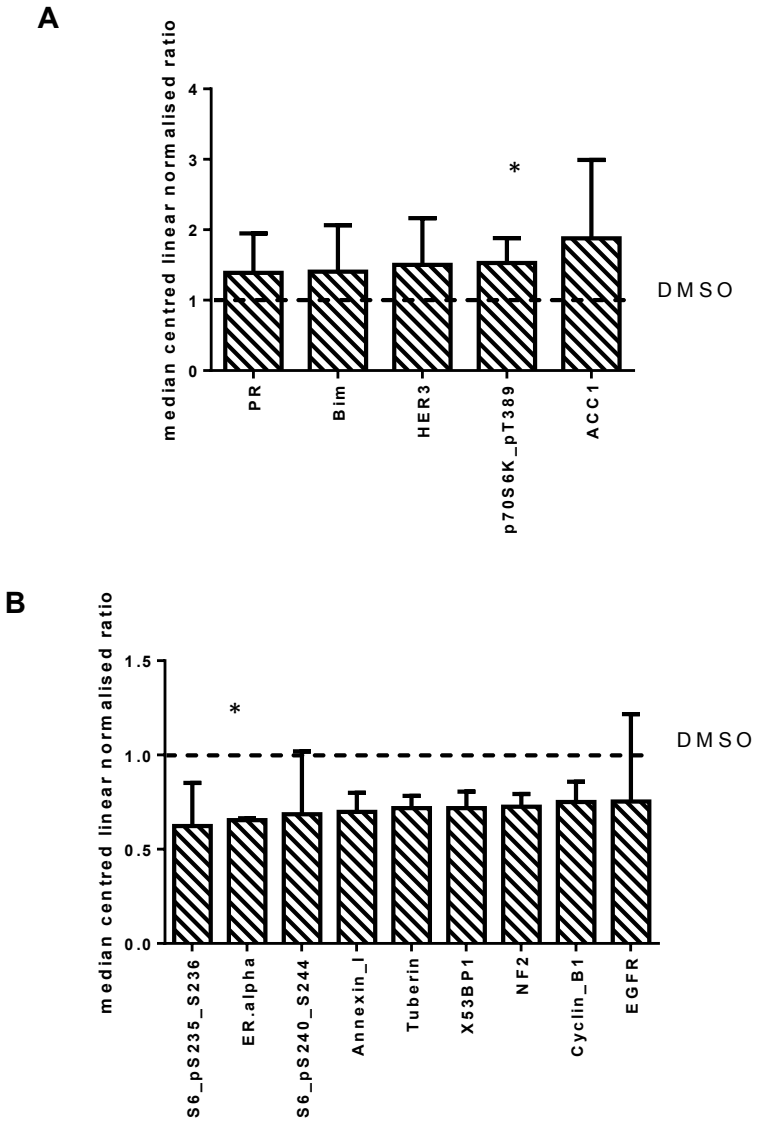


Figure 7-6 Proteins showing up-regulation A) or down-regulation B) in SKOV3 xenogrfts treated with cisplatin compared with vehicles

SKOV3 xenografts were treated with 1 mg/kg cisplatin and level of protein expression was compared with DMSO treated xenografts. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated xenografts. * indicates p values ≤ 0.05, Data shows mean of n=5 samples ± SD.

Table 7-4 Proteins showing ≥ 1.3-fold increase or decrease in expression in SKOV3 xenografts treated with cisplatin compared with DMSO treated samples (n=9)

Proteins	Ratio CDDP/DMSO	p-value
ACC1	1.8	0.1
p70S6K_pT389	1.5	0.006
HER3	1.5	0.08
Bim	1.4	0.1
PR	1.3	0.08
EGFR	0.75	0.4
Cyclin_B1	0.75	0.1
NF2	0.72	0.06
X53BP1	0.71	0.08
Tuberin	0.71	0.06
Annexin_I	0.69	0.1
S6_pS240_S244	0.68	0.3
ER.alpha	0.65	0.01
S6_pS235_S236	0.62	0.2

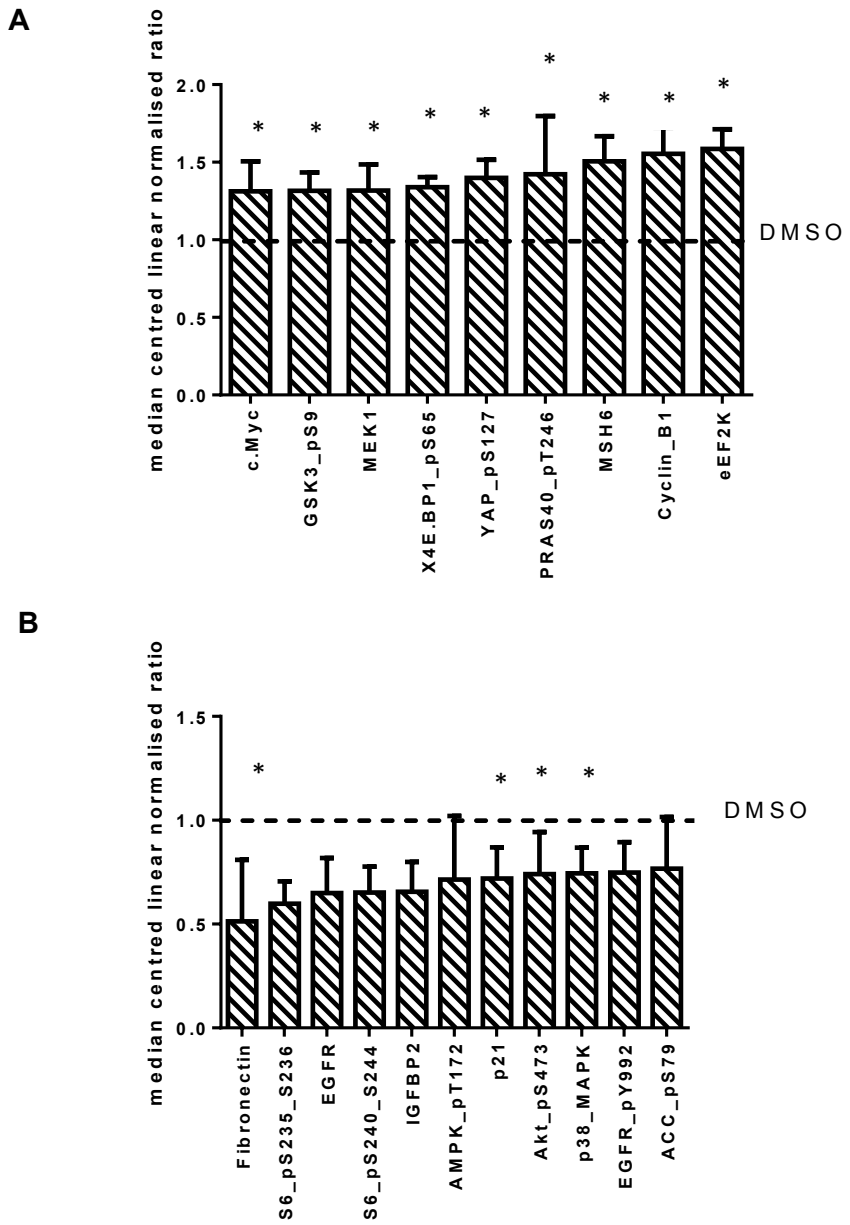


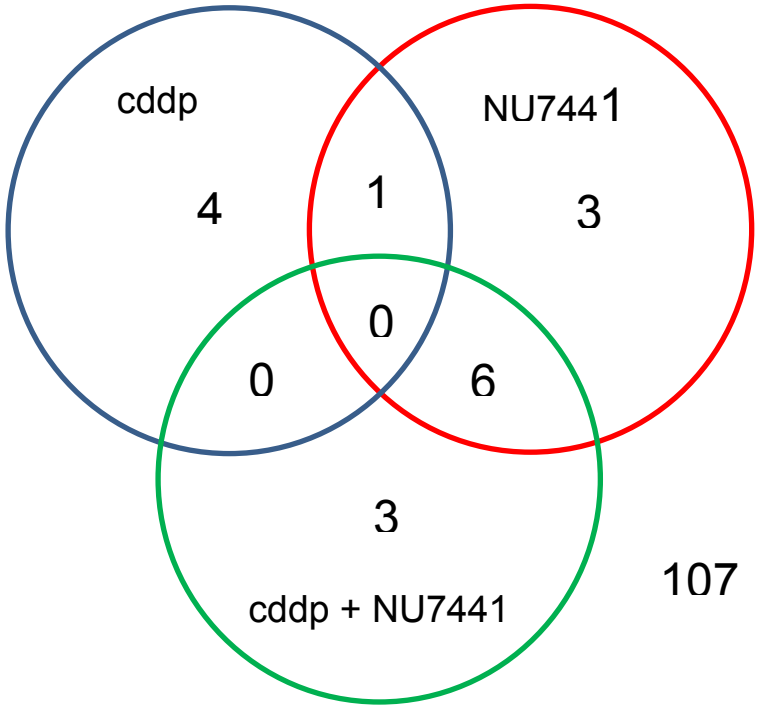
Figure 7-7 Proteins showing up-regulation A) or down-regulation B) in SKOV3 xenografts treated with combination of NU7441 and cisplatin compared with vehicles

SKOV3 xenografts were treated with 10 mg/kg NU7441 and 1 mg/kg cisplatin and level of protein expression was compared with DMSO treated xenografts. The graph demonstrates proteins showing ≥ 1.3-fold increase or decrease in expression compared with DMSO treated xenografts. * indicates P values ≤ 0.05, Data shows mean of n=9 samples ± SD.

Table 7-5 Proteins showing ≥ 1.3-fold increase or decrease in expression in SKOV3 xenografts treated with combination of NU7441 and cisplatin compared with DMSO treated samples (n=9)

Protein	Ratio NU7441+CDDP / DMSO	p-value
eEF2K	1.5	0.001
Cyclin_B1	1.5	0.000
MSH6	1.5	0.000
PRAS40_pT246	1.4	0.01
YAP_pS127	1.3	0.001
4EBP1_pS65	1.3	0.0001
MEK1	1.3	0.001
GSK3_pS9	1.3	0.001
c.Myc	1.3	0.005
ACC_pS79	0.76	0.1
EGFR_pY992	0.74	0.06
p38_MAPK	0.74	0.001
Akt_pS473	0.74	0.03
p21	0.71	0.001
AMPK_pT172	0.71	0.08
IGFBP2	0.65	0.2
S6_pS240_S244	0.65	0.2
EGFR	0.64	0.2
S6_pS235_S236	0.59	0.1
Fibronectin	0.51	0.02

A



B

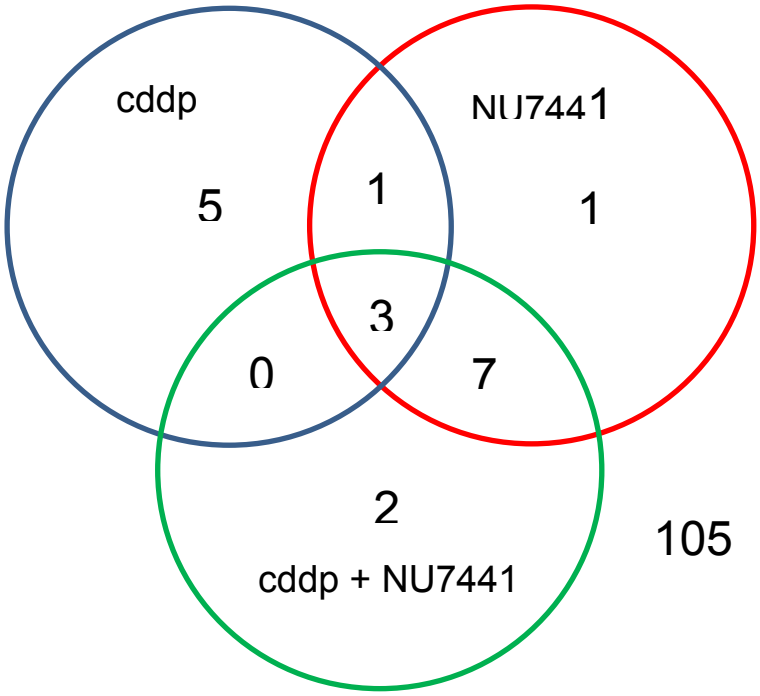


Figure 7-8 Comparison of up-regulations A) and down-regulations B) in protein expression in SKOV3 xenografts treated with NU7441 or cisplatin or combination of both

Three proteins were up-regulated and 2 proteins were down-regulated only in combination treatments but not in cddp and NU7441 treated samples.

Table 7-6Comparison of protein up-regulations in SKOV3 xenografts treated with NU7441 or cisplatin or combination of NU7441 and csiplatin.

Protein	CDDP	NU7441	NU7441+CDDP	Venn group
c.Myc	No	Yes	Yes	5
eEF2K	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
MSH6	No	Yes	Yes	5
PRAS40_pT246	No	Yes	Yes	5
YAP_pS127	No	Yes	Yes	5
p70S6K_pT389	Yes	Yes	No	4
Cyclin_B1	No	No	Yes	3
MEK1	No	No	Yes	3
4EBP1_pS65	No	No	Yes	3
eEF2	No	Yes	No	2
ER.alpha	No	Yes	No	2
Mre11	No	Yes	No	2
ACC1	Yes	No	No	1
Bim	Yes	No	No	1
HER3	Yes	No	No	1
PR	Yes	No	No	1

Table 7-7Comparison of protein down-regulations in SKOV3 xenografts treated with NU7441 or cisplatin or combination of NU7441 and cisplatin.

Sample	CDDP	NU7441	CDDP+NU7441	Venn group
EGFR	Yes	Yes	Yes	7
S6_pS235_S236	Yes	Yes	Yes	7
S6_pS240_S244	Yes	Yes	Yes	7
ACC_pS79	No	Yes	Yes	5
Akt_pS473	No	Yes	Yes	5
AMPK_pT172	No	Yes	Yes	5
Caspase-7_cleaved	No	Yes	Yes	5
Fibronectin	No	Yes	Yes	5
IGFBP2	No	Yes	Yes	5
p38_MAPK	No	Yes	Yes	5
Annexin_I	Yes	Yes	No	4
EGFR_pY992	No	No	Yes	3
p21	No	No	Yes	3
AMPK_alpha	No	Yes	No	2
Cyclin_B1	Yes	No	No	1
ER.alpha	Yes	No	No	1
NF2	Yes	No	No	1
Tuberin	Yes	No	No	1
53BP1	Yes	No	No	1

7.3 Discussion

In chapter 5, it was demonstrated that combination of the DNA-PK inhibitor NU7441 and cisplatin, increased apoptotic response in ovarian cancer monolayers compared to the cells treated with cisplatin only. Also it was shown in chapter 5 that combination of the DNA-PK inhibitor, NU7441, and cisplatin resulted in tumour shrinkage in SKOV3 xenografts. In this chapter, SKOV3 monolayers or xenografts treated with either NU7441 or cisplatin or combination of both drugs were assessed for alteration in protein expression in response to each treatment.

Single agent NU7441

Inhibition of DNA-PK with NU7441 led to a significant increase in expression of the mismatch repair protein MSH6 *in vivo*. As discussed in the introduction (Chapter 1), DNA-PK has an important role in DNA double strand break repair through the non-homologous end-joining (NEHJ) pathway. To initiate NEHJ, Ku70 binds to the broken end of DNA forming a complex together with Ku80. This complex recruits the catalytic subunit of DNA-PK_{CS} to the broken end of DNA leading to the activation of NEHJ pathway (Lieber, Ma et al. 2003). On the other hand MSH6 plays a fundamental role in the repair of mismatched DNA bases. Increase of MSH6 in response to DNA-PK inhibition suggests that in cancer cells when one DNA-repair pathway is disturbed, cells may rely on a different DNA repair pathway to repair DNA damages. Recently, Shahi et al have reported that MSH6 physically associates with Ku70 and regulates DNA double-strand break repair (Shahi, Lee et al. 2011). In addition, an increase in expression of Mre11, another protein involved in initiation of NEHJ, was observed in xenografts treated with NU7441 although this increase was not statistically significant.

A significant decrease in phosphorylation of AKT at S473 was found in response to DNA-PK inhibition. This decrease was expected as DNA-PK is one of the known protein kinases which phosphorylates AKT at S473 alongside with mTOR-Rictor complex 2 and integrin-linked kinase (ILK) (Dragoi, Fu et al. 2005). Interestingly, despite the decrease in phosphorylation of AKT at S473 in response to DNA-PK inhibition alone or in combination with cisplatin, a significant increase was found in expression of down-stream targets of AKT, pPRAS40 and pGSK3 suggesting that DNA-PK might not be involved in activation of AKT through the mTOR branch of the AKT pathway or for regulation of the glucose metabolic pathway mediated by AKT.

In addition, expression levels of AMPK and pAMPK were significantly decreased in xenografts treated with NU7441. The inhibitory effect of AKT for AMPK has already been reported (Lee and Park 2010) suggesting that inhibition of phosphorylation of AKT via DNA-PK inhibition in this study did not affect the inhibitory role of AKT on AMPK.

Cisplatin

Only a few proteins changed expression in response to 1 mg/kg cisplatin in SKOV3 xenografts, compared to the higher dose of cisplatin (1.5 mg/kg) in chapter 6, reflecting the lower dose of cisplatin used on cellular signalling. This finding was in agreement with the phenotypic observation in chapter 5, that no significant difference was seen in tumour growth in 14 days between xenografts treated with DMSO only and the ones treated with 1 mg/kg cisplatin.

Combination of NU7441 and cisplatin

MEK1 was significantly up-regulated in xenograft tumours treated with the combination of NU7441 and cisplatin, but not in tumours treated with either NU7441 or cisplatin. MEK1 has a key role in tumour cells survival through the RAS-MEK-ERK pathway, and cross talk between MEK and AKT pathways under cellular stress has been reported (Dai, Chen et al. 2009). Therefore, overexpression of MEK observed here might be due to loss of AKT mediated survival signalling cascade as a result of DNA-PK inhibition.

The cyclin-dependent kinase inhibitor, p21, was found to be significantly down-regulated only in xenograft tumours treated with the combination treatment. P21 is an inhibitor of proliferation, and is induced by both p53-dependent and -independent mechanisms following stress. Induction of p21 following cellular stress may cause cell cycle arrest. Cells are driven to apoptosis after caspase-3-induced cleavage of p21 (Gartel and Tyner 2002). Down-regulation of p21 in response to the combination treatment suggests DNA-PK mediated activation of p21 in response to DNA damage. On the other hand, cyclin B 1, an important component of cells cycle machinery that controls G2 to M transition, is inhibited by p21 and in this analysis, up-regulation of cyclin B1 was found in xenograft samples treated with combination of cisplatin and NU7441.

Up-regulation of proteins involved in apoptosis pathway such as cleaved caspase 3 and the pro-apoptotic protein BIM and also down-regulation of proteins involved in cell proliferation and survival pathways such as p38-MAPK, pmTOR and cyclin-B1 was found in SKOV3 monolayers treated with combination of NU7441 and cisplatin but not in samples treated with cisplatin or NU7441 only. This finding supports findings in chapter 5 demonstrating that DNA-PK inhibition using NU7441 combined with cisplatin results in increase of apoptosis and decrease in cell survival compared to single agent cisplatin or NU7441 treatments.

Interestingly, down-regulation of YAP (Yes-associated Protein) and TAZ phosphorylation found in response to the combination of cisplatin and DNAPK inhibition by NU7441 was also observed in response to the combination of AKT inhibition by GSK2141795 and cisplatin in chapter 6. As these protein expression alterations were both specific to the combination treatments and were not found in samples treated with either single agents, and both combinations resulted in the same phenotype (ie senstitisation of SKOV3 platinum resistant cells to cisplatin), this suggest a potential role for the Hippo pathway as a mechanism of sensitisation to cisplatin. The Hippo pathway has a key role in controlling organ size by inhibiting cell proliferation and promoting apoptosis through phosphorylation of YAP. Recent studies suggest a potential cross talk between the Hippo and PI3K/AKT/mTOR pathways that leads to coordination between the two pathways to control cell growth (Tumaneng, Schlegelmilch et al. 2012). Regulation of Hippo pathway is not fully understood yet so gaining further knowledge about this pathway may lead to development of novel therapies to overcome platinum resistance in ovarian cancer.

Conclusion

In this chapter a core set of PD biomarkers for NU7441 was identified *in vitro* and *in vivo*. However, those PD biomarkers were found to be different in cell monolayers and xenografts. This difference may be due to the limitations were already discussed for RPPA analysis in chapter 6 such as different microenvironment *in vitro* and *in vivo* and difference in time and schedule of treatments in the two different models. Down-regulation of pS6 and up-regulation of P38(p180) were identified as *in vitro* PD biomarkers while up-regulation of eEF2K, YAP(pS127), MSH6 and down-regulation of AKT(pS473) may represent *in vivo* PD biomarkers for NU7441. Although PD biomarkers reflect modulation of a defined biological target with a specific drug, biomarkers that correlate with the treatment outcome (such as predictive biomarkers) are required to translate biological changes into clinical benefits.

Therefore future clinical studies with DNA-PK inhibitors should include efforts to identify predictive biomarkers as well as PD biomarkers.

Also, in this chapter it was demonstrated while single agent cisplatin or NU7441 showed no effect in triggering apoptosis in SKOV3 cells, down-regulation of survival signalling pathways and up-regulation of apoptotic signalling were observed when cisplatin was combined with NU7441. These findings and findings from chapter 5 encourage further pre-clinical and clinical development of NU7441 or other DNA-PK inhibitors to overcome platinum resistance in ovarian cancer.

8 Summary and future directions

Epithelial ovarian cancer (EOC) is the most lethal form of gynaecological malignancy. The prognosis of patients with ovarian cancer is poor, with 30-40% overall 5-year survival, due to the development of platinum resistance; the most active agent in this disease. Novel therapeutic strategies for reversal of platinum resistance and/or active agents in platinum resistant patients are urgently required. In addition, improvements in the efficiency of cancer drug development are also needed if the potential of the large number of candidate drugs in development is to rapidly and economically realised. The development of drugs for treatment of cancer remains a slow and high-risk process with many compounds failing due to unfavourable pharmacokinetic profiles, toxicity and and/or lack of therapeutic efficacy (Neidle 2008). The high failure rate contributes to the high cost in both financial and human terms of drug development. One strategy, termed the PHAT (pharmacological audit trail) has advocated the preclinical development of effective pharmacodynamic (PD) biomarkers, to monitor drug activity and enable go/no-go decisions.

In this thesis pre-clinical evaluation and development of a specific, ATP-competitive AKT inhibitor, GSK2141795 as potential therapeutic strategy in ovarian cancer, as single agent and to restore platinum sensitivity was assessed. Single agent GSK2141795 showed growth inhibitory effect *in vitro* and *in vivo*. In addition, GSK2141795 was also shown to restore platinum sensitivity in platinum-resistant ovarian cancer cell lines and tumour xenografts.

A key area of increased focus aimed at improving the probability of success in the drug discovery process, is the identification and validation of reliable pharmacodynamic (PD) biomarkers of target modulation by the drug, that can be used both during pre-clinical and clinical development, and ideally can also be correlated with clinical outcome. One approach clinically is to use tumour biopsies taken before and after drug treatment to measure modulation of PD makers. PD biomarkers may include cellular, molecular, histopathological

and/or imaging parameters (Sarker, Pacey et al. 2007, Sarker and Workman 2007). It is anticipated that such PD biomarkers should clearly enable efficient and scientifically-driven "Go/NoGo" decisions allowing expediency of the drug development process.

In the case of AKT inhibitors, AKT substrates such as phospho-PRAS40, are being used as PD biomarkers (Yan, Serra et al. 2013). However, the invasive nature of serial tumour biopsies in patients limits its application and recruitment in clinical trials. In addition, intra-patient tumour heterogeneity can also limit the utility of tumour biopsies from single sites. The development of non- invasive PD biomarkers therefore has the potential to aid clinical drug development.

Positron emission tomography (PET) is a non-invasive imaging technology that measures the three dimensional spatial and temporal uptake of a radioligand *in vivo*. PET imaging using the glucose analogue [^{18}F]FDG as a radio-tracer is widely used in the field of oncology, to monitor the tumour response to the treatments by measuring tumour metabolism. However, given the role of AKT in the regulation of glucose metabolism, [^{18}F]FDG-PET can potentially be used as a non-invasive PD biomarker for AKT inhibitors. In this project, to enable the clinical development of GSK2141795, the utility of FDG-PET imaging as a PD biomarker of GSK2141795 induced AKT inhibition was explored. The changes in glucose metabolism (as measured by changes in FDG uptake) were shown to correlate with changes in downstream biomarkers (phospho-PRAS40) of AKT inhibition in cell lines *in vitro*, and similar effects were demonstrated *in vivo* in xenograft experiments. These results formed the basis of a clinical trial of GSK2141795 in ovarian cancer, to validate the utility of FDG-PET as a clinically useful PD biomarker for individualisation of inter-patient dosing, thereby optimising the risk/benefit profile of the drug.

In this dose escalation study of GSK2141795 in cancer patients, an inverse relationship between maximum GSK2141795 plasma concentrations with the maximum decrease in [^{18}F]FDG uptake in patient tumours was confirmed (Gungor H 2011). However, the lack of a clear dose response relationship in the latter study was likely due to a narrow dose range tested (25-75 mg, daily) coupled with large inter-patient variability in the pharmacokinetics of GSK2141795. Maximum GSK2141795 plasma concentrations observed in the clinical study ranged from approximately 300-800 ng/mL (0.70-1.86 μM), with >40% decrease in [^{18}F]FDG uptake in 5 out of 6 patients achieving >1 μM C_{max} on week 4 (Gungor, Saleem et al, 2011). The preclinical data reported in this thesis are in agreement with the clinical observations that AKT inhibition with GSK2141795 requires ≥1 μM concentration for optimal decreases in [^{18}F]FDG uptake.

PD biomarkers should ideally be incorporated as all stages of drug discovery from *in vitro* and preclinical studies to clinical trials. To identify additional PD biomarkers, the effect of inhibition of AKT using GSK2141795 as single agent or in combination with cisplatin on cellular signalling cascades in ovarian cancer cells was closely studied *in vitro*, *in vivo* and in tumour samples from ovarian cancer patients. This part of study was done to validate the PD biomarkers already known for AKT inhibition and also to find novel canonical and non-canonical PD markers for GSK2141795 as a single agent or in combination with cisplatin. In general, down-stream targets of AKT such as PRAS40, S6, GSK3, mTOR and 4EPB1 were down-regulated in response to AKT inhibition by GSK2141795. In particular, alterations in phosphorylation of AKT at T308, S6 at S235 and S240 and 4EPB1 at S65 was consistent across all the *in vitro*, *in vivo* and clinical samples and these proteins may represent stable molecular PD biomarkers of AKT inhibition.

In addition, proteins involved in compensatory survival signalling pathways, such as MAPK and EGFR, showed up-regulation in response to GSK2141795 treatment, and may represent resistance mechanisms. This finding emphasises the importance of considering cross talks between signalling pathways in targeted therapy drug development and has implication in development of rational combination therapies, such as the combination of AKT and MEK inhibitors.

Response biomarkers were also explored for GSK2141795 using pre and post-treatment clinical samples from the phase I trial of GSK2141795 (Gungor H 2011). Interestingly, some of these biomarkers such as YAP and TAZ were non-canonical biomarkers indicating the importance of signalling pathways other than PI3K/AKT/mTOR pathway on response to AKT inhibition. However, it should be considered that patients in this study were treated with daily GSK2141795 for only 4 weeks which may not be sufficient to observe the proteomic changes in response to the treatment. Later biopsies may be more informative to identify response biomarkers for GSK2141795. In addition, number of patients were relevantly low in this phase I clinical study and these markers merit further validation in larger Phase 2/3 trials.

In addition to PD biomarkers which are essential to provide reassurance that target and pathway modulation has been successful at all stages of drug development, predictive biomarkers are also required to identify a selected group of patients who may benefit the most from the treatment. Predictive biomarkers can be used to minimise the number of patients who may receive ineffective treatments. In this study predictive biomarkers for GSK2141795 were explored and pMAPK was found to be a potential predictive biomarker for clinical use of this drug.

The finding in this thesis that GSK2141795 synergises with platinum, to increase platinum sensitivity in *in vitro* and *in vivo* models, also merits further evaluation. A next step towards further clinical development of GSK2141795 may be the clinical assessment of safety and efficacy of this compound in combination with platinum based cytotoxic agents in recurrent ovarian cancer patients.

Another strategy for restoration of platinum sensitivity explored in this thesis was DNA-PK inhibition with NU7441. Like GSK2141795, NU7441 also increased sensitivity to platinum, but did not show significant single agent activity. Protein analysis of *in vitro* and *in vivo* platinum resistant ovarian cancer samples treated with combination of either the DNA-PK inhibitor NU7441 or the AKT inhibitor GSK2141795 with cisplatin, showed up-regulation of apoptotic proteins such as caspase and down-regulation of survival pathways in the combination treatment compared to the single agent treatments. Overall, findings from protein analysis using either single agent NU7441 or GSK2141795 or in combination with cisplatin were in agreement with phenotypic observations using the same treatments *in vitro* and *in vivo.* The results suggest further development of NU7441 in ovarian cancer, in combination with cisplatin.

Inhibition of PI3K was found to be less effective than inhibition of AKT in platinum resistant ovarian cancer cells lines. Knockdown of genes encoding for subunits of PI3K did not restore sensitivity to platinum in those cells lines. However, effect of PI3K inhibition on sensitivity to platinum has to be assessed in larger number of platinum resistant cell lines and also in patient derived tumour cells to validate findings from this study.

It has previously been shown in our group that following platinum induced DNA damage, AKT is translocated to the nucleus, where it is phosphorylated by DNA-PK at S473 and this

DNA-PK mediated activation of AKT causes resistance to platinum. On the other hand, PI3K mediated phosphorylation of AKT at the other phosphorylation site, T308 is believed to be necessary for activation of AKT. As inhibition of PI3K did not restore sensitivity to platinum in this study, it was further investigated if PI3K is involved in platinum induced DNA-PK mediated activation of AKT. It was demonstrated that inhibition of DNA-PK by NU7441 re-sensitised platinum resistant cells to cisplatin and knockdown of *PIK3CA* and *PIK3R1* genes encoding for catalytic and regulatory subunits of PI3K respectively, did not affect the restoration of platinum sensitivity by DNA-PK inhibition. These findings suggest that PI3K may not be involved in DNA-PK modulated response to cisplatin in platinum resistant cells. However, further investigation is required to support this hypothesis. For examples, effect of PI3K inhibition or knockdown on sensitisation to platinum should be assessed in larger number of *in vitro* and also *in vivo* and clinical samples. One experiment for the next step of this study to show if or if not phosphorylation at both T308 and S473 are required for platinum induced DNA-PK mediated activation of AKT, would be using GFP-tagged T308 and S473 mutant AKT constructs transfected into ovarian cancer cell lines. Effect of either of T308 and S473 mutations would be assessed on apoptotic response to cisplatin in platinum resistant and sensitive cells. In addition confocal microscopy will be used to locate AKT cisplatin treated cells. .

In this thesis, it was also demonstrated that 3D MTS may be more informative and reliable cellular model than traditional 2D monolayers to be used for predicting tracer uptake by xenografts and determining dose and schedule optimisation prior to pre-clinical *in vivo* experiments in PET oncology. This may be important in minimising the cost of the studies and the use of animals in translational approaches.

Together, the results of this study encourage further investigation into the potential clinical utility of AKT or DNA-PK inhibitors in combination with DNA-damage agents. Importantly,

these studies also demonstrate the value and potential of using 3D models in a fully translational preclinical setting in order to both predict and optimise dose and scheduling plans prior to translation of preclinical data such as these to the clinical setting.

One of the major challenges in drug development is translating efficacy determined during the pre-clinical studies into efficacy in clinical trials. Therefore, it is important for clinical models to take into account both of the molecular nature of the target and also the tumour micro-environment in patients. Currently, tumour cell lines grown as monolayers are used as the first line of study during lead optimisations because apoptosis, cell proliferation and other cellular end-points can be easily measured using different techniques in a high through-put format using 2D *in vitro* models. However, it is important to consider that adherent cells grown on a plastic dish may not reflect tumour biology. Phenotype, genetic and epigenetic alterations seen in these models may be induced by artificial environment of 2D cell culture. Therefore, *in vitro* models that mimic 3D tumour environment are required to bridge the gap between first line *in vitro* studies and pre-clinical *in vivo* experiments to reduce the *in vivo* failure and therefore to minimise the cost and use of animals in pre-clinical studies. Tumour cells grown as 3D spheroids may be more representative of tumour biology in the host organism. In this thesis, it was also demonstrated that 3D MTS may be more informative and reliable cellular model than traditional 2D monolayers to be used for predicting tracer uptake by xenografts and determining dose and schedule optimisation prior to pre-clinical *in vivo* experiments in PET oncology. This may be important in minimising the cost of the studies and the use of animals in translational approaches. Currently, pre-clinical tumour xenograft models are mostly generated by subcutaneous or intraperitoneal injection of human cancer cell lines into immune-compromised mice. These pre-clinical xenograft models are relatively easy to produce and they mimic tumour 3D micro-environment but they fail to mimic human microenvironment and key aspect of human malignancies such as metastasis. An alternative

model currently under development is xenografting of fresh human biopsy specimens onto immunocompromised mice (Aubard 2003, Oktem and Urman 2009)

Together, the results of this study encourage further investigation into the potential clinical utility of AKT or DNA-PK inhibitors in combination with DNA-damage agents. Importantly, these studies also demonstrate the value and potential of using 3D models in a fully translational preclinical setting in order to both predict and optimise dose and scheduling plans prior to translation of preclinical data such as these to the clinical setting.

9 References

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10 Appendix

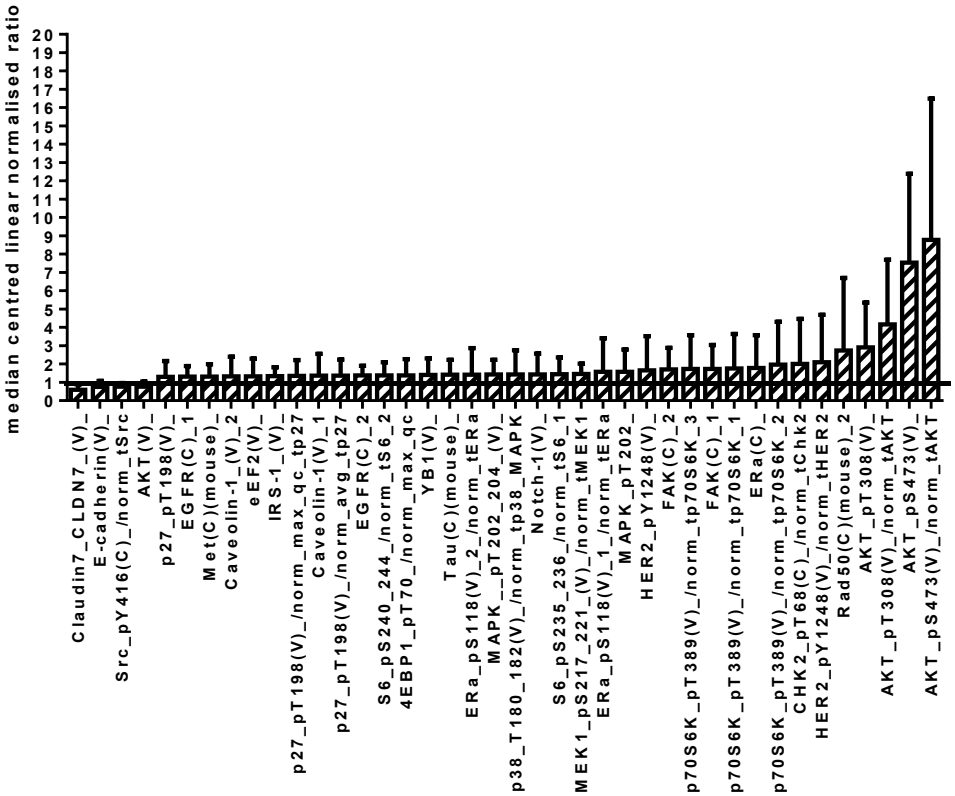


Fig. A.1 . Proteins showing different levels of expression in serous ovarian cancer tumours before and after treatment with GSK2141795 Biopsies from patients diagnosed with serous ovarian cancer were taken prior and following treatment with GSK2141795 for 4 weeks and differences in levels of protein expression between two samples were measured by RPPA analysis. The graph indicates proteins showing changes more than 1.3-fold in expression (n = 6). Data shows mean of samples for each protein ± SD.

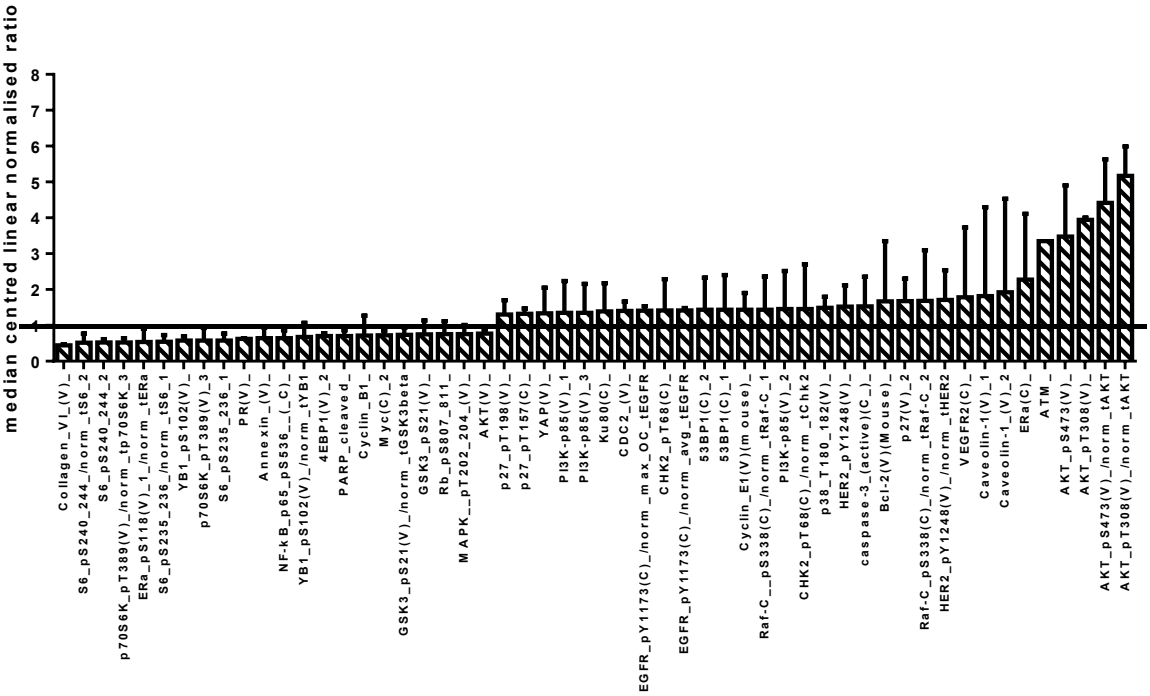
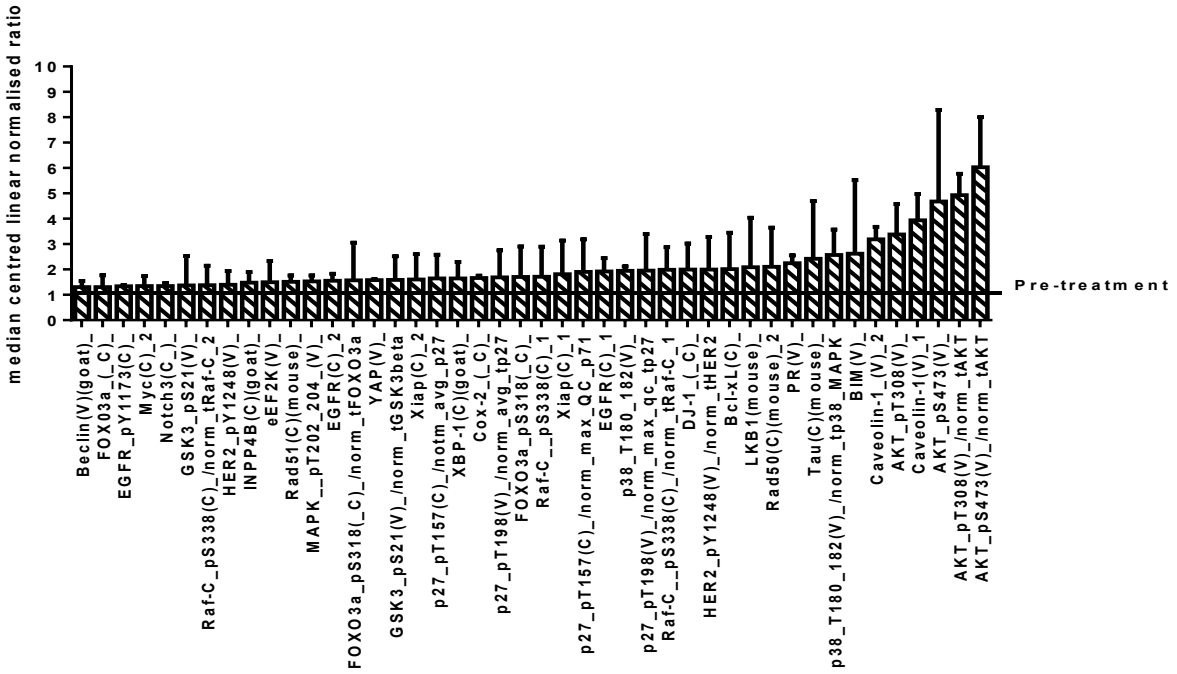


Fig. A.2 . Proteins showing different levels of expression in endometrioid ovarian cancer tumours before and after treatment with GSK2141795

Biopsies from patients diagnosed with endometrioid ovarian cancer were taken prior and following treatment with GSK2141795 for 4 weeks and differences in level of protein expression between two samples were measured by RPPA analysis. The graph indicates proteins showing changes more than 1.3-fold in expression (n = 2). Data shows mean of samples for each protein ± SD.

A



B

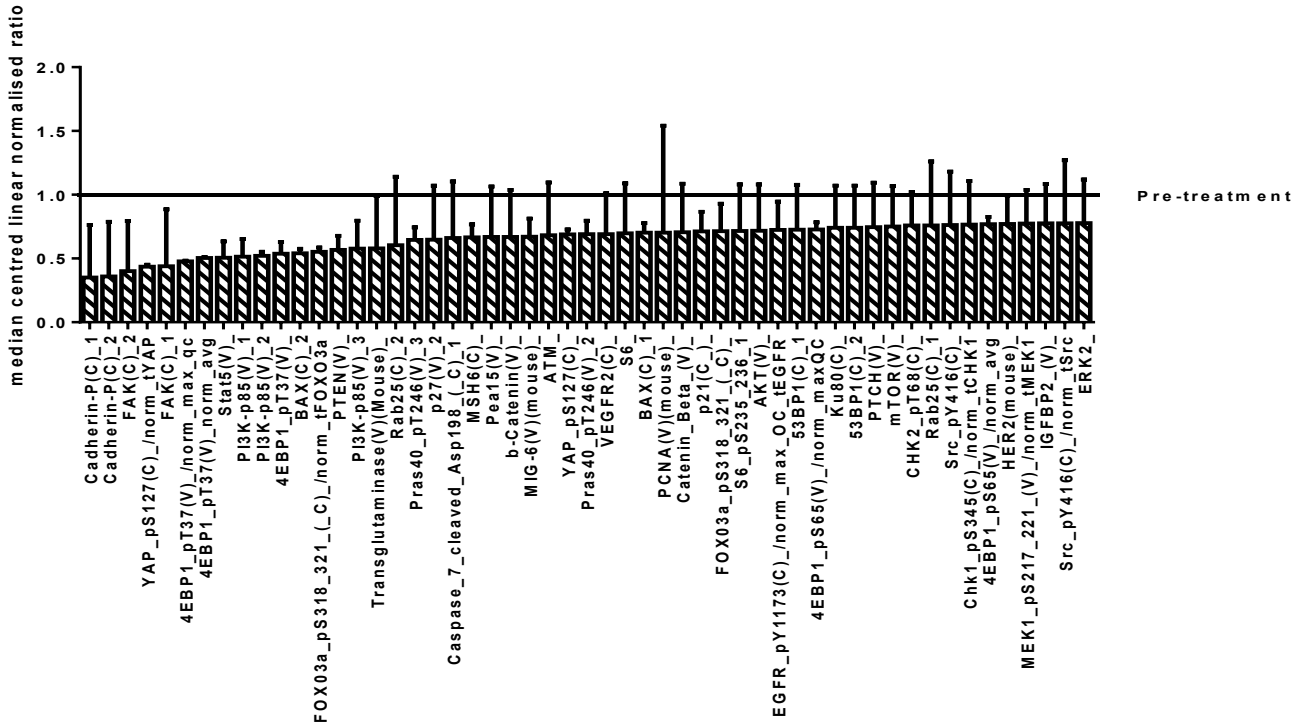


Fig. A.3 . Proteins showing different levels of expression in clear cell ovarian cancer tumours before and after treatment with GSK2141795.Biopsies from patients diagnosed with clear cell ovarian cancer were taken prior and following treatment with GSK2141795 for 4 weeks and differences in level of protein expression between two samples were measured by RPPA analysis. The graph indicates proteins showing A) up-regulations and B) down-regulations more than 1.3-fold in expression (n = 2). Data shows mean of samples for each protein ± SD.

Table. A.1 Proteins which their expression altered in more than one tumour type in response to treatment with GSK2141795.

Up-regulations				
Protein	SEROUS	Clear Cell	Endometrioid	Venn group*
AKT_pS473	Yes	Yes	Yes	7
AKT_pS473/norm	Yes	Yes	Yes	7
AKT_pT308_	Yes	Yes	Yes	7
AKT_pT308/norm	Yes	Yes	Yes	7
Caveolin-1	Yes	Yes	Yes	7
Caveolin-1	Yes	Yes	Yes	7
HER2_pY1248	Yes	Yes	Yes	7
HER2_pY1248/norm	Yes	Yes	Yes	7
p38_pT180_Y182	Yes	Yes	Yes	7
EGFR	Yes	Yes	No	4
MAPK__pT202_204	Yes	Yes	No	4
p27_pT198/norm	Yes	Yes	No	4
Rad50	Yes	Yes	No	4
Tau	Yes	Yes	No	4
CHK2_pT68/norm	Yes	No	Yes	6
Era	Yes	No	Yes	6
p27_pT198	Yes	No	Yes	6

Down-regulations				
Protein	SEROUS	Clear Cell	Endometrioid	Venn group*
AKT	Yes	Yes	No	4
Claudin7_CLDN7	Yes	No	No	1
E-cadherin	Yes	No	No	1
Src_pY416/norm	Yes	No	No	1
S6_pS235_236	No	Yes	Yes	5

*Venn group 7 = all three tumour type. Venn group 4 = serous and clear cell, Venn group 5 = endometrioid and clear cell. Venn group 6 = serous and endometrioid

Table. A.2 Proteins with common alternation in expression in both ovarian cancer clinical and pre-clinical samples from SKOV3 xenografts. .

Up-regulations		
Serous	Endometrioid	clear cell
Akt_pS473	Akt_pS473	Akt_pS473
Akt_pT308	Akt_pS473/norm	Akt_pT308
MAPK_pT202_Y204-	Akt_pT308	Bim
p38_pT180_Y182	Akt_pT308/norm	C-Raf_pS338
-	C-Raf_pS338	EGFR_pY992/norm
-	EGFR_pY1173/norm	GSK3-alpha-beta_pS21_S9
-	EGFR_pY992/norm	p38_pT180_Y182
-	p38_pT180_Y182/norm	YB-1
-	YB-1	-

Down-regulations		
Serous	Endometrioid	Clear cell
-	Cyclin_B1	4EBP1_pS65/norm
-	S6_pS235_236	4EBP1_pT37/norm
-	S6_pS240_244	b-Catenin
-	-	Caspase_7_cleaved_Asp198
-	-	Catenin_Beta
-	-	S6_pS235_236
-	-	VEGFR2

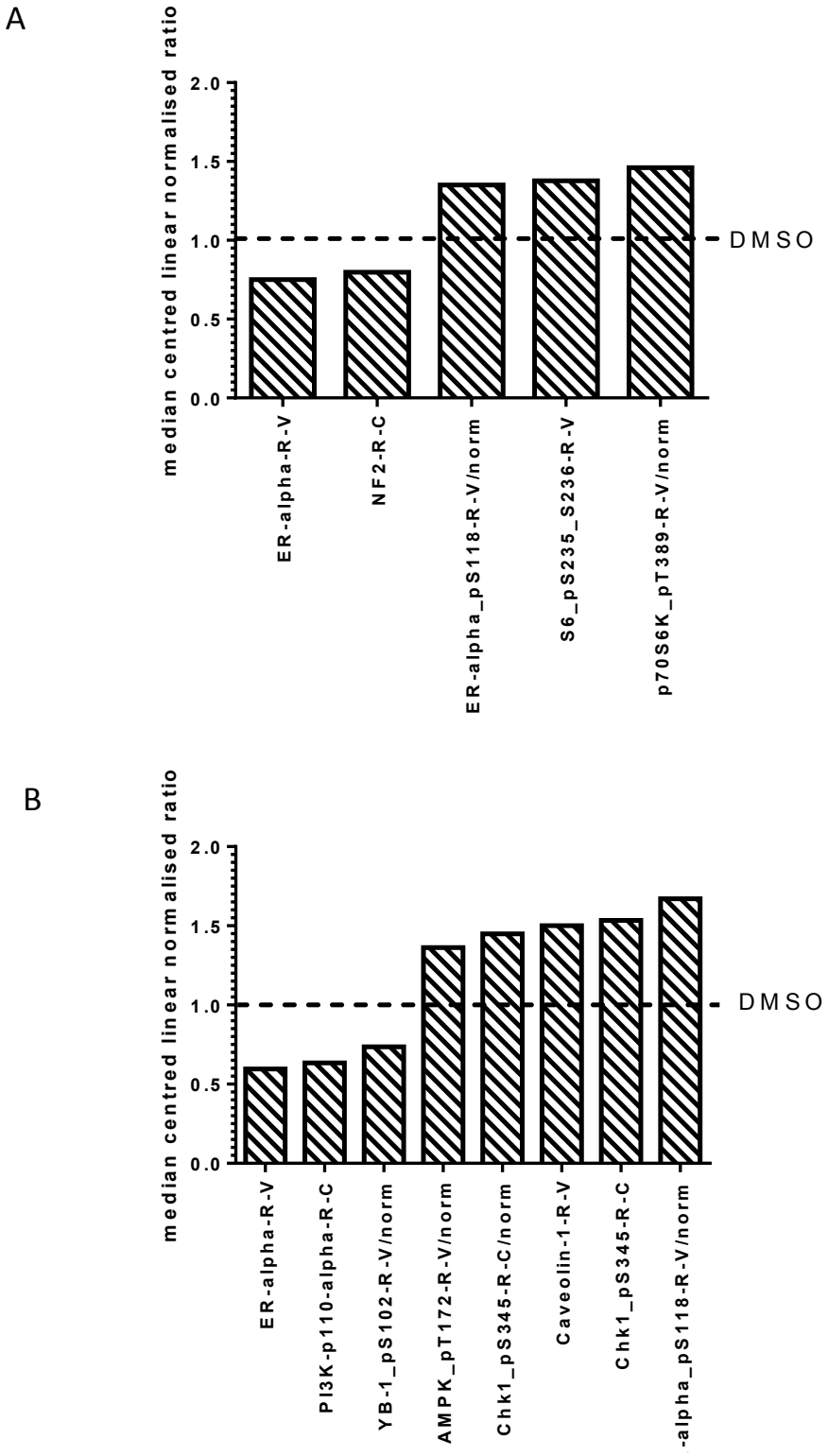


Figure A.4 Protein expression in SKOV3 monolayers treated with cisplatin in comparison with vehicle treated samples. SKOV3 monolayers were treated with 25 μM cisplatin for a) 0.5 hour and b) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and cisplatin.

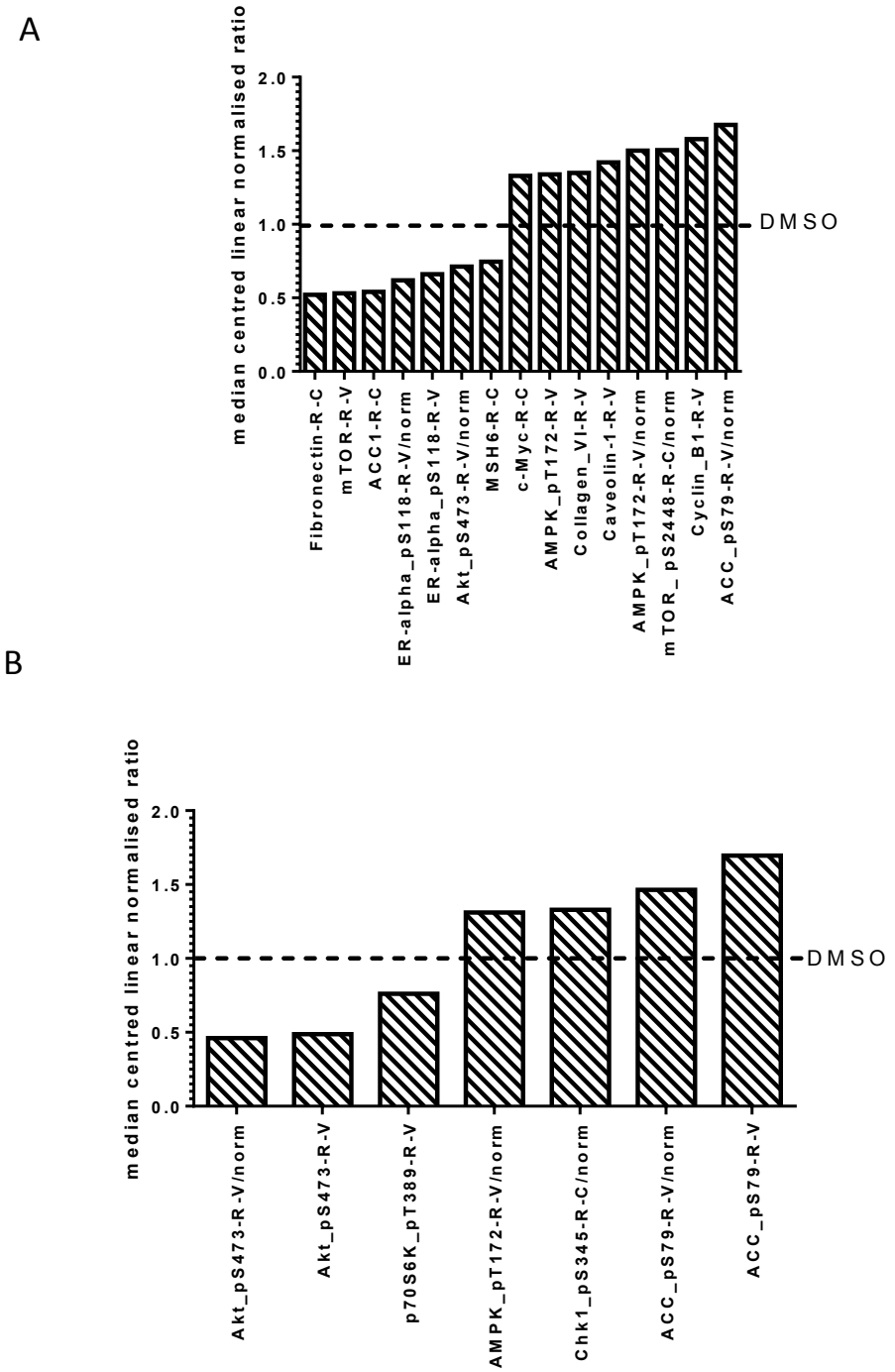
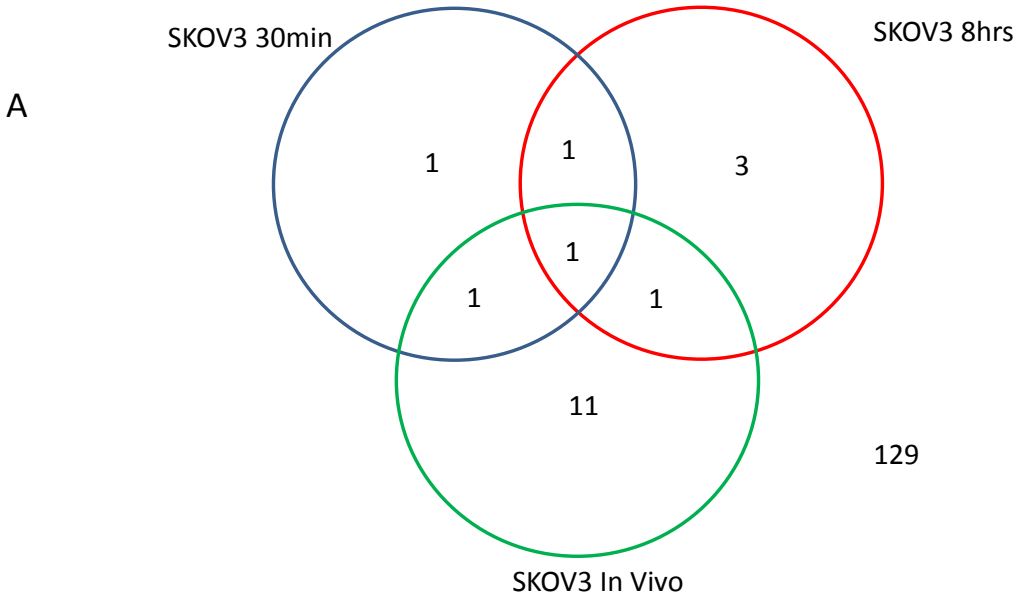


Figure A.5 Protein expression levels in PEO4 monolayers treated with cisplatin in comparison with vehicle treated samples. PEO4 monolayers were treated with 25 μM cisplatin for A) 0.5 hour and B) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and cisplatin.



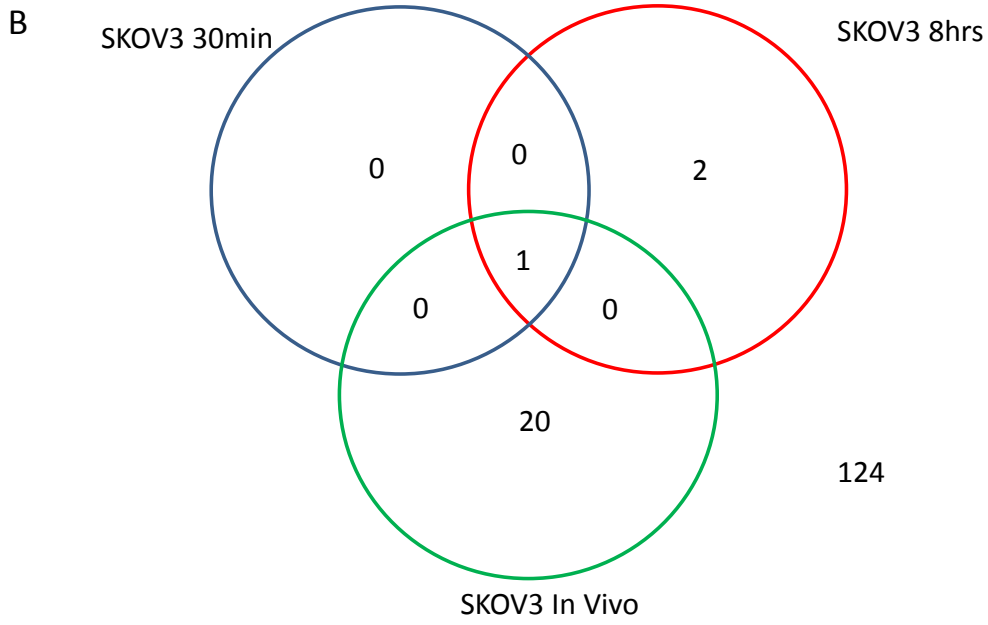


Figure A.6 Comparison of A) Up-regulation and B) down-regulation of 147 proteins in SKOV3 in vitro and *in vivo* models. Protein expression was compared in SKOV3 monolayers treated with cisplatin for 30 minutes and 8 hours and also 3 hours in SKOV3 xenografts

Table A.3 Proteins expression in SKOV3 xenografts in response to treatment with cisplatin compared with the same treatment in SKOV3 monolayers.

Protein expression up-regulation in SKOV3 models in response to GSK2141795				
Protein	SKOV3_cddp_0.5h	SKOV3_cddp_8h	SKOV3_vivo_cddp	Venn group
ER-alpha_pS118/norm	Yes	Yes	Yes	7
p70S6K_pT389/norm	Yes	No	Yes	6
AMPK_pT172/norm	No	Yes	Yes	5

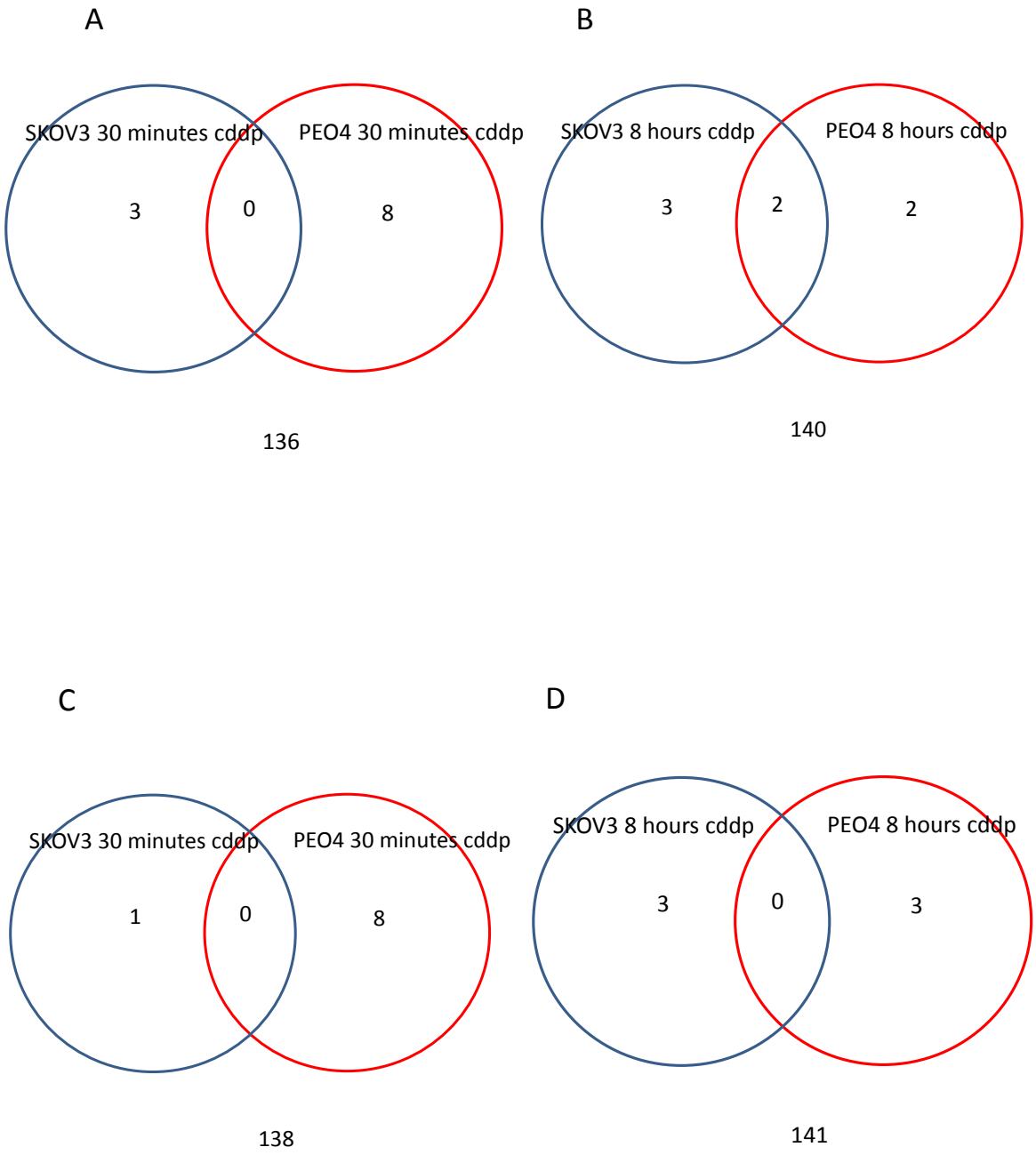


Figure A.7 Comparison of protein expression in SKOV3 and PEO4 monolayers in response to treatment with cisplatin. No protein was found to be upregulated A) or down-regulated C) in both SKOV3 and PEO4 monolayers treated with cisplatin for 30 minutes. Two proteins were upregulated B) and no proteins was down-regulated D) in both SKOV3 and PEO4 cells treated with cisplatin for 8 hours.

Table A.4 Proteins which showed increase in expression in both SKOV3 and PEO4 monolayers treated with cisplatin.

Protein	SKOV3_cddp_8h	PEO4_cddp_8h	Venn group
AMPK_pT172/norm	Yes	Yes	3
Chk1_pS345/norm	Yes	Yes	3

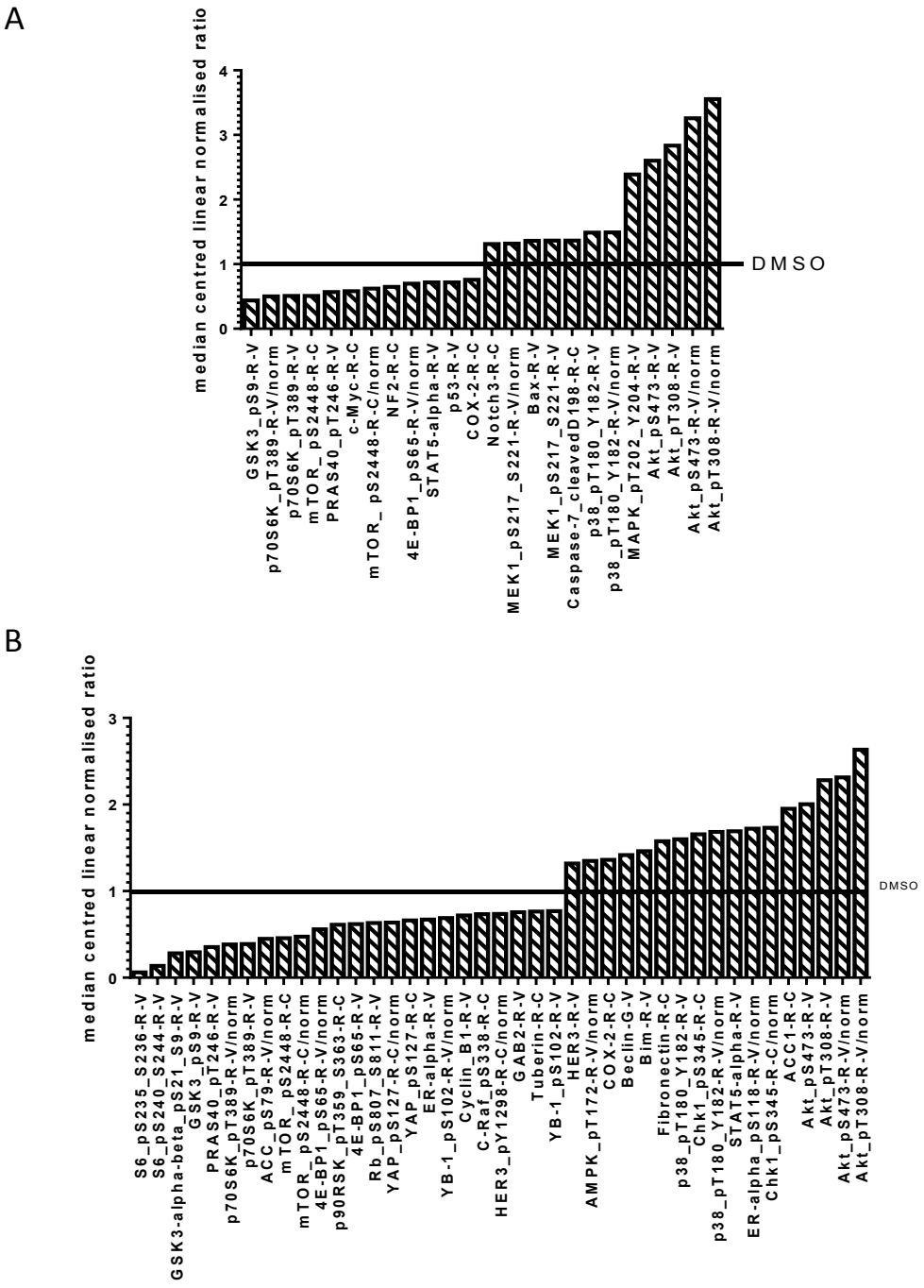


Figure.A.8 Protein expression in SKOV3 monolayers treated with combination of cisplatin and GSK2141795 in comparison with vehicle treated samples. SKOV3 monolayers were treated with combination of 5 μM GSK2141795 and 25 μM cisplatin for A) 0.5 hour and B) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and combination groups.

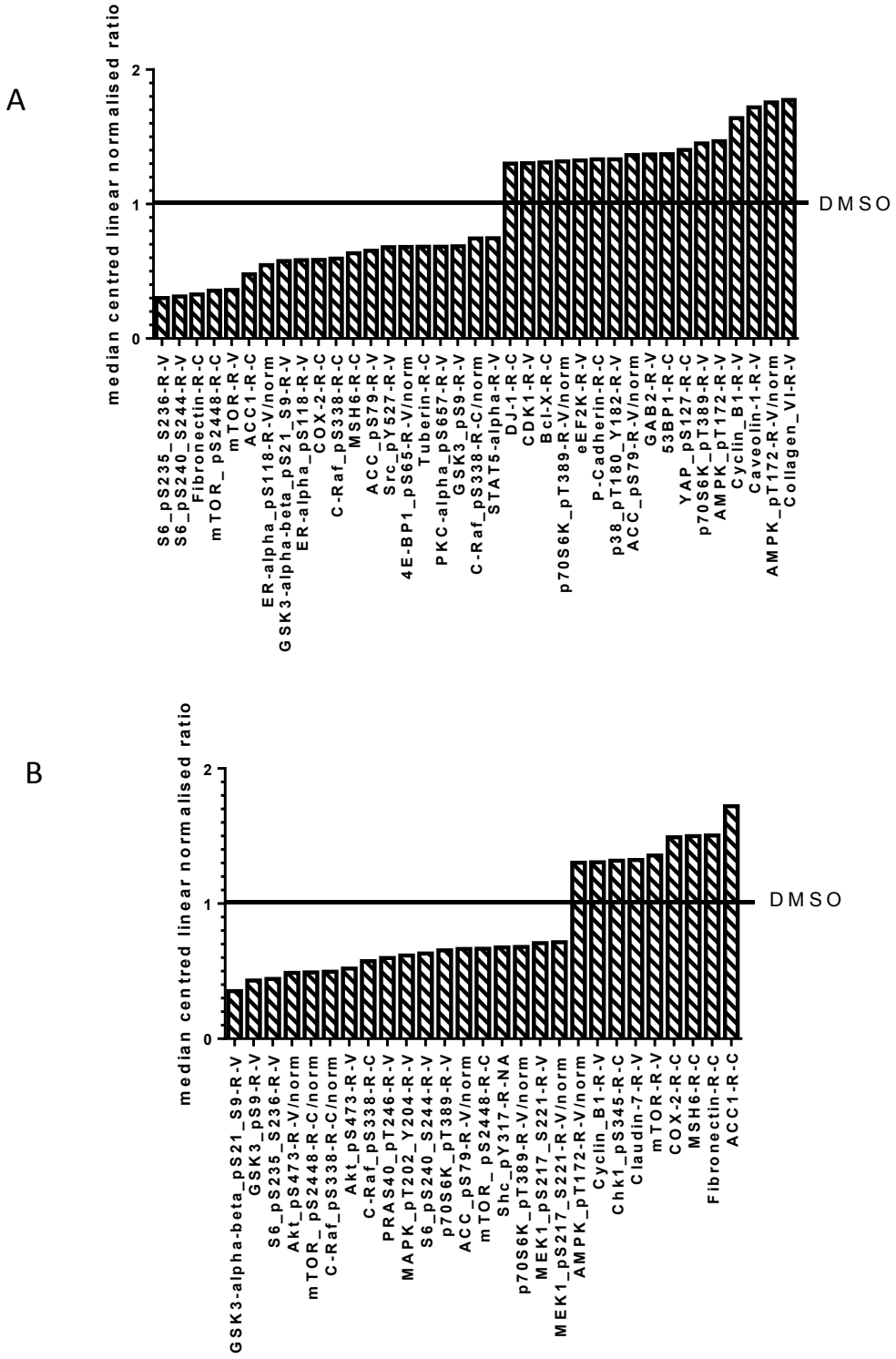
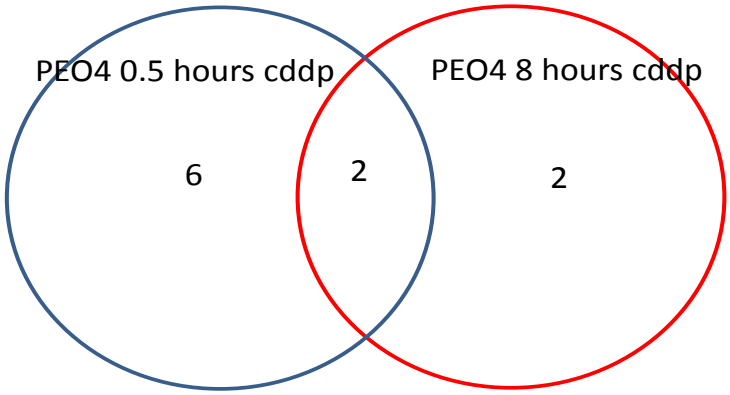


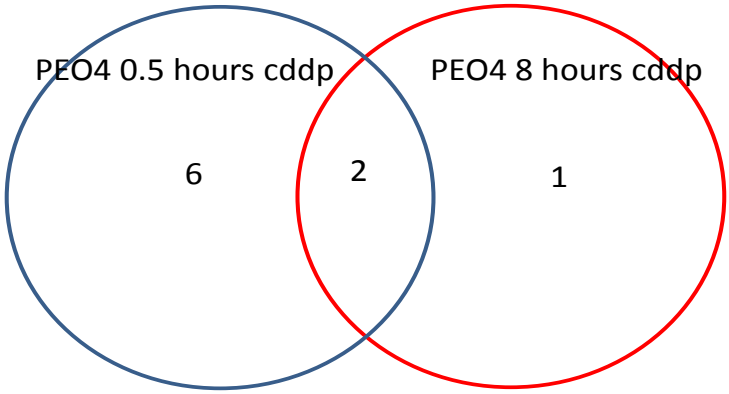
Figure.A.9 Protein expression in PEO4 monolayers treated with combination of cisplatin and GSK2141795 in comparison with vehicle treated samples. PEO4 monolayers were treated with combination of 5 μM GSK2141795 and 25 μM cisplatin for A) 0.5 hour and B) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and combination groups.

A



137

B



138

Figure A.10 Comparison of protein expression alternation in PEO4 monolayers treated with cisplatin for 30 minutes or 8 hours. Two proteins were up-regulated A) and 2 proteins were down-regulated B) in both time-points.

Table A.5 Proteins which their expressions altered in response to cisplatin in PEO4 monolayers in both 30 minutes and 8 hours.

Protein	PEO4_cddp_0.5h	PEO4_cddp_8h	Venn group
ACC_pS79/norm	Yes	Yes	3
AMPK_pT172/norm	Yes	Yes	3

Protein	PEO4_cddp_0.5h	PEO4_cddp_8h	Venn group
Akt_pS473	Yes	Yes	3
Akt_pS473/norm	Yes	Yes	3

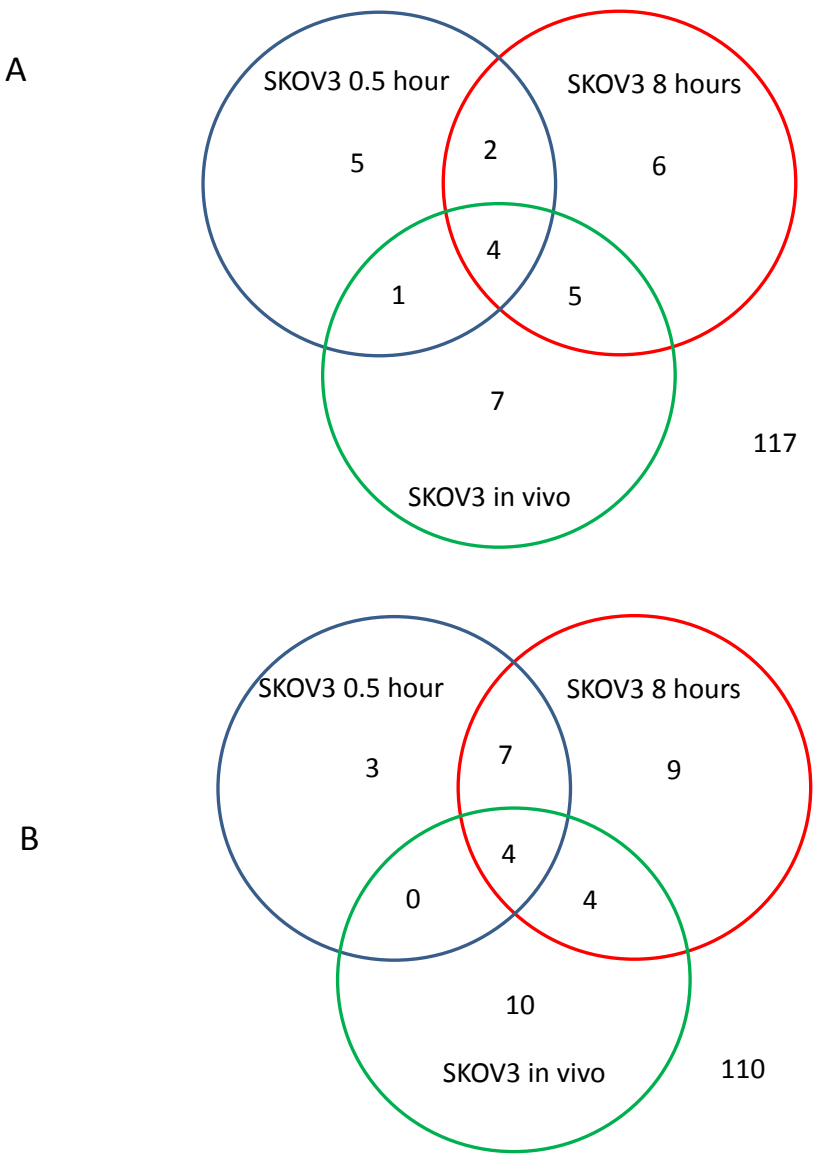


Figure A.11 Comparison of A) Up-regulation and B) down-regulation of 147 proteins in SKOV3 *in vitro* and *in vivo* models. Protein expression was compared in SKOV3 xenografts treated with combination of GSK2141795 and SKOV3 monolayers treated with combination of cisplatin and GSK2141795 for 30 minutes and 8 hours.

Table.A.6 Up-regulation of proteins expression in SKOV3 xenografts in response to combination treatment with cisplatin and GSK2141795 compared with the same treatment in SKOV3 monolayers.

Protein	SKOV3_cddp_gsk795_0.5h	SKOV3_cddp_gsk795_8h	SKOV3_vivo_cddp_795
Akt_pS473	Yes	Yes	Yes
Akt_pS473/norm	Yes	Yes	Yes
Akt_pT308	Yes	Yes	Yes
Akt_pT308/norm	Yes	Yes	Yes
p38_pT180_Y182	Yes	Yes	No
p38_pT180_Y182/norm	Yes	Yes	No
Caspase-7_cleavedD198	Yes	No	Yes
Bax	Yes	No	No
MAPK_pT202_Y204	Yes	No	No
MEK1_pS217_S221	Yes	No	No
MEK1_pS217_S221-/norm	Yes	No	No
Notch3	Yes	No	No
AMPK_pT172/norm	No	Yes	Yes
Beclin	No	Yes	Yes
Bim	No	Yes	Yes
HER3	No	Yes	Yes
STAT5-alpha	No	Yes	Yes

Table.A.7 Down-regulation of proteins expression in SKOV3 xenografts in response to combination treatment with cisplatin and GSK2141795 compared with the same treatment in SKOV3 monolayers.

Protein	SKOV3_gsk795_0.5h	SKOV3_cddp_gsk795_8h	sSKOV3_vivo_cddp+795
4E-BP1_pS65	Yes	Yes	Yes
4E-BPYes_pS65/norm	Yes	Yes	Yes
S6_pS235_S236	Yes	Yes	Yes
S6_pS240_S244	Yes	Yes	Yes
GSK3_pS9	Yes	Yes	No
GSK3-alpha-beta_pS21_S9	Yes	Yes	No
mTOR_ pS2448	Yes	Yes	No
mTOR_ pS2448/norm	Yes	Yes	No
p70S6K_pT389	Yes	Yes	No
p70S6K_pT389/norm	Yes	Yes	No
PRAS40_pT246	Yes	Yes	No
c-Myc	Yes	No	No
JNK_ pT183-T185	Yes	No	No
p53	Yes	No	No
Cyclin_B1	No	Yes	Yes
ER-alpha	No	Yes	Yes
Rb_pS807_S811	No	Yes	Yes
Tuberin	No	Yes	Yes

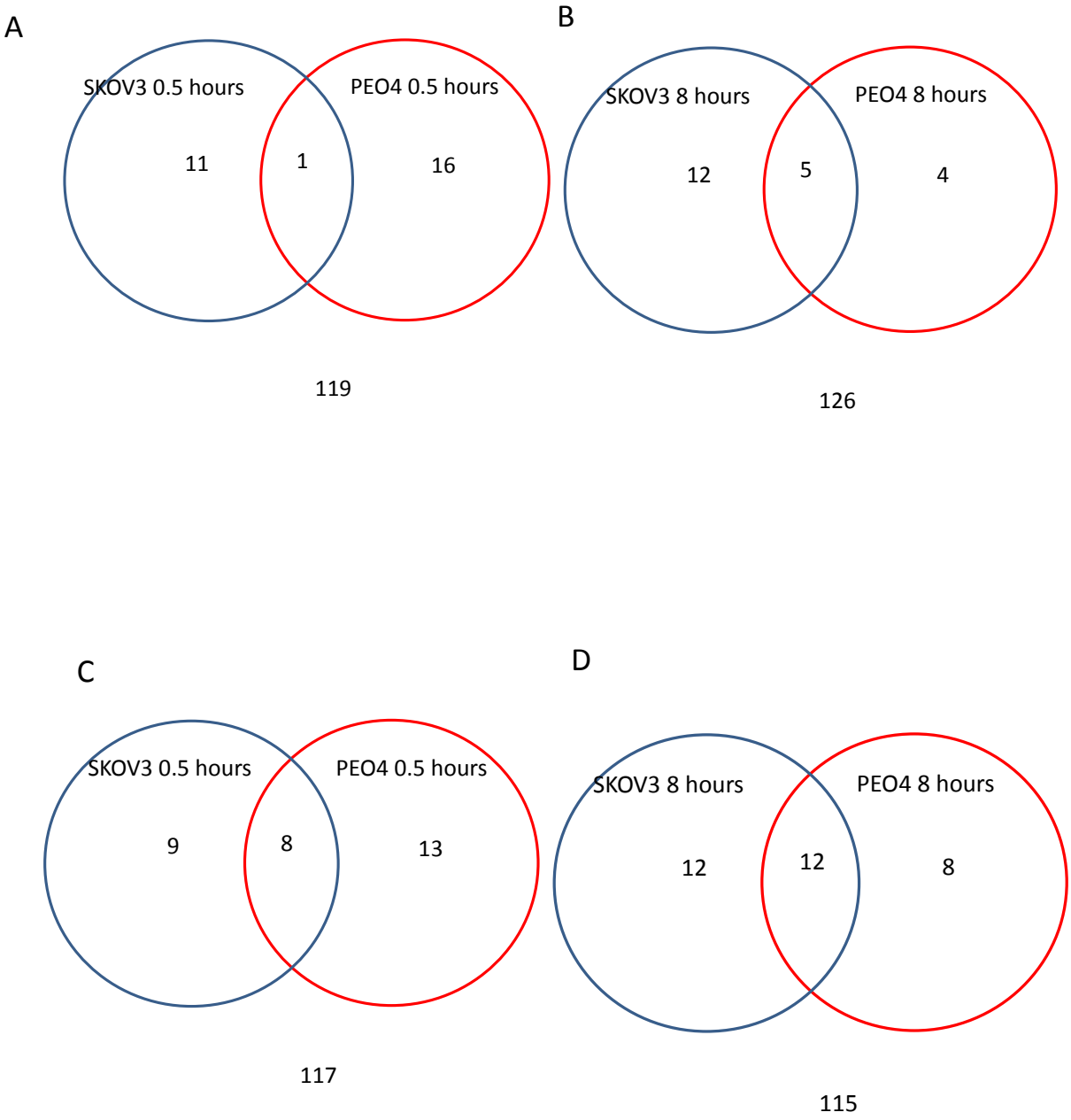


Figure A.12 Comparison of protein expression in SKOV3 and PEO4 monolayers in response to combination treatment with cisplatin and GSK2141795. One protein was found to be upregulated A) and 8 to be down-regulated C) in common in both SKOV3 and PEO4 monolayers treated with cisplatin and GSK2141795 for 30 minutes. Five proteins were upregulated B) and 12 proteins were down-regulated D) in both SKOV3 and PEO4 cells treated with cisplatin and GSK2141795 for 8 hours.

Table. A.8 Alternations in protein expression in response to combination of cisplatin and GSK2141795 in SKOV3 monolayers compared with the response to the same treatment in PEO4 cells.

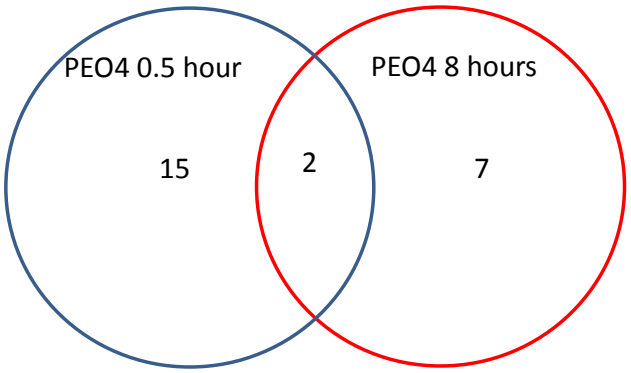
Up-regulation in SKOV3 v PEO4 cells treated with GSK795 and cddp (30 minutes)			
Protein	SKOV3_cddp_gsk795_0.5h	PEO4_cddp_gsk795_0.5h	Venn group
p38_pT180_Y182	Yes	Yes	3

Down-regulation in SKOV3 v PEO4 cells treated with GSK795 and cddp (30 minutes)			
Protein	SKOV3_cddp_gsk795_0.5h	PEO4_cddp_gsk795_0.5h	Venn group
4E-BP1_pS65/norm	Yes	Yes	3
COX-2	Yes	Yes	3
GSK3_pS9	Yes	Yes	3
GSK3-alpha-beta_pS21_S9	Yes	Yes	3
mTOR_ pS2448	Yes	Yes	3
S6_pS235_S236	Yes	Yes	3
S6_pS240_S244	Yes	Yes	3
STAT5-alpha	Yes	Yes	3

Up-regulation in SKOV3 v PEO4 cells treated with GSK795 and cddp (8 hours)			
Protein	SKOV3_cddp_gsk795_8h	PEO4_cddp_gsk795_8h	Venn group
ACC1	Yes	Yes	3
AMPK_pT172/norm	Yes	Yes	3
Chk1_pS345	Yes	Yes	3
COX-2	Yes	Yes	3
Fibronectin-R	Yes	Yes	3

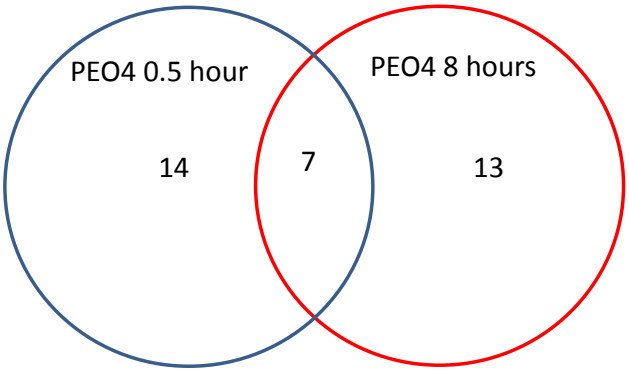
Down-regulation in SKOV3 v PEO4 cells treated with GSK795 and cddp (8 hours)			
Protein	SKOV3_cddp_gsk795_8h	PEO4_cddp_gsk795_8h	Venn group
4E-BP1_pS65	Yes	Yes	3
ACC_pS79/norm	Yes	Yes	3
C-Raf_pS338	Yes	Yes	3
GSK3_pS9	Yes	Yes	3
GSK3-alpha-beta_pS21_S9	Yes	Yes	3
mTOR_ pS2448	Yes	Yes	3
mTOR_ pS2448/norm	Yes	Yes	3
p70S6K_pT389	Yes	Yes	3
p70S6K_pT389/norm	Yes	Yes	3
PRAS40_pT246	Yes	Yes	3
S6_pS235_S236	Yes	Yes	3
S6_pS240_S244	Yes	Yes	3

A



123

B



113

Figure A.13 Comparison of protein expression alternation in PEO4 monolayers treated with combination of cisplatin and GSK2141795 for 30 minutes or 8 hours. Two proteins were up-regulated A) and 7 proteins were down-regulated B) in common in both time-points

Table A.7 Proteins which showed alternation in expression in response to combination of cisplatin and GSK2141795 in PEO4 monolayers in both 30 minutes and 8 hours

Up-regulation in protein expression in PEO4 cells treated with GSK795 and cddp for 30 minutes v 8 hrs			
Protein	PEO4_cddp_gsk795_0.5h	PEO4_cddp_gsk795_8h	Venn group
AMPK_pT172-R-V/norm	Yes	Yes	3
Cyclin_B1-R-V	Yes	Yes	3

Down-regulation in protein expression in PEO4 cells treated with GSK795 and cddp for 30 minutes v 8 hrs			
Protein	PEO4_cddp_gsk795_0.5h	PEO4_cddp_gsk795_8h	Venn group
C-Raf_pS338-R-C	Yes	Yes	3
C-Raf_pS338-R-C/norm	Yes	Yes	3
GSK3_pS9-R-V	Yes	Yes	3
GSK3-alpha-beta_pS21_S9-R-V	Yes	Yes	3
mTOR_ pS2448-R-C	Yes	Yes	3
S6_pS235_S236-R-V	Yes	Yes	3
S6_pS240_S244-R-V	Yes	Yes	3

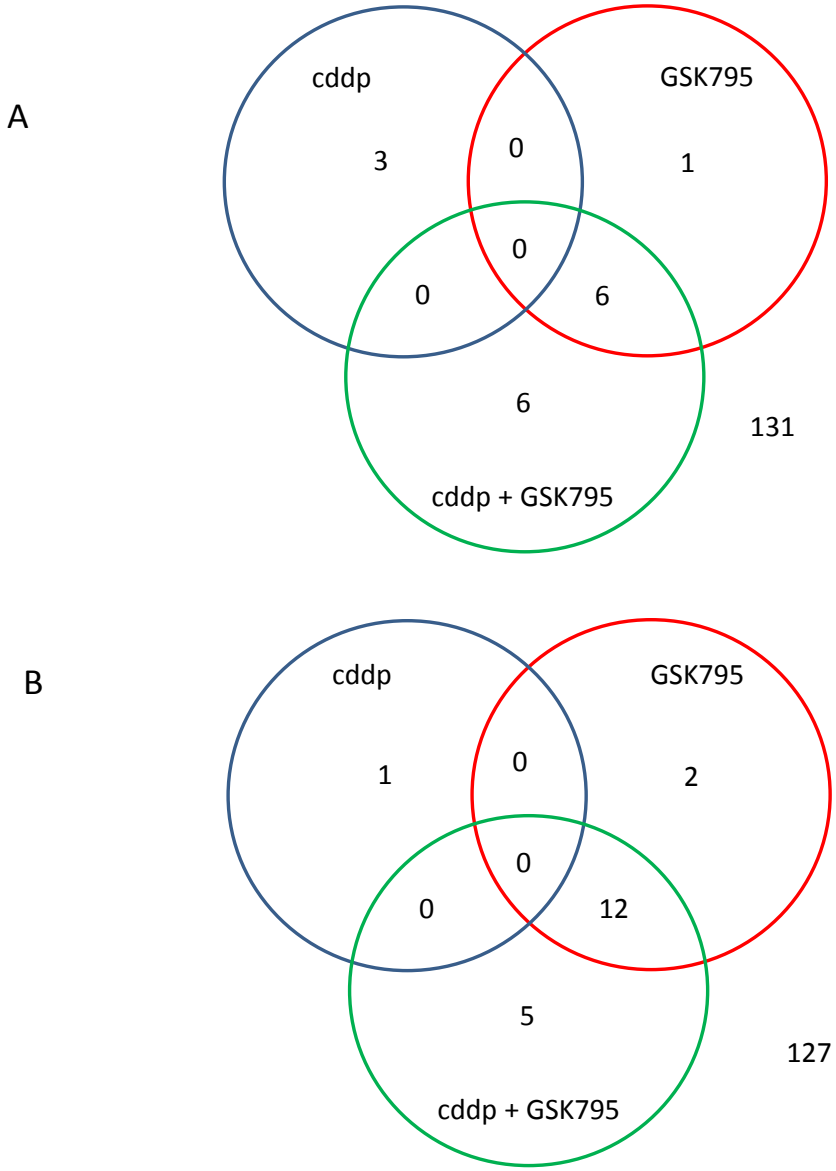


Figure A.13 Comparison of changes in protein expression level in response to cddp or GSK2141795 as single agents or in combination in SKOV3 monolayers treated for 30 minutes. SKOV3 monolayers were treated with cddp or GSK2141795 or combination of two drugs for 30 minutes. Up-regulations (A) or down-regulations (B) of protein expression in response to the three treatments were compared in Venn diagrams.

Table. A.9 Comparison of up-regulations and down-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination in SKOV3 monolayers (30 minutes treatment)

Up-regulation in protein expression level				
Protein	SKOV3_cddp_0.5h	SKOV3_gsk795_0.5h	SKOV3_cddp_gsk795_0.5h	Venn group
4E-BP1_pS65/norm	0	Yes	Yes	5
c-Myc	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
GSK3-alpha-beta_pS21	No	Yes	Yes	5
mTOR_ pS2448	No	Yes	Yes	5
mTOR_ pS2448/norm	No	Yes	Yes	5
p53	No	Yes	Yes	5
p70S6K_pT389	No	Yes	Yes	5
p70S6K_pT389/norm	No	Yes	Yes	5
PRAS40_pT246	No	Yes	Yes	5
S6_pS235_S236	No	Yes	Yes	5
S6_pS240_S244	No	Yes	Yes	5
4E-BP1_pS65	No	Yes	No	2
JNK_ pT183-T185	No	Yes	No	2
Caveolin-1	No	No	Yes	3
COX-2	No	No	Yes	3
NF2	No	No	Yes	3
Paxillin	No	No	Yes	3
STAT5-alpha	No	No	Yes	3

Down-regulation in protein expression level				
Protein	SKOV3_cddp_0.5h	SKOV3_gsk795_0.5h	SKOV3_cddp_gsk795_0.5h	Venn group
ER-alpha_pS118/norm	Yes	No	No	1
p70S6K_pT389/norm	Yes	No	No	1
S6_pS235_S236	Yes	No	No	1
Akt_pS473	No	Yes	Yes	5
Akt_pS473/norm	No	Yes	Yes	5
Akt_pT308	No	Yes	Yes	5
Akt_pT308/norm	No	Yes	Yes	5
p38_pT180_Y182	No	Yes	Yes	5
p38_pT180_Y182/norm	No	Yes	Yes	5
Beclin	No	Yes	No	2
Bax	No	No	Yes	3
Caspase-7_cleavedD198	No	No	Yes	3
MAPK_pT202_Y204	No	No	Yes	3
MEK1_pS217_S221	No	No	Yes	3
MEK1_pS217_S221/norm	No	No	Yes	3
Notch3-R-C	No	No	Yes	3

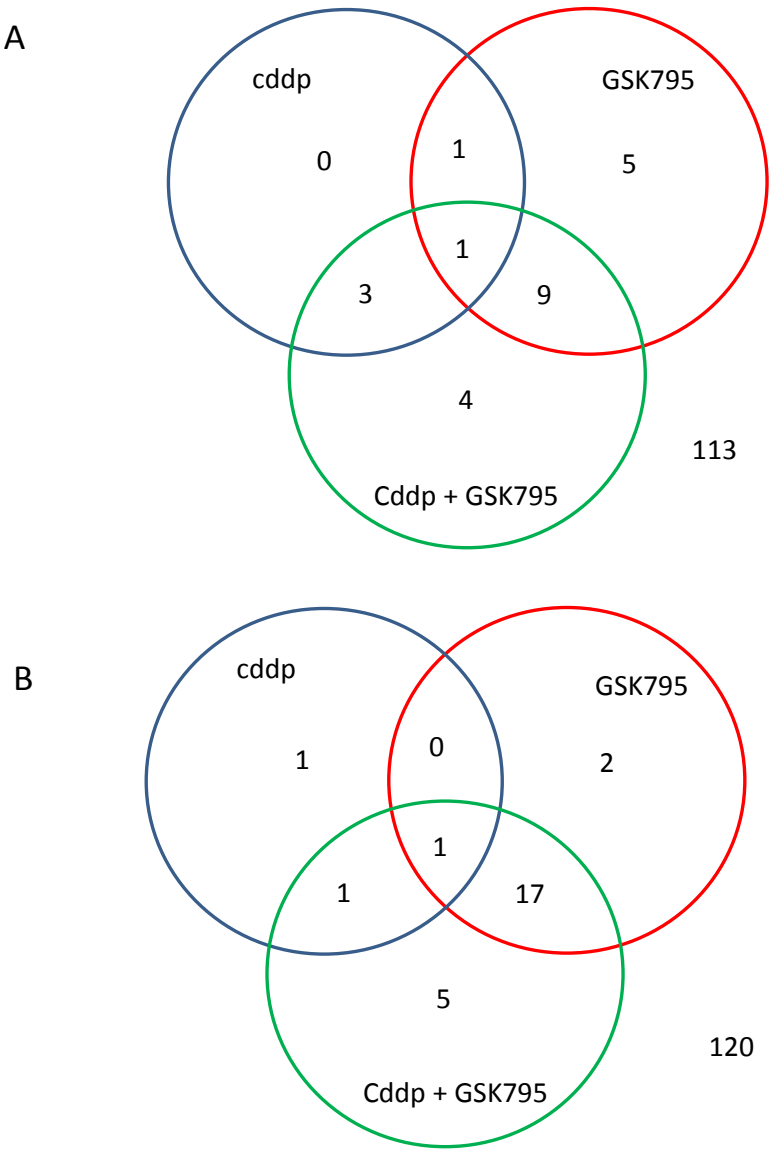


Figure A.14 Comparison of changes in protein expression levels in response to cddp or GSK2141795 as single agents or in combination in SKOV3 monolayers treated for 8 hours. SKOV3 monolayers were treated with cddp or GSK2141795 or combination of two drugs for 8 hours. Up-regulations (A) or down-regulations (B) of protein expression in response to the three treatments were compared in Venn diagrams.

Table. A.10 Comparison of up-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination at 8 hours in SKOV3

Protein	SKOV3_cddp_8h	SKOV3_gsk795_8h	SKOV3_cddp_gsk795_8h	Venn group
AMPK_pT172/norm	Yes	Yes	Yes	7
Caveolin-1	Yes	Yes	No	4
Chk1_pS345	Yes	No	Yes	6
Chk1_pS345/norm	Yes	No	Yes	6
ER-alpha_pS118/norm	Yes	No	Yes	6
ACC1	No	Yes	Yes	5
Akt_pS473	No	Yes	Yes	5
Akt_pS473/norm	No	Yes	Yes	5
Akt_pT308	No	Yes	Yes	5
Akt_pT308/norm	No	Yes	Yes	5
Bim	No	Yes	Yes	5
Fibronectin	No	Yes	Yes	5
p38_pT180_Y182	No	Yes	Yes	5
p38_pT180_Y182/norm	No	Yes	Yes	5
EGFR_pY1068/norm	No	Yes	No	2
EGFR_pY992/norm	No	Yes	No	2
N-Cadherin	No	Yes	No	2
p53	No	Yes	No	2
YB-1	No	Yes	No	2
Beclin	No	No	Yes	3
COX-2	No	No	Yes	3
HER3	No	No	Yes	3
STAT5-alpha	No	No	Yes	3

Table. A.11 Comparison of down-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination at 8 hours in SKOV3 monolayers

Protein	SKOV3_cddp_8h	SKOV3_gsk795_8h	SKOV3_cddp_gsk795_8h	Venn group
YB-1_pS102/norm	Yes	Yes	Yes	7
ER-alpha	Yes	No	Yes	6
PI3K-p110-alpha	Yes	No	No	1
4E-BP1_pS65	No	Yes	Yes	5
4E-BP1_pS65/norm	No	Yes	Yes	5
ACC_pS79/norm	No	Yes	Yes	5
C-Raf_pS338	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
GSK3-alpha-beta_pS21_S9	No	Yes	Yes	5
mTOR_ pS2448	No	Yes	Yes	5
mTOR_ pS2448/norm	No	Yes	Yes	5
p70S6K_pT389	No	Yes	Yes	5
p70S6K_pT389/norm	No	Yes	Yes	5
p90RSK_pT359_S363	No	Yes	Yes	5
PRAS40_pT246	No	Yes	Yes	5
Rb_pS807_S811	No	Yes	Yes	5
S6_pS235_S236	No	Yes	Yes	5
S6_pS240_S244	No	Yes	Yes	5
Tuberin	No	Yes	Yes	5
YAP_pS127/norm	No	Yes	Yes	5
4EBP1_pT37-T46/norm	No	Yes	No	2
ACC_pS79	No	Yes	No	2
Cyclin_B1	No	No	Yes	3
GAB2	No	No	Yes	3
HER3_pY1298/norm	No	No	Yes	3
YAP_pS127	No	No	Yes	3
YB-1_pS102	No	No	Yes	3

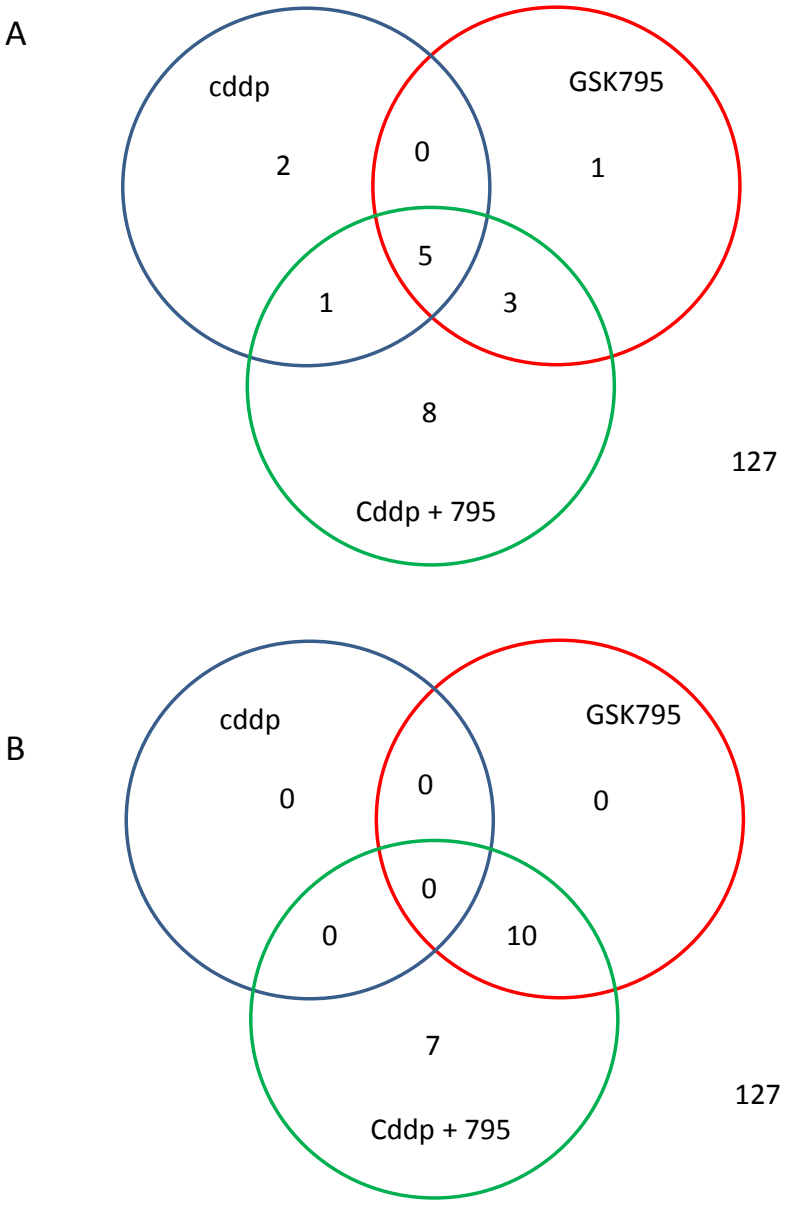


Figure A.15 Comparison of changes in protein expression level in response to cddp or GSK2141795 as single agents or in combination in PEO4 monolayers treated for 30 minutes. PEO4 monolayers were treated with cddp or GSK2141795 or combination of two drugs for 30 minutes. Up-regulations (A) or down-regulations (B) of protein expression in response to the three treatments were compared in Venn diagrams.

Table. A.12 Comparison of up-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination at 30 minutes in PEO4 monolayers

Protein	PEO4_cddp_0.5h	PEO4_gsk795_0.5h	PEO4_cddp_gsk795_0.5h	Venn group
ACC1	Yes	Yes	Yes	7
ER-alpha_pS118	Yes	Yes	Yes	7
ER-alpha_pS118/norm	Yes	Yes	Yes	7
Fibronectin	Yes	Yes	Yes	7
MSH6	Yes	Yes	Yes	7
mTOR	Yes	Yes	Yes	7
Akt_pS473	Yes	No	No	1
Akt_pS473/norm	Yes	No	No	1
4E-BP1_pS65/norm	No	Yes	Yes	5
COX-2	No	Yes	Yes	5
C-Raf_pS338	No	Yes	Yes	5
C-Raf_pS338/norm	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
GSK3-alpha-beta_pS21_S9	No	Yes	Yes	5
mTOR_ pS2448	No	Yes	Yes	5
S6_pS235_S236	No	Yes	Yes	5
S6_pS240_S244	No	Yes	Yes	5
4E-BP1_pS65	No	Yes	No	2
eEF2	No	Yes	No	2
NF2	No	Yes	No	2
ACC_pS79	No	No	Yes	3
HER2_pY1248	No	No	Yes	3
PKC-alpha_pS657	No	No	Yes	3
Src_pY527	No	No	Yes	3
STAT5-alpha	No	No	Yes	3
Tuberin	No	No	Yes	3

Table. A.13 Comparison of down-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination at 30 minutes in PEO4 monolayers

Protein	PEO4_cddp_0.5h	PEO4_gsk795_0.5h	PEO4_cddp_gsk795_0.5h	Venn group
ACC1	Yes	Yes	Yes	7
ER-alpha_pS118	Yes	Yes	Yes	7
ER-alpha_pS118/norm	Yes	Yes	Yes	7
Fibronectin	Yes	Yes	Yes	7
MSH6	Yes	Yes	Yes	7
mTOR	Yes	Yes	Yes	7
Akt_pS473	Yes	No	No	1
Akt_pS473/norm	Yes	No	No	1
4E-BP1_pS65/norm	No	Yes	Yes	5
COX-2	No	Yes	Yes	5
C-Raf_pS338	No	Yes	Yes	5
C-Raf_pS338/norm	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
GSK3-alpha-beta_pS21_S9	No	Yes	Yes	5
mTOR_ pS2448	No	Yes	Yes	5
S6_pS235_S236	No	Yes	Yes	5
S6_pS240_S244	No	Yes	Yes	5
4E-BP1_pS65	No	Yes	No	2
eEF2	No	Yes	No	2
NF2	No	Yes	No	2
ACC_pS79	No	No	Yes	3
HER2_pY1248	No	No	Yes	3
PKC-alpha_pS657	No	No	Yes	3
Src_pY527	No	No	Yes	3
STAT5-alpha	No	No	Yes	3
Tuberin	No	No	Yes	3

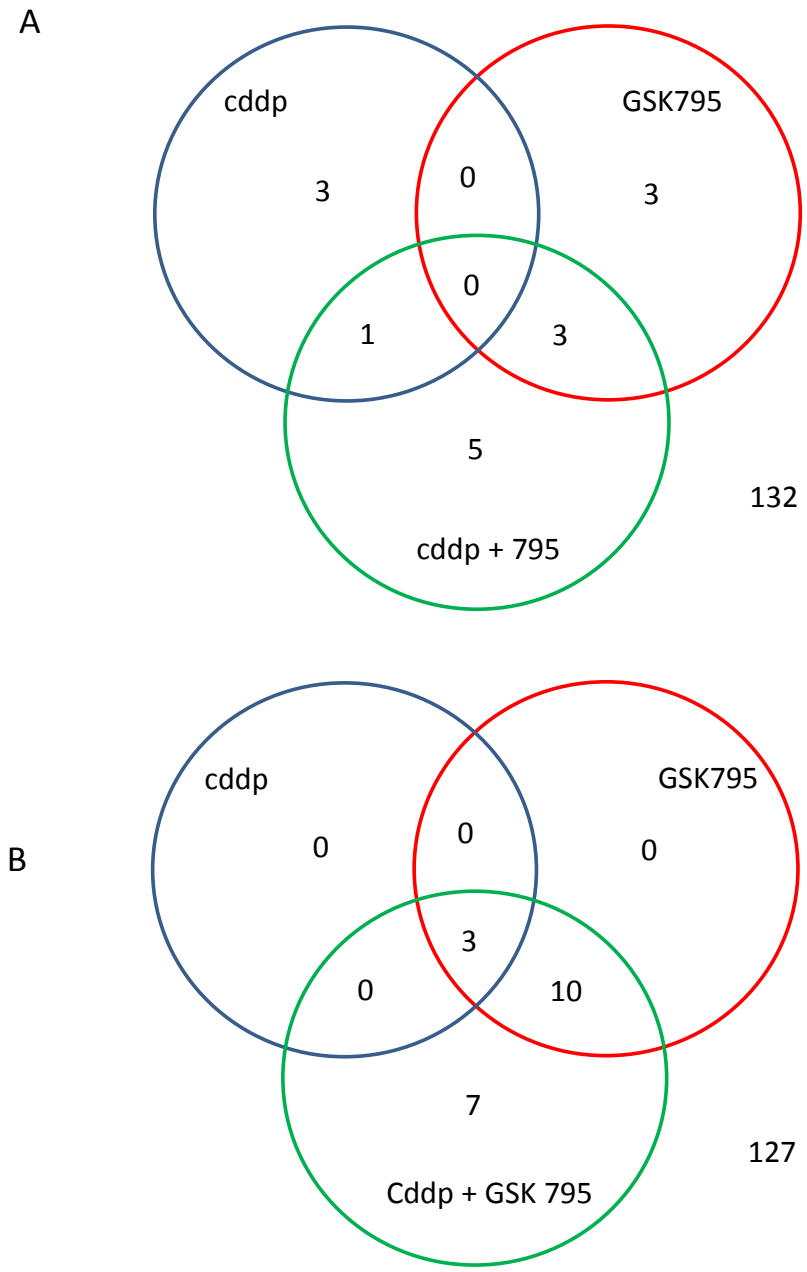


Figure A.16 Comparison of changes in protein expression level in response to cddp or GSK2141795 as single agents or in combination in PEO4 monolayers treated for 8 hours. PEO4 monolayers were treated with cddp or GSK2141795 or combination of two drugs for 30 minutes. Up-regulations (A) or down-regulations (B) of protein expression in response to the three treatments were compared in Venn diagrams.

Table. A.14 Comparison of up-regulations and down-regulations in protein expression levels in response to cddp and GSK2141795 as single agents or in combination for 8 hours in PEO4 monolayers

Up-regulations				
Protein	PEO4_cddp_8h	PEO4_gsk795_8h	PEO4_cddp_gsk795_8h	Venn group
AMPK_pT172/norm	Yes	No	Yes	6
ACC_pS79	Yes	No	No	1
ACC_pS79/norm	Yes	No	No	1
Chk1_pS345/norm	Yes	No	No	1
ACC1	No	Yes	Yes	5
Claudin-7	No	Yes	Yes	5
COX-2	No	Yes	Yes	5
Akt_pT308	No	Yes	No	2
Akt_pT308/norm	No	Yes	No	2
VEGFR2	No	Yes	No	2
Chk1_pS345	No	No	Yes	3
Cyclin_B1	No	No	Yes	3
Fibronectin	No	No	Yes	3
MSH6	No	No	Yes	3
mTOR	No	No	Yes	3

Down-regulations				
Protein	PEO4_cddp_8h	PEO4_gsk795_8h	PEO4_cddp_gsk795_8h	Venn group
Akt_pS473	Yes	Yes	Yes	7
Akt_pS473/norm	Yes	Yes	Yes	7
p70S6K_pT389	Yes	Yes	Yes	7
C-Raf_pS338	No	Yes	Yes	5
C-Raf_pS338/norm	No	Yes	Yes	5
GSK3_pS9	No	Yes	Yes	5
GSK3-alpha-beta_pS21_S9	No	Yes	Yes	5
mTOR_ pS2448	No	Yes	Yes	5
mTOR_ pS2448/norm	No	Yes	Yes	5
p70S6K_pT389/norm	No	Yes	Yes	5
PRAS40_pT246	No	Yes	Yes	5
S6_pS235_S236	No	Yes	Yes	5
S6_pS240_S244	No	Yes	Yes	5
4E-BP1_pS65	No	No	Yes	3
ACC_pS79/norm	No	No	Yes	3
MAPK_pT202_Y204	No	No	Yes	3
MEK1_pS217_S221	No	No	Yes	3
MEK1_pS217_S221/norm	No	No	Yes	3
p38_pT180_Y182	No	No	Yes	3
Shc_pY317	No	No	Yes	3

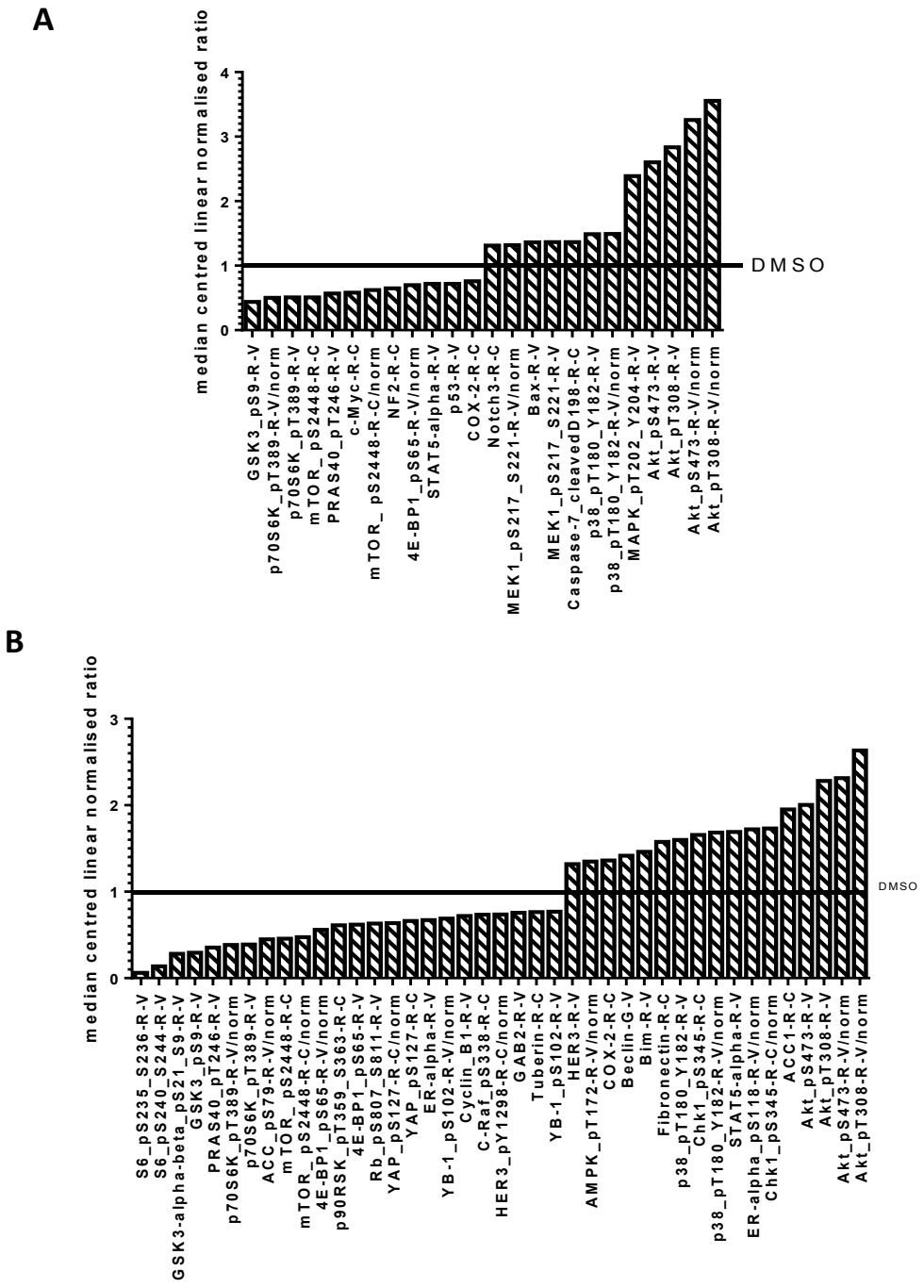


Figure.A.17 Protein expression in SKOV3 monolayers treated with combination of cisplatin and GSK2141795 in comparison with vehicle treated samples. SKOV3 monolayers were treated with combination of 5 μM GSK2141795 and 25 μM cisplatin for a) 0.5 hour and b) 8 hours. Proteins were filtered using 1.3-fold cut off between vehicle and combination groups.

Table A.15. Differences in protein expression between biopsies taken from responder and non-responder patients prior to the daily treatment with GSK2141795

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
MAPK__pT202_204	-2.77	-0.29	0.11	0.02
Xiap(C)_1	-2.76	-0.17	0.06	0.02
PARP_cleaved	2.42	0.40	0.16	0.04
PKC_pS657	-2.08	-0.19	0.09	0.06
MAPK_pT202	-2.03	-0.42	0.21	0.07
PKC_pS657	-1.99	-0.23	0.12	0.08
4EBP1_pT70_/norm	-1.97	-0.21	0.11	0.08
Stat3_pS705	-1.96	-0.21	0.11	0.08
p90RSK_pT359	-1.89	-0.13	0.07	0.09
PR	1.85	2.39	1.29	0.09
ACC1	1.75	0.57	0.33	0.11
YAP	1.67	0.52	0.31	0.13
CHK2_pT68	-1.62	-0.20	0.12	0.14
IGFBP2	-1.61	-1.59	0.99	0.14
Myc_2	-1.59	-0.27	0.17	0.14
p27_1	1.59	0.41	0.26	0.14
4EBP1_pT70_/norm	-1.58	-0.16	0.10	0.15
MSH2	1.55	0.50	0.32	0.15
4EBP1	1.53	0.19	0.13	0.16
Gata3	-1.50	-0.14	0.09	0.16
LKB1	-1.50	-0.17	0.12	0.17
Shc_pY317	-1.46	-0.17	0.12	0.17
Rad50	-1.45	-0.15	0.10	0.18
AKT	-1.44	-0.27	0.19	0.18
p70S6K_pT389_1	1.44	0.54	0.37	0.18
CDC2	1.43	0.35	0.25	0.18

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
S6_pS240_244_2	1.36	0.45	0.33	0.20
mTOR	-1.33	-0.21	0.16	0.21
BIM	1.32	0.54	0.41	0.22
S6_pS235_236_1	1.30	0.52	0.40	0.22
Rad50	-1.27	-0.21	0.17	0.23
Myc_1	-1.24	-0.19	0.15	0.24
p70S6K_pT389/norm_tp70S6K_1	1.23	0.59	0.48	0.25
YAP_pS127_/norm_tYAP	-1.22	-0.31	0.25	0.25
Rb(4H1)	-1.20	-0.10	0.08	0.26
p38___MAPK	1.18	0.14	0.12	0.26
4EBP1_pS65(V)_/norm	-1.17	-0.18	0.16	0.27
MEK1_pS217_221	-1.17	-0.09	0.08	0.27
Notch3	1.16	0.15	0.13	0.27
eIF4E	1.15	0.24	0.21	0.28
INPP4B	-1.15	-0.32	0.28	0.28
AR	-1.11	-0.24	0.21	0.29
MSH6	1.10	0.39	0.35	0.30
Tau©	1.07	0.20	0.19	0.31
Caveolin-1	1.06	0.64	0.60	0.31
ERK2	-1.05	-0.20	0.19	0.32
Cadherin-P	1.04	2.84	2.73	0.32
53BP1	-1.04	-0.22	0.21	0.32
PTEN	-1.03	-0.23	0.23	0.33
PCNA(V)	1.02	1.72	1.68	0.33
Cadherin-P	1.01	2.14	2.12	0.34
NF-kB_p65_pS536	-1.01	-0.17	0.17	0.34
Bcl-xL	-0.99	-0.05	0.05	0.34
Snail	-0.99	-0.20	0.21	0.35
EGFR	-0.99	-0.15	0.15	0.35

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
Src_pY527_/norm_tSrc	0.99	0.16	0.16	0.35
Src(V)	-0.98	-0.40	0.41	0.35
b-Catenin	-0.98	-0.36	0.37	0.35
Stathmin	0.96	0.14	0.15	0.36
Beclin	-0.94	-0.36	0.39	0.37
p38_T180_182_/norm_tp38_MAPK	-0.94	-0.33	0.35	0.37
VEGFR2	-0.93	-0.24	0.26	0.37
p27_pT157_/notm__p27	-0.93	-0.36	0.39	0.37
MEK1_pS217_221_/norm_tMEK1	-0.91	-0.07	0.08	0.39
S6_	0.90	0.18	0.20	0.39
YB1	0.90	0.12	0.13	0.39
Tuberrin(TSC2)	-0.89	-0.10	0.11	0.39
XBP-1	-0.89	-0.26	0.30	0.39
4EBP1_pT37	0.89	0.13	0.15	0.40
	0.90	0.12	0.13	0.40
FOX03a_	0.86	0.13	0.15	0.41
PI3K-p85	0.86	0.15	0.18	0.41
Cyclin_B1_	0.85	0.37	0.43	0.41
Bcl-2	0.84	0.59	0.70	0.42
eEF2K	-0.84	-0.33	0.39	0.42
cJun_pS73	-0.83	-0.07	0.08	0.43
Bak	0.83	0.13	0.16	0.43
Src_pY416_/norm_tSrc	0.82	0.27	0.33	0.43
FOXO3a_pS318/norm_tFOXO3a	-0.81	-0.12	0.14	0.44
Collagen_VI	0.80	1.81	2.26	0.44
EGFR_pY1173	-0.80	-0.08	0.10	0.44
PKCa	-0.79	-0.09	0.11	0.45
Catenin_Beta	-0.79	-0.25	0.31	0.45
Chk1_pS345	-0.79	-0.10	0.13	0.45

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
p27_pT198_/norm__tp27	-0.78	-0.48	0.62	0.46
PTCH	-0.77	-0.15	0.20	0.46
PARP_Cleaved	-0.75	-0.12	0.16	0.47
HER2_pY1248_/norm_tHER2	-0.75	-0.92	1.23	0.47
BAX	0.74	0.08	0.11	0.48
Cyclin_E1	0.74	0.31	0.42	0.48
Rb_pS807_811_/norm_tRb	0.74	0.21	0.29	0.48
p21	0.73	0.15	0.21	0.48
PI3K-p85	0.73	0.15	0.21	0.48
IGFR1b	-0.73	-0.14	0.20	0.48
Notch-1	-0.73	-0.58	0.80	0.48
ERa_pS118_1_/norm_tERa	-0.72	-0.34	0.47	0.49
ERa_pS118	-0.72	-0.27	0.37	0.49
Met©	-0.71	-0.50	0.71	0.50
Mre11(31H)	-0.70	-0.29	0.41	0.50
Pea15	0.69	0.19	0.27	0.50
Cyclin_D1	-0.67	-0.09	0.14	0.52
Pras40_pT246	0.66	0.05	0.07	0.52
GSK3_pS21/norm_tGSK3beta	-0.66	-0.25	0.38	0.52
p70S6K_pT389_2	-0.65	-0.08	0.13	0.53
c-Met_pY1235	-0.65	-0.17	0.27	0.53
Rab25	0.64	0.25	0.39	0.54
HER2_pY1248	-0.63	-0.73	1.16	0.55
p27_2	0.61	0.14	0.22	0.55
p27_pT157	-0.61	-0.24	0.39	0.56
AKT_pS473	-0.61	-0.27	0.44	0.56
Caspase_7_cleaved_Asp198	-0.61	-0.26	0.43	0.56
Rab25	0.60	0.40	0.67	0.56
p53(CS9282)_	0.58	0.12	0.20	0.57

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
FOX03a_pS318_321_/norm_tFOXO3a	-0.58	-0.09	0.15	0.58
p70S6K_pT389_3	0.56	0.08	0.15	0.59
p53_	-0.55	-0.34	0.62	0.59
Src_pY416	0.55	0.14	0.26	0.60
p27_pT198	-0.55	-0.31	0.58	0.60
Pras40_pT246	0.54	0.04	0.08	0.60
YB1_pS102(V)_	0.54	0.08	0.16	0.60
FOXO3a_pS318	-0.53	-0.04	0.08	0.61
eEF2	-0.52	-0.34	0.66	0.62
Taz	0.52	0.07	0.14	0.62
MIG-6(V)	-0.51	-0.10	0.20	0.62
CHK2_pT68_/norm	-0.50	-0.15	0.30	0.63
S6_pS235_236_/norm_tS6_1	0.50	0.17	0.34	0.63
Raf-B©	-0.50	-0.09	0.17	0.63
4EBP1_pT37(V)_norm	-0.49	-0.08	0.16	0.64
4EBP1_pS65	0.49	0.09	0.19	0.64
Raf-C_pS338_2	-0.48	-0.06	0.12	0.64
p38_T180_182	-0.48	-0.18	0.38	0.64
Kit-_1	0.48	0.09	0.20	0.64
Raf-C__pS338_1	-0.48	-0.07	0.14	0.64
caspase-3_(active)	-0.47	-0.05	0.10	0.65
ACC_pS79	-0.46	-0.28	0.61	0.65
NF2	-0.46	-0.09	0.19	0.66
Smad4	-0.45	-0.24	0.52	0.66
Smad3	-0.44	-0.03	0.07	0.67
p70S6K_pT389_/norm_tp70S6K_2	-0.44	-0.09	0.19	0.67
FAK	0.43	0.46	1.09	0.68
Taz_pS89	-0.42	-0.03	0.08	0.68
Taz_pS89_/norm_tTaz	-0.42	-0.06	0.13	0.69

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
S6_pS240_244_/norm_tS6_2	0.41	0.10	0.25	0.69
E-cadherin	-0.38	-0.21	0.54	0.71
AKT_pS473_/norm_tAKT	-0.38	-0.19	0.49	0.71
Raf-C_pS338_/norm_tRaf-C_2	0.38	0.12	0.33	0.71
Bcl-X	-0.37	-0.04	0.11	0.72
Rb_pS807_811_	0.35	0.12	0.34	0.73
Raf-C	-0.35	-0.04	0.12	0.73
c-Jun	0.34	0.05	0.16	0.74
PI3K_P110a	0.34	0.04	0.10	0.74
XRCC1	0.34	0.02	0.07	0.74
Raf-C__pS338_/norm_tRaf-C_1	0.33	0.08	0.25	0.75
p90RSK_pT359_S363	0.33	0.04	0.12	0.75
ERa_pS118	0.33	0.04	0.13	0.75
ERCC1©	0.32	0.01	0.05	0.76
GSK3_pS21	-0.31	-0.11	0.35	0.76
p70S6K_pT389_/norm_tp70S6K_3	0.30	0.08	0.26	0.77
PDK1_pS241	-0.29	-0.04	0.14	0.77
p70S6K	0.27	0.04	0.15	0.79
AKT_pT308/norm_tAKT	0.26	0.08	0.31	0.80
Vasp	0.26	0.05	0.21	0.80
EGFR_pY1173_/norm_tEGFR	0.23	0.02	0.08	0.82
ATM_	-0.23	-0.13	0.54	0.82
CD31(V)	-0.23	-0.03	0.13	0.82
Ku80	0.23	0.04	0.16	0.82
GSK3-beta	0.23	0.03	0.12	0.82
IRS-1	0.22	0.04	0.17	0.83
Stat5	-0.21	-0.13	0.64	0.84
Src_pY527	0.21	0.02	0.10	0.84
Cox-2	0.19	0.03	0.18	0.85

protein	t-test for Equality of Means	Mean Difference between Responders and Non-responders	Std. Error Difference between Responders and Non-responders	p-value
CHK1	0.19	0.03	0.16	0.86
HER2	0.18	0.05	0.25	0.86
AIB-1	-0.17	-0.02	0.12	0.87
p53_	0.15	0.04	0.28	0.89
Chk1_pS345_/norm	-0.15	-0.03	0.19	0.89
AKT_pT308	0.14	0.04	0.28	0.89
FOX03a_pS318_321	-0.10	-0.01	0.11	0.92
4EBP1_pT70_	0.08	0.01	0.07	0.94
YB1_pS102(V)_/norm_tYB1	0.08	0.02	0.23	0.94
MEK1	-0.06	0.00	0.08	0.95
DJ-1	-0.06	-0.01	0.11	0.95
Transglutaminase	-0.05	-0.02	0.42	0.96
YAP_pS127	0.05	0.01	0.26	0.96
Annexin	0.03	0.01	0.35	0.97
Claudin7_CLDN7	-0.03	-0.01	0.44	0.98
Pras40_pT246	0.03	0.00	0.16	0.98
PI3K-p85	-0.03	-0.01	0.25	0.98
Rad51	0.02	0.00	0.17	0.98
Chk2(1C12)	-0.02	0.00	0.18	0.99
Paxillin	0.01	0.00	0.13	0.99