

To my girls Constance and Amélie

And

My paternal grandfather the late Charan Singh Dhillon

Defining the clinical role of the mammalian target of rapamycin (mTOR) in lung cancer

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Declaration of Originality

Unless otherwise stated in the text, this thesis is the result of my own work.

ABSTRACT

Lung cancer is the biggest cancer killer. Most patients present with advanced disease. These diseases are not amenable to surgery which currently provides the best chance of cure. New treatment approaches are urgently needed, and these will come from a greater understanding of the disease biology. Our group has identified growth factor signaling molecules, downstream of growth factor receptors, which are overexpressed in all SCLC tumours examined compared to lung epithelial cells. These include the growth controlling kinase the mammalian target of rapamycin (mTOR). Here the importance of mTOR in lung cancer was examined.

We examined the expression of mTOR in NSCLC and SCLC patients. We demonstrated that total mTOR was overexpressed in early stage NSCLC patients and in SCLC patients. More specifically mTOR predicts poor outcome in early stage NSCLC patients. It appears to identify poor outcome in lymph node negative and stage IA patients where previously other markers have failed to consistently yield prognostic benefit. This information may help identify those at high risk of relapse.

We demonstrate that using total protein immunohistochemistry may be more useful than using phospho-protein expression in assessing the contribution of a signaling protein to cancer biology in patient specimens. We show that in cell lines, formalin fixed cell line and in tumour biopsies phospho-mTOR signals varies in intensity with time from removing from media/blood supply. We suggest that no conclusions can be drawn from commonly used phospho-protein immunohistochemical studies and total protein studies may be more reliable.

Previously our laboratory has shown that high concentrations of rapamycin, an mTOR inhibitor, block MEK/ERK signaling which is necessary for pro-survival/chemoresistance effects induced by FGF-2 in SCLC cells. We demonstrate that this phenomenon is also observed with the clinically relevant mTOR inhibitor- RAD001 and other growth factors/chemicals in other SCLC cell lines, but not in NSCLC cell lines. It is therefore possible that high concentrations of RAD001 might help to reduce FGF-2-induced chemoresistance in SCLC. Moreover, by impairing growth factor induced ERK signaling; RAD001 has an additional mechanism of blocking growth factor induced proliferation. This could be a potentially novel therapeutic method.

We investigated the mechanism of how mTOR mediates the ability of high concentrations of rapalogues to suppress MEK/ERK signaling and FGF-2 triggered chemoresistance. Using siRNAi and a PP2A inhibitor to various components of the pathways involved, we have found that neither FKBP12 nor mTOR is directly involved whilst the phosphatase PP2A may aid the mTOR regulation of MEK/ERK signaling.

We established, using combination analysis, that the H69 SCLC cell line is more sensitive to combined mTOR inhibitor/cytotoxic chemotherapy killing compared to the H510 SCLC cell line and the order of drug exposure may not be important.

Collectively, our results show that mTOR is important in NSCLC as well as SCLC biology providing a possible biomarker for poor outcome and a suitable target for new therapies such as RAD001.

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ABBREVIATIONS

4E-BP1	4E Binding Protein 1
ADH	Vasopressin
AIDS	Acquired Immune Deficiency Syndrome
AKT	Protein kinase B
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate –activated protein kinase
APAF-1	Apoptotic protease activating factor-1
ATP	Adenosine Triphosphate
BAD	BCL-2 associated death promoter
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2- associated X protein
BCL-2	B-cell leukemia-2 protein
BRAC1	Breast cancer 1
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CDK	Cyclin-dependent Kinase
CI	Combination Index
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DR	Death Receptor
ECL	Enhanced chemo-luminescence

ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eIF4	Eukaryotic Translation Initiation Factor 4E
ERCC1	Excision Repair Cross-Complementation Group 1 Protein
ERK	Extracellular signal-regulated kinase
FasL	Fas Ligand
FAT	FRAP-ATM-TTRAP
FCS	Foetal Calf Serum
FDA	Federal Drug Administration
FGF	Fibroblast Growth Factor
FHTT	Fragile Histidine Triad
FKHR	Forkhead homolog 1 rhabdomyosarcoma
FRAP	FK506 binding protein 12-rapamycin associated protein
FRB	FKBP-Rapamycin Binding domain
FTI	Farnesyl Transferase inhibition
GAP	GTPase-activating protein
GDP	Guanine Diphosphate
Grb2	Growth Factor Receptor-bound Protein2
GRP	Gastrin releasing peptide
GTP	Guanine Triphosphate
HEAT	Huntington, Elongation factor 3
HGF	Hepatocyte Growth Factor
HIF1 α	Hypoxia Inducible Factor 1 α

HRP	Horseradish Peroxidase
IACD	Inhibitor of Caspase-3- activated DNase
IAG	Immediate Early Genes
IAP	Inhibitor of apoptosis protein
IC	Inhibitory Concentration
IGF-I	Insulin-like Growth Factor I
IHC	Immunohistochemistry
IHS	Immunohistochemistry Score
JNK	Jun N-terminal kinase
LAT-1	L-type amino acid transporter 1
LDH	Lactate Dehydrogenase
LOH	Loss of Heterozygosity
MAPK	Mitogen-activated Protein Kinase
MEK	Mitogen activated protein/extracellular signal-regulated kinase kinase
mLST8	mTOR associated protein, LST8 homolog
mSIN1	Mammalian Stress-Activated Protein Kinase interacting Protein 1
mTOR	mammalian Target of Rapamycin
NaSe	Sodium Selenite
NCI	National Cancer Institute
NSE	Non-Specific Enolase
NSCLC	Non-small Cell Lung Cancer
ODC1	Ornithine Decarboxylase 1
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffer Solution

PCI	Prophylactic Cranial Irridiation
PDB	Phorbol Ester
PDCD	Phosphorylating Programmed Cell Death
PDGFR	Platelet Derived Growth Factor Receptor
PK1	Phosphoinositide-Dependent Kinase-1
PH	Plekstrin Homology
PI	Phosphoinositides
PI3K	Phosphatidylinositol-3-Kinase
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PIKK	Phosphatidylinositol Kinases
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PPR5	Proline Rich Protein 5
PRAS40	Proline Rich AKT Substrate 40kDa
PS	Performance Status
PtdIns	Phosphatidylinositol-4, 5-Bisphosphate
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyacrylamide Fluoride
RAPTOR	Regulatory Associated Protein of mTOR
RASSF1A	RAS association family 1 gene
RHEB	Ras Homolog Enriched in Brain
Rb	Retinoblastoma
RICTOR	Rapamycin-Insensitive Companion of mTOR
RPMI	Roswell Park Memorial Institute

RSK	Ribosomal S6 Kinases
RTK	Receptor Tyrosine Kinases
S6K1	S6 Kinase 1
S6K2	S6 Kinase 2
SCC	Squamous Cell Carcinoma
SCF	Stem Cell Factor
SCLC	Small Cell Lung Cancer
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SFM	Serum Free Medium
SH2	Src Homology
SNP	Single Nucleotide Polymorphism
SoS	Son of Sevenless
SPSS	Statistical Package for Social Sciences
STATs	Signal Transducers and Activators of Transcription Factors
TBST	Tris Buffered Saline-Tween
TK	Tyrosine Kinase
TMA	Tissue Microarray
TNF	Tumour Necrosis Factor
TNM	Tumour Node Metastasis
TOP	Terminal Oligopyrimidine
TOR	Target of Rapamycin
TOS	Target of Rapamycin signaling
TRAIL	TNF-related apoptosis-inducing ligand

TSC	Tuberose Sclerosis Complex
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

CHAPTER I

INTRODUCTION

I.1 Lung cancer

Lung cancer is a major global health problem. It accounts for more deaths worldwide than any other malignant disease (Parkin et al., 2005). About 1.2 million people die of lung cancer each year and the incidence is increasing. In western countries the incidence is declining but in Eastern Europe and the developing world where the number of smokers remain high, lung cancer rates are increasing.

Only 13% of lung cancer patients survive more than 5 years. The disease has no particular symptoms or signs for its detection at an early stage. Most patients therefore present with advanced disease and hence are inoperable. Clearly advances are needed in early diagnosis and in new therapeutic strategies.

Lung cancer is histologically divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) and within each type many sub-divisions (Travis et al., 1999) (Table 1.1). However the main distinction is between SCLC and NSCLC because of the manner of how SCLC presents its metastatic spread and response to treatment.

Histological sub-classification of NSCLC includes adenocarcinoma (30% of cases) (whose incidence is rising at the expense of squamous cell), squamous cell (45% of cases) and large cell (<10% of cases) whilst SCLC comprises small cell carcinomas, mixed small cell and combined small cell carcinomas. Approximately 80-85% of presenting lung cancer cases are NSCLC and 15-20% are SCLC.

Small Cell Lung Cancer
Variant Combined small cell carcinoma Mixed small cell/large cell carcinoma
Non- Small Cell Lung Cancer
Adenocarcinoma Acinar Papillary Bronchioloalveolar Solid adenocarcinoma with mucin subtypes Adenocarcinoma with mixed subtypes Squamous cell carcinoma Papillary Clear cell Small cell Basoloid Large Cell carcinoma Adenosquamous carcinoma Carcinomas with pleomorphic sarcomatoid, or sarcomatous elements Carcinoid tumour Typical Atypical Carcinomas of salivary gland type Mucoepidermoid carcinoma Adenoid cystic carcinoma

Table 1.1 Histological classification of lung cancer (WHO 1999)

I.1.1 Aetiology

Most lung cancer patients are smokers (85-90%) (Parkin et al., 1994). In fact, smokers are 22 times more likely to die from lung cancer than non-smokers, and 90% of the major histological types of lung cancer cases, including SCLC are attributed to smoking (Chua et al., 2004). Genetic damage to the respiratory epithelium caused by smoking persists for years after smoking cessation and 50% of American lung cancer cases occur in ex-smokers (Hecht, 1999; Wistuba et al., 1997).

The 10-15% who do not smoke implies that other factors are also involved such as passive smoke, air pollution, work related risk factors (exposure to arsenic, chromium and nickel (Siemiatycki et al., 2004)) and environmental radon exposure.

The fact that only 1 in 10 of lifetime smokers develop lung cancer suggests genetic susceptibility is important too. For example, three large genome wide association studies identified an association between single-nucleotide polymorphism (SNP) variation at 15q24-15q25.1 and susceptibility to lung cancer (Amos et al., 2008). Host specific factors such as differences in carcinogen metabolism and detoxification, DNA repair e.g. germ line alterations in nucleotide excision repair genes such as excision repair cross-complementation group 1 protein (ERCC1) (Yu et al., 2008), cell cycle control, cell signaling and apoptosis may play a part.

I.1.2 Treatment of Lung Cancer

NSCLC

Most NSCLC patients present with advanced disease (stage IV) or locally advanced disease (Stage IIIB) which is inoperable however those that present at an earlier stage (Stages I-III A), for selected patients surgical resection is the only chance of cure. Stage is determined

by the tumour, node and metastasis system (TNM). Table 1.2 shows the 5-year percentage survival of each stage.

Patients with stage I disease (T1-2N0) usually present with no symptoms and most are cured with surgical excision of the affected lobe of the lung. These patients do not benefit from adjuvant chemotherapy i.e. chemotherapy given after surgical resection (Douillard et al., 2006; Wakelee et al., 2007). Five-year survival rates are only around 70% (Tsuchiya et al., 2007).

Stage II disease (T1-2 N1 and T3N0) is treated with surgery (lobectomy or pneumonectomy). Current data indicate that these patients gain additional benefit from adjuvant platinum based chemotherapy (Arriagada et al., 2004; Douillard et al., 2006). Those with incomplete resection and/or mediastinal lymph nodes should receive post-operative radiotherapy to reduce local recurrence (Group, 1998).

Stage IIIA disease (T3N1, T3N2) patients if suitable will have complete resection via lobectomy with mediastinal lymph node dissection. Adjuvant platinum based chemotherapy is again offered post operatively with radiotherapy (Arriagada et al., 2004).

NSCLC patients with advanced disease (stage IIIB/IV) with a good performance status are treated with up to 6 cycles of platinum-containing combination chemotherapy (Group, 1995). Platinum-based regimes, reduces risk of death by 27% and leads to a 10% absolute increase in one-year survival compared to best supportive care. Three drug based regimes do not perform better than 2 drug based regimes (Alberola et al., 2003; Laack et al., 2004). However recent trial data looking at the combination of cisplatin and the multi-targeted antifolate drug pemetrexed has superior survival compared to cisplatin and gemcitabine with respect to adenocarcinoma and large-cell histology (Scagliotti et al., 2008).

For patients who progress to 2nd line treatment with a good performance status (PS) drugs such as docetaxol and pemetrexed have been given approval after trials showed improved survival against best supportive care for docetaxol and similar survival for pemetrexed against docetaxol but with less febrile neutropenia (Hanna et al., 2004; Shepherd et al., 2000). Inhibition of the epidermal growth factor receptor (EGFR) is at the forefront of targeted small molecule therapies in advanced NSCLC, with drugs such as gefitinib, cetuximab and erlotinib, with inhibition of angiogenesis via vascular endothelial growth factor (VEGF) inhibition with the monoclonal antibody bevacizumab also showing promising results.

Erlotinib has been used in clinical trials in patients who have failed one previous line of chemotherapy, in patients who also express the EGFR. In clinical trials, survival was superior in the erlotinib treated group compared to the placebo group (Shepherd et al., 2005). Sub-group analysis showed that patients with adenocarcinoma histology, female, non-smokers and with an eastern ethnicity responded well to erlotinib. These patients tended to have somatic mutations in the EGFR. A recent study showed that roughly 16% of NSCLC patients have activating mutations in the EGFR (Rosell et al., 2009).

Recently the combination of cetuximab, cisplatin and vinorelbine was found to have superior survival (in advanced EGFR-expressing stage IIIB or stage IV NSCLC) compared to just chemotherapy alone (Pirker et al., 2009).

Stage	5-year survival (%)
Stage I	76
T1N0M0	84
T2N0M0	68
Stage II	
T1-2 N1M0	47
T3N0M0	40
Stage III	21
Stage IV	2

Table 1.2 5-year percentage survival for NSCLC stages I-IV

Bevacizumab when added to platinum based chemotherapy, in the first line advanced setting showed a significant progression free and overall survival benefit against chemotherapy alone. Squamous cell patients were excluded from the study due to safety reasons (Sandler et al., 2005).

SCLC

The TNM staging system is widely used to determine therapy for NSCLC. However, in SCLC this system does not appear to be superior to the much simpler Veterans Administration Lung Group classification based on suitability of patients for thoracic radiotherapy (Simon & Wagner, 2003). In this system patients are divided into two groups, limited or extensive stage, depending on whether the disease is confined to one hemi-thorax which can be encompassed within a single radiotherapy field. About 30% of patients present with limited disease and this in conjunction with performance status are good independent predictors of prognosis. Other features which may predict prognosis include lactate dehydrogenase (LDH), albumin, non specific enolase (NSE) and fibroblast growth factor-2 (FGF-2) levels, ERCC1 expression and gender (Bremnes et al., 2003; Ruotsalainen et al., 2002; Wakelee et al., 2007). Treatment is currently determined by stage. The median and overall survival for limited stage disease is about 18 months and 15% at 3 years, respectively. For extensive stage disease these figures are 8 months and <3% (Kurup & Hanna, 2004).

Approximately 30% of patients present with limited disease and roughly 80% respond to combination chemotherapy. In the last 30 years, there have been two important advances in the management of SCLC. The first has been the recognition that early thoracic radiotherapy concurrent with chemotherapy improves local control and survival. A meta analyses demonstrated that conventionally fractionated thoracic radiation added to chemotherapy

improved survival by 5.4% and local disease control by 25.3% (Pignon et al., 1992). These survival advantages were observed with old regimens containing cyclophosphamide, adriamycin and vincristine. More recent studies (Bremnes et al., 2003) have demonstrated even better survival and less oesophageal toxicity when combining thoracic radiotherapy with etoposide and a platinum chemotherapy drug. The addition of a third drug to the etoposide-platinum combination did not further improve survival (Ettinger et al., 2005). Etoposide and platinum chemotherapy combined concurrently with thoracic radiotherapy has emerged as the standard of care for patients with limited disease SCLC. A question as to whether thoracic radiotherapy should be given with early or later cycles of chemotherapy has recently resolved by a systematic review of seven randomised trials. This review found a survival benefit of 5% at two years for early (<9 weeks from starting chemotherapy), compared to late (>9 weeks) irradiation (Fried et al., 2004).

The second advance was the discovery that prophylactic cranial radiotherapy (PCI) also improves survival by halving the risk of brain relapse. A meta-analysis of seven trials found a 5.4% survival increase at 3 years together with a reduction in incidence of brain metastasis from 59% to 33 % using PCI (Auperin et al., 1999). Thus all patients with limited stage disease who are free of brain metastases at the end of therapy should have PCI.

Disease beyond one hemi-thorax is present at diagnosis in more than 60-70% of patients. Platinum/etoposide combinations have become the current standard of care (Pujol et al., 2001; Sundstrom et al., 2002). Unlike other disease settings such as NSCLC and testicular cancer where cisplatin produces superior survival results than carboplatin, at the current time there is insufficient data to indicate inferiority of carboplatin in SCLC (Okamoto et al., 2007). Prophylactic cranial radiotherapy in this group shows a reduction in the incidence of symptomatic brain metastases and prolonged disease free and overall survival (Slotman et al.,

2007). Importantly and in contrast to NSCLC, therapy should not be withheld in SCLC patients whose PS is over 2 as they can still be salvaged with treatment (Johnson et al., 1996). Treatment of relapsed SCLC is a major problem for oncologists. Prognosis is very poor at this stage. Predicting who will respond is important. The response-complete/partial, the duration of response after 1st line treatment (>3 months) and PS are helpful predictors. If patients relapse at least 3 months after conclusion of 1st line treatment then they have a reasonable chance to respond to the same or a different treatment. Patients relapsing within 1-2 months have a poor chance of responding. Nevertheless, even this cohort can benefit as demonstrated by a phase III randomised study of oral topotecan versus best supportive care. Survival was 25.9 weeks with topotecan versus 13.9 weeks for best supportive care (O'Brien et al., 2006). If patients do not respond or are 'refractory'; clinical trials and best supportive care should be considered.

For both NSCLC and SCLC despite present treatments survival is still poor. In particular, for SCLC new treatments have not been forthcoming. New treatments and novel therapeutic strategies are urgently needed. These will come from a better understanding of the disease biology. The biology of lung cancer will now be reviewed.

I.2 Biology of Lung cancer

Growth Signals

Within an organism, growth is a tightly regulated process. It is the result of a dynamic equilibrium between two forces, one tending to increase the cell population (cell proliferation) and one that tends to reduce it (cell death). In most tissues, cell turnover is slow, most cells remaining in the non proliferative G_0/G_1 phase of the cell cycle. However, many cells retain the capacity to re-enter the cell cycle and undergo cell division. This property is essential for many biological processes including tissue repair and immunological responses.

In the last thirty years, growth factors most of which act externally via specific cell surface receptors have been shown to regulate growth and apoptosis. This is achieved through complex intracellular signaling, which once integrated, are translated into cellular responses.

In cancer, these intracellular signaling pathways are modified leading to an escape from the normal control of cellular proliferation causing excessive growth. Proto-oncogenes encode normal cellular proteins which are highly conserved and are often of crucial importance in controlling cell proliferation. Activated proto-oncogenes encode molecules involved in aberrant growth factor signaling, either by directly promoting cell growth, by mimicking other growth factors, or by neutralizing growth inhibitory signals. Also activating mutations in intracellular signaling pathways downstream of these cell surface receptors can affect many pathways simultaneously.

As mentioned, growth factors are proteins that bind to cell surface receptors to elicit their cellular activities. They can be separated into two main classes: polypeptide growth factors and neuropeptide growth factors. Multiple signaling pathways branch out from their respective activated receptors, to the nucleus or other cellular compartments where further

events regulating cell functions occur. The initiation of the signaling cascade is dependent on tyrosine kinase activity for polypeptide growth factor receptors whereas neuropeptide receptors which lack tyrosine kinase activity depend on heterotrimeric G proteins to elicit the signaling cascade.

Tumour cells have lost their dependency to growth factor stimulatory signals from the external environment and can proliferate independently. This phenotype of growth autonomy is achieved by many molecular changes and gene mutations within the cell, typically by being self-sufficient in growth factors. These growth factors can be secreted by and act on the same cell (autocrine) or can be secreted by one cell type and act on another (paracrine). Aberrant expression and signaling by a number of growth factors and receptors have been identified in lung cancer. In NSCLC these include upregulation/mutation of certain receptor tyrosine kinases (RTKs), in particular the EGFR. In SCLC overexpression of insulin-like growth factor I (IGF-I) and its receptor as well as a number of neuronal growth factors are frequently observed. (Table 1.3)

Epidermal growth factor receptor and signaling in NSCLC

Overexpression of EGFR is common in NSCLC and in particular in squamous cell carcinoma (SCC), whereas EGFR overexpression is rare in SCLC (Rusch et al., 1993; Selvaggi et al., 2004). The EGFR is membrane bound and contains three main regions: an extracellular ligand binding domain, a hydrophobic membrane spanning region and a cytoplasmic region holding the catalytic tyrosine kinase activity. Ligand binding triggers receptor dimerization, promoting the autophosphorylation of specific tyrosine residues within the intracellular domain, creating binding sites for transduction proteins containing Src-homology 2 or phosphotyrosine-binding domains (Hodkinson et al., 2008). Major

signaling pathways/proteins are activated by EGFR activation including the Ras/Raf/mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), and the signal transducers and activators of transcription factors (STATs). These pathways promote cell proliferation, prevent apoptosis, angiogenesis and increase cell migration (Figure 1.1).

As mentioned, the Ras/Raf/MAPK pathway is activated on ligand binding to EGFR. The effects of this pathway are diverse, with a large numbers of Ras effectors, but in general Ras signaling upon Epidermal Growth Factor (EGF) stimulation has been associated with increase cell growth and proliferation. One of the three Ras genes, Kirsten-Ras (K-Ras, p21-Ras), is mutated in ~ 30% of NSCLC.

In NSCLC, increased EGFR signaling is obtained by an increase gene copy number and by activating mutations within the EGFR gene (Hirsch & Witta, 2005). One mutated EGFR variant termed EGFRvIII, found in ~16% of NSCLC, has gained increasing interest in recent years. EGFRvIII lacks the extracellular ligand-binding domain, preventing ligand binding, yet the receptor is constitutively active and capable of activating downstream modulators (Pedersen et al., 2001). In recent years, novel activating mutations within the tyrosine kinase (TK) domain of EGFR have been identified in NSCLC. Mutations in the first four exons of the EGFR TK domain have been observed. These include in-frame deletions in exon 19 (up to 60% of EGFR mutations) and the L858R missense mutation in exon 21 (up to 35% of EGFR mutations). These mutations have gained massive interest, since they have been found to correlate with increased response to treatment with EGFR tyrosine kinase inhibitors (Paez et al., 2004) and are present in 16% of all NSCLC patients (Rosell et al., 2009). These responders are more likely to be women, have adenocarcinoma histology, non smokers and to be asian (Herbst et al., 2008).

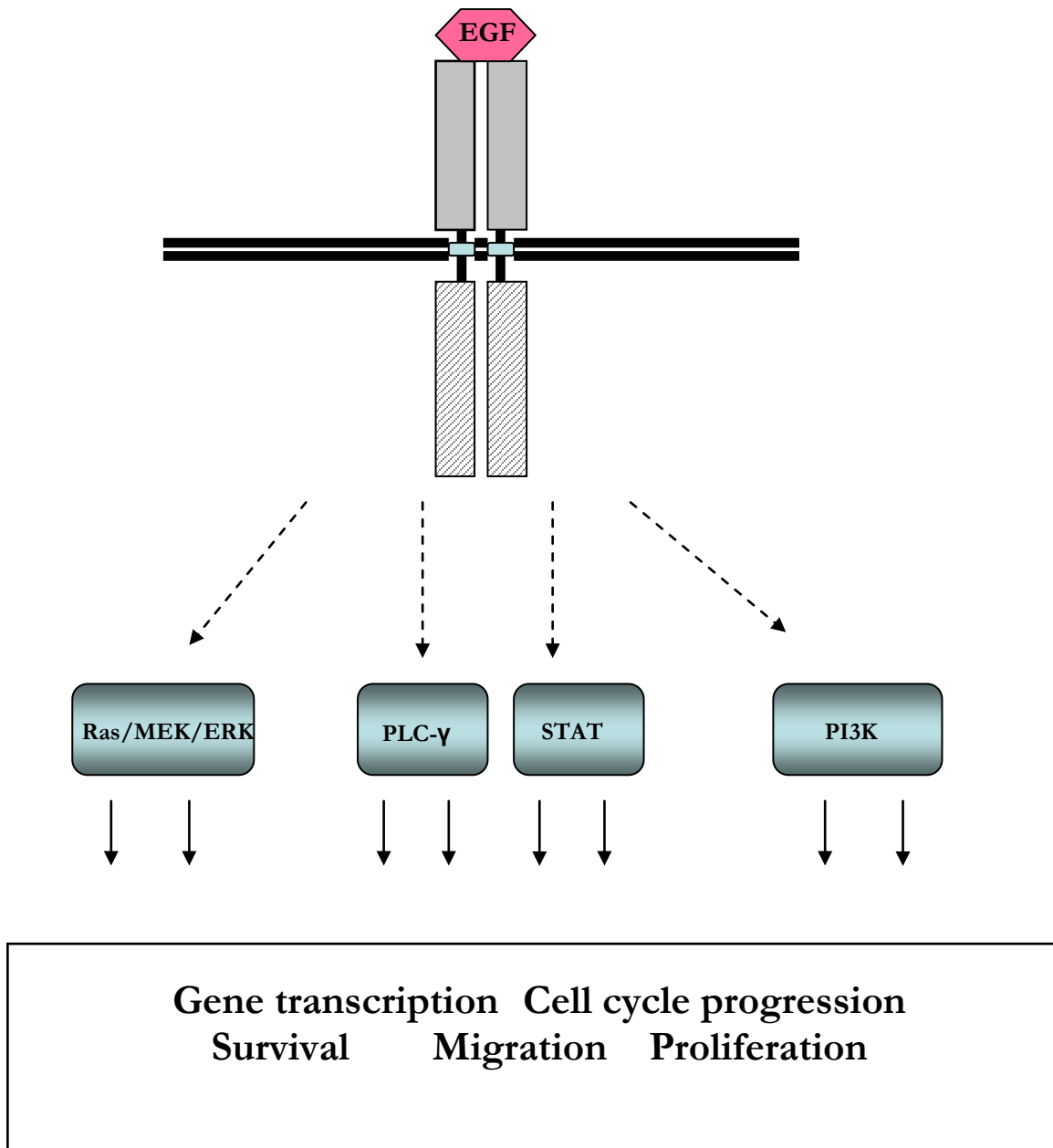


Figure 1.1 Highly simplified summary of EGFR-signaling pathway and their most important effects on biological processes

Oncogene	NSCLC %	SCLC %
EGFR	50-90	-
HER2-Neu	30	-
IGF-I	-	>95
C-Kit/C-Met	-	70
K-Ras	20-30	-
Neuropeptides Bombesin Bradykinin CCK Endothelin-1 Galanin Neurotensin Somatostatin Vasopressin VIP	16-47	100
C-MYC	50	10-40

Table 1.3 Signaling mediators involved in activating growth factor signaling in lung cancer.

Percentages are for aberrant activity/mutation frequency (Hansen, 2008)

Activating Ras mutations

Ras proteins are guanine nucleotide-binding proteins that belong to the small guanosine triphosphate (GTP)-binding protein (G protein) superfamily (Takai et al., 2001). It is a signal transduction protein. As previously mentioned, mutations in the intracellular membrane associated signaling mediator Ras (K-Ras) are detected in up to 30% of NSCLC (Gao et al., 1997) but rarely in SCLC. Ras protein becomes activated by the binding of guanine triphosphate (GTP), allowing for transmission of growth stimulatory signals to the cell nucleus. Downregulation of Ras signaling occurs by hydrolysis of GTP to guanine diphosphate (GDP), mediated by the GTPase-activating protein (GAP). In NSCLC, activating point mutations in the K-Ras gene result in resistance to GAP activity, thereby trapping the Ras protein in a constitutively active state and capable of continuous growth promoting signaling. A recent global meta-analysis correlated the presence of point mutated constitutively active Ras in NSCLC with a poor prognosis (Mascaux et al., 2005).

C-MYC

This proto oncogene encodes nuclear products which are the targets of Ras signaling. The myc family of proto oncogenes have been implicated in various human cancers (Nesbit et al., 1999). Through its role as transcription factor, c-MYC alters the expression of hundreds of target genes, many of which are themselves oncogenes or tumor suppressors (Prochownik, 2008). Protein overexpression is due to gene amplification or by transcriptional dysregulation. In SCLC 18-31% had amplification of c-MYC as compared to 8-20% of NSCLC. DNA amplification of myc family genes and in particular c-MYC has been associated with shortened survival in SCLC patients (Brennan et al., 1991; Noguchi et al., 1990). MYC mRNA is more stable in lung cancers (Richardson & Johnson, 1993).

Growth factors and SCLC

The expression level of the mitogen IGF-I is elevated in most cases of SCLC, resulting in a self-stimulatory autocrine loop involving the IGF-I receptor which is commonly co-expressed in SCLC (Reeve et al., 1990). IGF signaling acts by binding of IGF ligands (IGF-I and II) to cell surface RTKs (IGF-IR and IIR). IGF-IR signaling is complex but primarily occurs through the Ras/Raf/MAPK and the PI3-kinase/AKT pathways. A correlation between significantly elevated IGF-I serum levels and lung cancer risk has been reported but results of other studies are conflicting (Lukanova et al., 2001; Yu et al., 1999).

The proto oncogene c-Kit and its ligand stem cell factor (SCF) are highly expressed in SCLC (Rygaard et al., 1993). Despite the suppression of SCLC proliferation in vitro with the small molecule inhibitor of c-kit, imatinib, there have been conflicting reports as to the prognostic significance of c-kit expression in SCLC tumours and several phase II trials with imatinib in SCLC patients have showed no tumour responses (Krug et al., 2005; Rohr et al., 2004).

c-Met is another RTK that is overexpressed in SCLC. In contrast to the c-Kit/SCF system, the c-Met ligand-hepatocyte growth factor (HGF) is rarely co-expressed with the receptor in SCLC (Rygaard et al., 1993) but is expressed and secreted from surrounding normal lung fibroblasts, suggesting a paracrine action. The importance of paracrine c-Met signaling for lung cancer pathogenesis has been investigated further in a study where c-Met expressing lung cancer cells were transplanted into HGF overexpressing mice, resulting in increased metastatic potential of the transplanted cells (Yu & Merlino, 2002).

Another RTK growth factor involved in SCLC and NSCLC biology is the fibroblast growth factor 2 (FGF-2), I will discuss this particular growth factor in depth later.

Highly elevated expression of different neuropeptides is a characteristic of SCLC and also in some (mainly poorly differentiated) NSCLC tumours (Kiriakogiani-Psaropoulou et al., 1994).

Neuropeptides signal via heterotrimeric (i.e. 3 subunits) G-proteins. Sequence comparison for these receptors reveals that they all possess a seven transmembrane domain structure leading to activation of downstream signaling targets including PI3-kinase/AKT/mTOR and Ras/Raf/MAPK mediating proliferation, cell cycle regulation, cell migration and angiogenesis (Molina & Adjei, 2006). Interestingly, the development of resistance to cytotoxic chemotherapy in SCLC is accompanied by an increase in the expression of neuropeptide growth factor receptors (MacKinnon et al., 2005; Waters et al., 2003). Gastrin releasing peptide/bombesin (GRP) signaling via its receptor is one of the most intensively studied neuropeptides in SCLC. This is because of the concentration of the GRP precursor-Pro-GRP is highly elevated in the majority of SCLC cases and serum concentrations decrease on surgical resection (Weber et al., 1985). Other neuropeptides highly expressed in SCLC include bradykinin, vasopressin (ADH) and neuron specific enolase (Sattler & Salgia, 2003; Simon & Wagner, 2003).

Apoptosis and Lung Cancer

Apoptosis is a physiological cell death mechanism that first occurs during early development of a living organism (Brill et al., 1999). It allows for the elimination of dysfunctional or damaged cells so that the organism can carry on unaffected. The ability to apoptose is present in a latent form in virtually every cell in the body, and when initiated by a variety of stimuli, it unfolds in a well defined and organized series of events. This leads to biochemical and physiological alterations that ultimately lead to cell death, typically within 24 hours. Alterations include cell membrane disruption, breakdown of cytoplasmic and nuclear skeletons, nuclear fragmentation and chromosome degradation (Kerr, 2002; Wyllie et al., 1980). Evasion of apoptosis is a hallmark of cancer.

Two main apoptosis pathways exist-the death receptor and mitochondrial pathway (Figure 1.2). The former has an important role in the maintenance of tissue homeostasis and is activated by the interaction of cell surface death receptors with their ligands. However, in most physiological situations apoptosis is started via the mitochondrial pathway.

Apoptosis is activated by a family of intracellular cysteine proteases called caspases. They are divided into two classes- initiator caspases including caspases P8, P9 and P10 and effector caspases including caspases P3, P6 and P7.

The death receptor pathway is activated by ligands: tumour necrosis factor (TNF), Fas Ligand (FasL) and TRAIL (TNF-related apoptosis-inducing ligand) binding to their respective receptors: TNFRI, FAS and Death Receptor 4 (DR4) and Death Receptor 5 (DR5). This triggers initiator caspase activation through a death inducing signaling complex (DISC), resulting in caspase-P8 activation of effector caspases, either directly or through B-cell leukemia-2 protein (BCL-2) interacting domain (BID)-mediated release of cytochrome c from mitochondria. The mitochondrial pathway is activated with the release of cytochrome c from the intermembrane space of mitochondria. Two proapoptotic family members, BCL-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) facilitate cytochrome c release by participating in the formation of a pore that releases mitochondrial intermembrane space proteins. After its release, cytochrome c binds to apoptotic protease activating factor-1 (APAF-1). APAF-1 binds to procaspase-9 forming a multiprotein complex, called an apoptosome, which activates effector caspases through caspase-9.

Anti-apoptotic factors such as Bcl-2 related proteins antagonize BAX and BAK. Also, IAP (inhibitor of apoptosis protein) binds and inhibits apoptosome-related caspases. However this inhibition can be relieved by the release of another mitochondrial protein called

Smac/Diablo, which binds IAP and releases active caspases. The most extensively studied IAP is survivin (Shivapurkar et al., 2003).

Recent data suggest that DR4 and DR5 are upregulated in NSCLC and overexpression of DR5 correlates with poor prognosis. Bcl-2 expression is higher in SCLC compared to NSCLC. It has also been shown that the BAX: Bcl-2 ratio might be of importance for resistance to apoptosis. Caspase-8 and caspase 10 might be deregulated and differences in deregulation in SCLC compared to NSCLC especially concerning caspase-8 have been observed, resulting in deregulation of DISC. In addition, it has been shown that downregulation of caspase-3 is correlated with a poor prognosis in NSCLC (Fennell, 2005; Shivapurkar et al., 2003).

Survivin expression is increased in NSCLC and lack of expression could be associated with a good prognosis. Also microarray data show that survivin is upregulated in SCLC cell lines and patient tumours (Pedersen et al., 2003).

Much focus is on BCL-2 inhibition, with some drugs in the pre-clinical trial stage.

Tumour suppressor inactivation

As previously mentioned proto oncogene activation in lung cancer is common and so is inhibition of tumour suppressor activity. Tumour suppressor genes act to prevent and control cell growth, mostly by regulation of cell cycle progression. Inactivation of both alleles is required and this occurs by a two step process involving a chromosomal translocation or deletion resulting in loss of heterozygosity (LOH), followed by an inactivating point mutation of the remaining allele. LOH of certain chromosomal regions is common in lung cancer, and in these regions tumour suppressors involved in

tumourogenesis are frequently found. I will focus now on two important tumour suppressors in lung cancer- p53 and retinoblastoma (RB).

P53

P53 is one of the most studied tumour suppressors. P53 is a key player in the cellular response to stress, such as ultraviolet light and chemotherapy, acting as a gatekeeper of the cell cycle. Active p53 regulates transcription of a number of genes involved in cell cycle control (such as cyclin-dependent kinase inhibitors) leading to cell cycle arrest, allowing for repair of damaged DNA. Activation also leads to apoptosis via activation of a number of apoptotic mediators and activates genes encoding anti-angiogenic factors. The p53 gene is located on chromosome 17 (17p13) which is mutated in the majority of lung cancer cases, in particular SCLC and SCC (Chmara et al., 2004; Petersen et al., 1997). p53 mutations result in a poorer prognosis in NSCLC. Since so few SCLC patients lack mutations in p53 it is difficult to assess prognosis in these patients (Campling & El-Deiry, 2003).

Retinoblastoma (RB)

The gene for this tumour suppressor encodes a transcription factor involved in the regulation of G1 to S-phase transition in the cell cycle. Activation depends on phosphorylation, leading to E2F release and then progression of the cell cycle from G1 to S. Phosphorylation of Rb is dependent on complex formation with cell cycle proteins like cyclin D and Cyclin Dependent Kinase 4 and 6 (CDK4/6). These complexes are inhibited by p16. LOH or mutation is very common (90%) in SCLC (Hensel et al., 1990), whilst the p16 gene is frequently inactivated in NSCLC (Otterson et al., 1994), leading to lack of Rb

activity. Animal models of p53 and Rb null mice who develop SCLC provide further evidence that Rb is essential in lung cancer (Minna et al., 2003).

Chromosome 3p

The most frequent chromosomal abnormality in lung cancer is loss of regions in chromosome 3p. LOH at this site is prevalent in 70-100% of NSCLC and more than 50% of SCLC (Kok et al., 1987; Naylor et al., 1987). Many genes in this region are considered tumour suppressors and are inactivated in both SCLC and NSCLC such as fragile histidine triad (FHIT) and RASSF1A (RAS association family 1 gene) (Zabarovsky et al., 2002).

Angiogenesis

New blood vessel formation (angiogenesis) is a carefully regulated normal physiological process. Tumours must develop angiogenic ability to survive since oxygen and nutrients cannot be supplied by simple diffusion alone once a certain size is attained. Angiogenesis is regulated by pro-angiogenic factors and anti-angiogenic factors. Activation is by a change in the balance of pro- and anti- factors (de Castro Junior et al., 2006). The most studied pro-angiogenic factors are VEGF and acidic and basic FGF-1/FGF-2 which bind to RTKs on endothelial cells (Veikkola et al., 2000). Whilst the typical anti-angiogenic factor is thrombospondin-1 which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinases (Bull et al., 1994). There are over 100 different pro- and anti- angiogenic factors. VEGF levels have been demonstrated to determine predict poor prognosis in NSCLC, in particular SCC (Imoto et al., 1998).

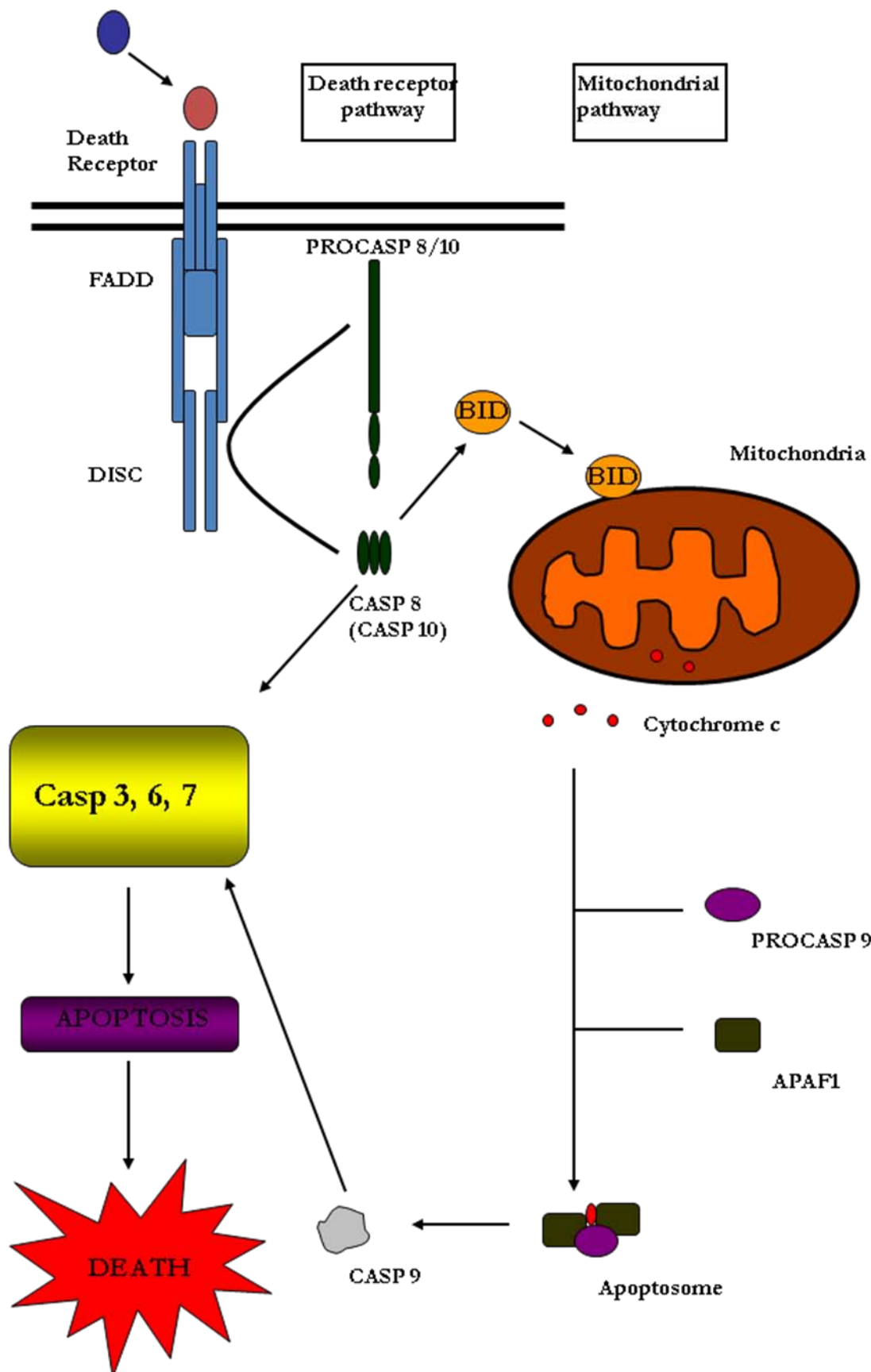


Figure 1.2 Simplified diagram of major pathways in apoptosis

Invasion and metastasis

Another defining characteristic of the malignant phenotype is invasion into local tissues and then spread to metastatic deposits. The dissemination of cancer is a complex process depending on many interactions between the cancer cell and the host environment. There are many steps including detachment from the primary, penetration of the basement membrane, invasion of surrounding stromal tissue, gaining access to the blood/lymphatic system by crossing the subendothelial basement membrane, seed in an organ and survival in that organ (Hart & Saini, 1992). Integrins are heterodimeric cell surface receptors which are expressed on all cell types. They mediate adhesion and cell spreading by linking the extracellular matrix (ECM) with the cytoskeleton (Martini, 1994). Laminins are heterotrimeric ECM proteins which are prominent constituents of the basement membrane. Laminin-integrin interactions are common and key markers of tissue invasion through the basement membrane and leading to metastasis. The expression of laminin α chains ($\alpha 3$ and $\alpha 5$) is often reduced in lung cancer leading to fragmentation of basement membrane elements and hence invasion (Akashi et al., 2001). Laminin 5 and its binding receptor $\alpha 6\beta 4$ -integrin, is only expressed in NSCLC and not in SCLC. Laminin 5 overexpression is associated with a poor prognosis in NSCLC and is an independent prognostic factor in NSCLC (Manda et al., 2000; Moriya et al., 2001). Resistance to chemotherapy is a principal problem in the treatment of SCLC. It has been shown that SCLC is surrounded by an extensive stroma of ECM at both primary and metastatic sites. Adhesion of SCLC cells to ECM enhances tumorigenicity and confers resistance to chemotherapeutic agents as a result of $\beta 1$ integrin-stimulated tyrosine kinase activation suppressing chemotherapy-induced apoptosis (Sethi et al., 1999).

I.3 Intracellular signaling pathways

Since many of the genetic alterations found in cancer involve genes which code for proteins involved with modulation of signal transduction (Hanahan & Weinberg, 2000) recent research has focused on the inhibition of molecules involved with signal transduction and carcinogenesis. These intracellular signaling pathways transduce signals from the cell surface to the nucleus. They are involved with cell proliferation, apoptosis and a variety of other biological processes (Brivanlou & Darnell, 2002) involved with cancer. Many of the key proteins involved with signal transduction have kinase activity. Protein kinases mediate most signal transduction in eukaryotic cells.

Two critical pathways I will now focus on are the RAF/MAPK mitogen activated kinase pathway and the PI3-Kinase/AKT/mTOR pathway.

The RAF/MAPK signaling pathway

The Ras-Raf-MEK-ERK (MEK for MAPKK and ERK kinase, ERK for extracellular signal-regulated kinase) signal transduction pathway is present in all eukaryotic cells (Kolch, 2000). This important pathway relays extracellular signals to the nucleus via a cascade of highly specific phosphorylation events involving Ras, RAF, MEK and ERK to regulate fundamental cellular processes such as proliferation, differentiation and cell survival (Kolch, 2000).

Mechanisms of activation

The mammalian MEK/ERK signal cascade consists of the MAPKs- ERK1 and ERK2, the MAPK kinases (MAPKKs) - MEK1 and MEK2, and the MAPKK kinases (MAPKKKs) - Raf family of kinases. Raf kinases phosphorylate and activate the MEK1 and MEK2 dual

specificity protein kinases MEK1/2 then phosphorylate and activate ERK1/2 MAPKs. Activated ERK phosphorylate and regulate the activities of over 160 proteins (Yoon & Seger, 2006). Most ERK substrates are nuclear proteins. Activated ERKs can translocate to the nucleus, where they phosphorylate and regulate various transcription factors, ultimately leading to changes in gene expression (Zuber et al., 2000) (Figure 1.3).

The pathway is initially activated by the binding of growth factors such as EGF, Vasopressin and VEGF to their respective receptors be that tyrosine kinases or G-protein coupled receptors leading to the activation of a kinase cascade that relays signals to the nucleus.

Typically Ras protein is activated by recruitment of the adaptor protein growth factor receptor-bound protein2 (Grb2) to the afore mentioned activated receptor. Grb2 contains one Src Homology 2 (SH2) domain and two SH3 domains. Its two SH3 domains direct complex formation with proline-rich regions of other proteins and its SH2 domain binds tyrosine phosphorylated sequences present on activated RTKs. The proline rich guanine nucleotide exchange protein Sos (Son of sevenless) binds to Grb2 when complexed with the receptor. Sos is an exchange factor that facilitates the exchange of GDP for GTP on Ras and thereby activating this GDP binding protein (Rozakis-Adcock et al., 1993). As previously mentioned Ras GTPases consist of a large family of membrane-associated proteins that switch between GTP-bound active and GDP-bound inactive conformation (Wittinghofer & Pai, 1991). Ras oncogenes are the most frequently mutated in human cancers (Campbell & Der, 2004). The mutations leading to a malignant phenotype prevent Ras proteins from hydrolysing GTP, therefore maintaining an active state. The active Ras-GTP binds in turn to the amino-terminal Ras binding domain of Raf, a family of serine/threonine kinases (A-Raf, B-Raf and Raf-1), and recruit them to the cell membrane for activation. Activated Raf kinases are the point of entry into the subsequent MEK/ERK

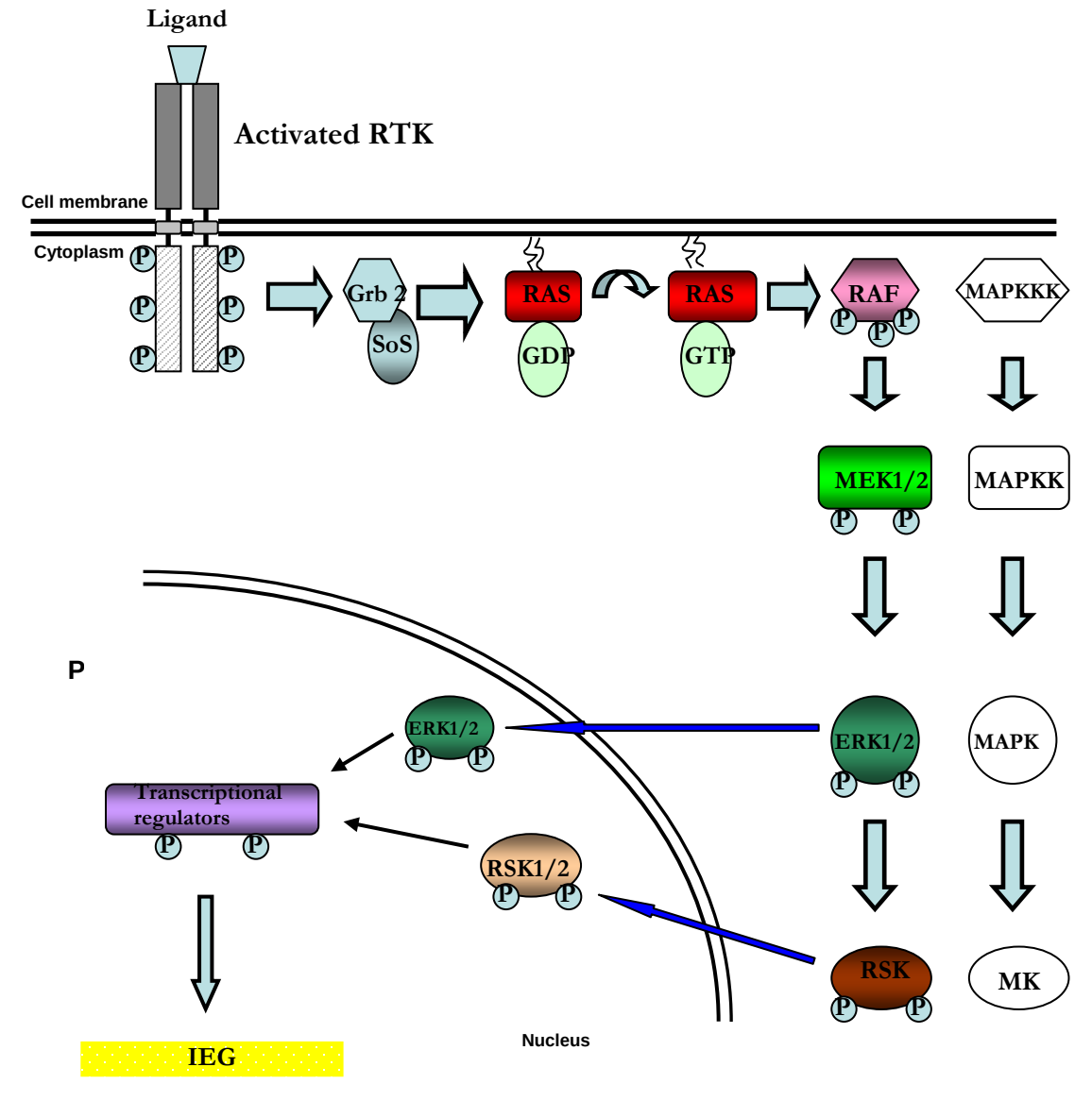


Figure 1.3 The MEK/ERK signaling pathway

The MEK/ERK cascade consists of the MAPKs ERK1 and ERK2, the MAPK kinases (MAPKKs) MEK1 and MEK2, and the MAPKK kinases (MAPKKKs) Raf family of kinases. ERK1/2 phosphorylates several MAPK-activated protein kinases (MKs), including the family of ribosomal S6 kinases (RSK). In the nucleus, ERK1/2 and RSK1/2 phosphorylate transcriptional regulators, resulting in the transcriptional activation of immediate early genes (IEGs).

kinase cascade, initiated by activation of MEK1 and MEK2 proteins through phosphorylation of serine residues in the activation loop (S217/S221) (Alessi et al., 1994).

B-Raf has been shown to be the most efficient at activating MEK1/2 (Huser et al., 2001; Jaiswal et al., 1994). Moreover B-Raf somatic mutations have been identified in many malignancies, especially malignant melanoma, and less so in thyroid, colon and ovary (Davies et al., 2002; Garnett & Marais, 2004).

Activated dual specificity MEK1/2 kinase subsequently phosphorylates and activates p42/44 mitogen activated kinases, ERK1 and ERK2, by threonine and tyrosine phosphorylation within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop (Kolch, 2000). ERK1 and ERK2 are serine/threonine kinases, share 83% amino acid identity and are expressed to various extents in all tissues. Activated ERK1/2 can then catalyze the phosphorylation and as mentioned activation of several substrates and, thereby, exert their effects.

Substrates and functions

Activated ERK1/2 phosphorylate substrates in all cellular compartments, including various membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2 (Myocyte enhancing factor 2), c-Fos, c-Myc, and STAT3), cytoskeletal proteins (neurofilaments and paxillin), and several protein kinases, termed MAPK-activated protein kinases (MKs) (Jacobs et al., 1999; Robinson & Cobb, 1997).

The MK family of ERK 1/2 substrates consists of related kinases that mediate a wide range of biological functions in response to mitogens and stress stimuli. Members of the family include the ~90 kDa ribosomal S6 kinases (RSKs), the mitogen- and stress-activated kinases and MAPK interacting kinases (Roux & Blenis, 2004). Among these, RSK family members (RSK1-4) are exclusively activated by the ERKs. ERK 1/2 and RSK 1/2 can translocate into

the nucleus, where, together with nuclear members of RSK family, they phosphorylate several important transcriptional regulators including E1k-1, the cyclic AMP (cAMP) response element binding protein (CREB) and histone H3 (Frodin & Gammeltoft, 1999). The result is rapid transcriptional activation of immediate early genes (IEGs), including transcriptional factors that control the cell cycle and/or cell survival.

Ras/Raf/MAPK inhibitors

Since the MAPK signaling pathway plays a critical role in transmitting signals from the cell surface to the nucleus, thereby influencing proliferation and cell survival, inhibitors of specific components of the pathway have been developed and evaluated in the clinic with varying degrees of success in cancer.

At least three different approaches have been developed to target the activated Ras pathway. Firstly, by blocking upstream activation of Ras at cell surface receptors e.g. RTKs. Secondly, targeting Ras itself or interrupting protein processing e.g. Farnesyl Transferase inhibition (FTI) and thirdly targeting downstream effector pathways e.g. Raf kinase and MEK inhibitors (Marshall, 1996). Most focus has been on targeting Ras by inhibiting farnesylation of Ras protein using FTIs. Tipifarnib, a specific FTI has made it to phase III trials. Gastrointestinal tumours were chosen in the trials because of the high prevalence of Ras mutations in pancreatic (90%) and colorectal cancer (50%). In combination with gemcitabine in pancreatic cancer and as a single agent after two prior chemotherapy regimes in colorectal cancer, no progression or overall survival benefit was observed (Rao et al., 2004; Van Cutsem et al., 2004). Further studies are assessing Tipifarnib in NSCLC.

Sorafenib is a novel bi-aryl urea inhibitor of Raf-1, suppressing both wild type and V599E mutant BRAF activity in vitro, and several RTKs involved in angiogenesis and tumour

progression, including VEGFR-2, VEGFR-3, PDGFR-beta (Platelet Derived Growth Factor), Flt-3 (FMS-like tyrosine kinase 3) and c-KIT (Strumberg, 2005). Sorafenib has made it to phase III clinical trials in unresectable renal cell carcinoma who have received at least one prior systemic therapy. Clear progression free survival benefits were seen versus placebo which led to approval by the US Food and Drug administration (FDA) for Sorafenib in renal cell carcinoma (Gore & Escudier, 2006).

CI-1040 is the first MEK-targeted agent to reach clinical trials. It is a highly potent and selective non-competitive inhibitor of both MEK isoforms, MEK1 and MEK2. CI-1040 bind to the hydrophobic binding pocket of MEK1 and MEK2 that is adjacent to but distinct from the magnesium adenosine triphosphate (ATP)-binding site, which induces a conformational change in unphosphorylated MEK that locks it into a closed but catalytically inactive form (Sebolt-Leopold et al., 1999). Unfortunately phase II clinical studies in breast, colon, pancreas and lung cancer showed insufficient antitumour activity to warrant further development (Rinehart et al., 2004). Further studies with PD0325901, a second generation MEK inhibitor have subsequently been undertaken but have recently been stopped because of toxicity issues and a lack of efficacy. Other MEK inhibitors are currently being investigated in the clinic.

The PI3K/AKT/mTOR signaling pathway

The PI3K signaling pathway is another signaling cascade that is important for migration, cell growth and survival. The importance of this pathway in cancer comes from the extensive evidence that it is frequently dysregulated by various genetic and epigenetic mechanisms in many tumour types (Wymann et al., 2003) (Table 1.4). Thus pharmacological inhibition of

this activated pathway in cancer provides a reasonable therapeutic strategy to kill or arrest cancer cells (Weinstein, 2002; Weinstein & Joe, 2006).

PI3kinase (PI3Ks) - Structure and Activation

The Phosphatidylinositol-3-Kinase (PI3Ks) are a family of lipid and serine/threonine kinases that when activated, catalyse the phosphorylation of the 3'-hydroxy group of phosphoinositides (PI) (membrane associated phospholipids, with a modifiable hydrophilic inositol head, that can act as a second messenger) (Fruman et al., 1998). There are three classes of PI3Ks but only class IA and II has been implicated in human cancer (Arcaro et al., 2002). Due to the focus of this thesis only Class 1A PI3ks will be discussed. Class 1A PI3Ks are composed of heterodimers of an inhibitory adaptor/regulatory (p85 α , p55 α , p50 α , p85 β , p55 γ) and a catalytic (p110, p110 β , p110 δ) subunit. Only p110 α and p85 α have been found to be mutated and p85 α to be translocated in cancer. The p85 protein binds, via two SH2 domains and integrates signals from activated receptors via phosphorylated tyrosine residues (certain receptors directly e.g. PDGFR (Hu et al., 1992) and others e.g. EGFR, FGFR and nerve growth factor indirectly via docking proteins such as GRB2-associated binding protein 1 (Gab1) and Gab2 (Holgado-Madruga et al., 1996; Holgado-Madruga et al., 1997; Ong et al., 2001) and intracellular proteins like protein kinase C (PKC), SHP1, Rac, Rho, hormone receptors, mutated Ras and Src, providing an integration point for activation of p110 and subsequent downstream molecules (Otsu et al., 1991; Pawson & Nash, 2000; Yuan & Cantley, 2008). Upon activation, PI3Ks phosphorylate phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P2 (PIP₂) to form PtdIns (3, 4, 5) P3 (PIP₃) in the membrane which functions as a second messenger to activate downstream targets. This is achieved by

recruitment of proteins that contain a subset of pleckstrin homology (PH) domains (Bottomley et al., 1998).

PIP₃ levels are tightly controlled under non-stimulated conditions, due to tight regulation of PI3K and the function of PIP₃ phosphatases PTEN (phosphatase and tensin homolog gene on chromosome 10), SHIP1 and SHIP2 (Huber et al., 1999; Maehama & Dixon, 1998; Pesesse et al., 1997).

PTEN is one the most commonly mutated tumour suppressors in human cancer. Frequent genetic inactivation of PTEN occurs in glioblastoma, endometrial cancer, prostate cancer, and reduced expression is found in many other tumor types such as NSCLC and breast cancer (Steck et al., 1997). In SCLC, PTEN mutations are not common (Forgacs et al., 1998). PTEN acts as a phosphatase to dephosphorylate PIP₃ to PIP₂ resulting in inhibition of the AKT signaling pathway.

Genetic screens have identified AKT (Protein Kinase B) as the primary downstream mediator of the effects of PI3K. Upon activation; PI3Ks phosphorylate phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P₂ (PIP₂) to form PtdIns (3, 4, 5) P₃ (PIP₃) in the membrane which functions as a second messenger to activate downstream targets. This is achieved by recruitment of proteins that contain a subset of pleckstrin homology (PH) domains (Bottomley et al., 1998). PIP₃ levels are tightly controlled under non-stimulated conditions, due to tight regulation of PI3K and the function of PIP₃ phosphatases PTEN (phosphatase and tensin homolog gene on chromosome 10) SHIP1 and SHIP2 (Huber et al., 1999; Maehama & Dixon, 1998; Pesesse et al., 1997).

I.4 Downstream effectors of PI3Ks- AKT and mTOR

AKT

Akt is the cellular homologue of the viral oncogene v-akt (Bellacosa et al., 1991). It is a 57kDa serine/threonine kinase that is related to human protein kinase A (PKA) and PKC (Coffer & Woodgett, 1991). There are three known AKT isoforms- AKT1-3, which are derived from three distinct genes. It has a PH domain that allows it to bind to PIP₂ and PIP₃ in the inner surface of the plasma membrane, over other PIs (James et al., 1996). However for full activation, two amino acids must be phosphorylated: One in the kinase domain (threonine 308 in AKT1), phosphorylated by constitutively active phosphoinositide-dependent kinase 1 (PDK1), which allows for stabilizing the activation loop, whilst phosphorylation of the other in the hydrophobic C-terminal domain (serine 473 in AKT1) by PDK2 is necessary for full activation (Alessi et al., 1996). Until recently the identity of PDK2 has been a mystery. It was found to be the mammalian target of rapamycin (mTOR)-rictor (rapamycin-insensitive companion of mTOR) complex (see later) (Sarbasov et al., 2005). Hence mTOR lies above and below AKT in the signaling pathway.

Once activated, AKT translocates to the cytoplasm to activate many downstream effectors with a wide range of cellular effects (Figure 1.4). AKT has been implicated in human cancer. AKT2 is amplified in pancreatic, breast and ovarian cancers and point mutations in AKT2 have been found in colorectal cancer (Bellacosa et al., 1995; Cheng et al., 1992; Cheng et al., 1996; Parsons et al., 2005). AKT3 is overexpressed in hormone-insensitive breast and prostate cancers (Testa & Bellacosa, 2001).

The discussion will now focus in depth on one of the downstream targets of AKT, the subject of this thesis- mTOR.

The mammalian Target of Rapamycin- mTOR

mTOR was discovered 16 years ago, whilst investigating the mechanism of action of its original inhibitor–Rapamycin. Rapamycin, which is a macrolide antibiotic product, was isolated from a soil sample on the south Pacific island of Easter Island (known as Rapa Nui to the inhabitants). Rapamycin is produced by *Streptomyces hygroscopicus* and was initially used as an antifungal agent (Vezina et al., 1975).

Later the drug was found to have immunosuppressive activity which led to its use with Cyclosporine A against organ transplant rejection. Rapamycin was then evaluated by the Developmental Therapeutic Branch of the National Cancer Institute (NCI), it was found to be a non-cytotoxic agent, with cytostatic activity against several human cancers in vitro and in vivo. Rapamycin's exact mechanism of action was not known at the time, however in the early 1990s, it was found that it acted as an inhibitor of cellular proliferation and cell cycle progression (Dumont et al., 1990; Flanagan & Crabtree, 1993). In 1991 a group in Switzerland discovered two new genes in *Saccharomyces cerevisiae*, the target of rapamycin (TOR) genes (TOR1 and TOR2) (Heitman et al., 1991). These genes were then discovered in mammals and named mTOR (Brown et al., 1994). TOR was found to have structural similarities with several known phosphatidylinositol kinases (PIKK) including PI3K (Helliwell et al., 1994). mTOR is a 289 kDa serine/threonine kinase, which plays a critical role in regulating basic cellular functions including growth and proliferation. It also acts as a central controller, integrating a plethora of signaling pathways which responds to growth factors and nutritional status. The signaling pathways that activate mTOR are altered in many cancers.

Structure

mTOR consists of several conserved motifs (Figure 1.5A). At the N-terminus are two tandem arrays of HEAT repeats (Huntington, Elongation factor 3 (EF3), A subunit of

protein phosphatase 2A (PP2A), TOR1), that are considered to be protein-protein interaction domains (Andrade & Bork, 1995). Downstream of the HEAT repeats, is a region conserved in PIKK family members called the FRAP–ATM–TTRAP (FAT) domain. Phosphoinositide-3-kinase-related protein kinase (PIKK) members with a FAT domain also possess a c-terminal FATC domain. The FAT and FATC might contribute to the active conformation of the intervening catalytic kinase domain (Bosotti et al., 2000). The FKBP-12 rapamycin-binding domain (FRB) is required for the binding of the rapamycin-FKBP12 complex to mTOR (Chen et al., 1995; Choi et al., 1996).

Rapamycin has a unique mechanism of action. It first binds the cytosolic 12-kDa immunophilin FK506-binding protein (FKBP12) and this complex as mentioned binds to the FRB domain of mTOR. Point mutation in the FRB domain result in rapamycin resistance (Chen et al., 1995). It ‘normally’ plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking (Avila et al., 2003). The catalytic domain (CD) of mTOR contains autophosphorylation sites as well as the ATP-binding site. The regulatory domain region of mTOR contains sites that are phosphorylated in response to growth factors, but it is not clear how this affects mTOR function (Nave et al., 1999; Scott et al., 1998; Sekulic et al., 2000).

Molecule	Alteration in tumours	Frequency	Tumour type
PTEN	Mutations (somatic)	>50%	Glioma, melanoma, prostate, endometrial, variable in sporadic breast cancer
PTEN	Decreased expression, methylation, LOH	>50%	Breast, melanoma, prostate, microsatellite instability-high colorectal cancer, endometrial cancer, Leukaemia
PTEN	Germline mutations	80% of Cowdens	High risk of breast, thyroid and endometrial carcinomas
P85	Activating mutations	Rare	Ovary, colon, glioma
P85	Fusion	Very rare	Lymphoma
P55 γ (p85 isoform)	Deletion mutations	Unknown	Lung cancer cell line (HCC15)
PIK3CA	Amplification	Up to 50%	Ovary, cervix, lung, breast
PIK3CA	Activating mutation	>50%	Bowel
		>25%	Breast
AKT1	Amplification	Low	Gastric
AKT2	Amplification	Low	Ovary, Pancreas
AKT2	Mutation	Low	Colorectal
AKT3	Overexpression	Low	Hormone-resistant prostate, breast
PDK1	Mutation	Low	Colorectal
S6 Kinase 1	Amplification	30%	Breast
TSC1/2	Mutation	>50%	Tuberous sclerosis
Forkhead family	Translocations	>50%	Alveolar rhabdomyosarcoma
TCL1	Rearrangement	Low	Acute leukaemia
		Unclear	T-cell leukaemia
			Chronic lymphocytic leukaemia

Table 1.4 Abnormalities in the PI3K/AKT signaling pathway in cancer

PIK3CA, gene encoding the p110 α PI3K subunit; PDK1 phosphoinositide-dependent kinase-1; PTEN, Phosphatase and Tensin Homolog; TCL1, T-cell leukaemia 1; TSC, tuberous sclerosis complex.

Adapted from Hennessy et al Nat.Rev. Drug Discov 4:12 988-1004 (Hennessy et al., 2005)

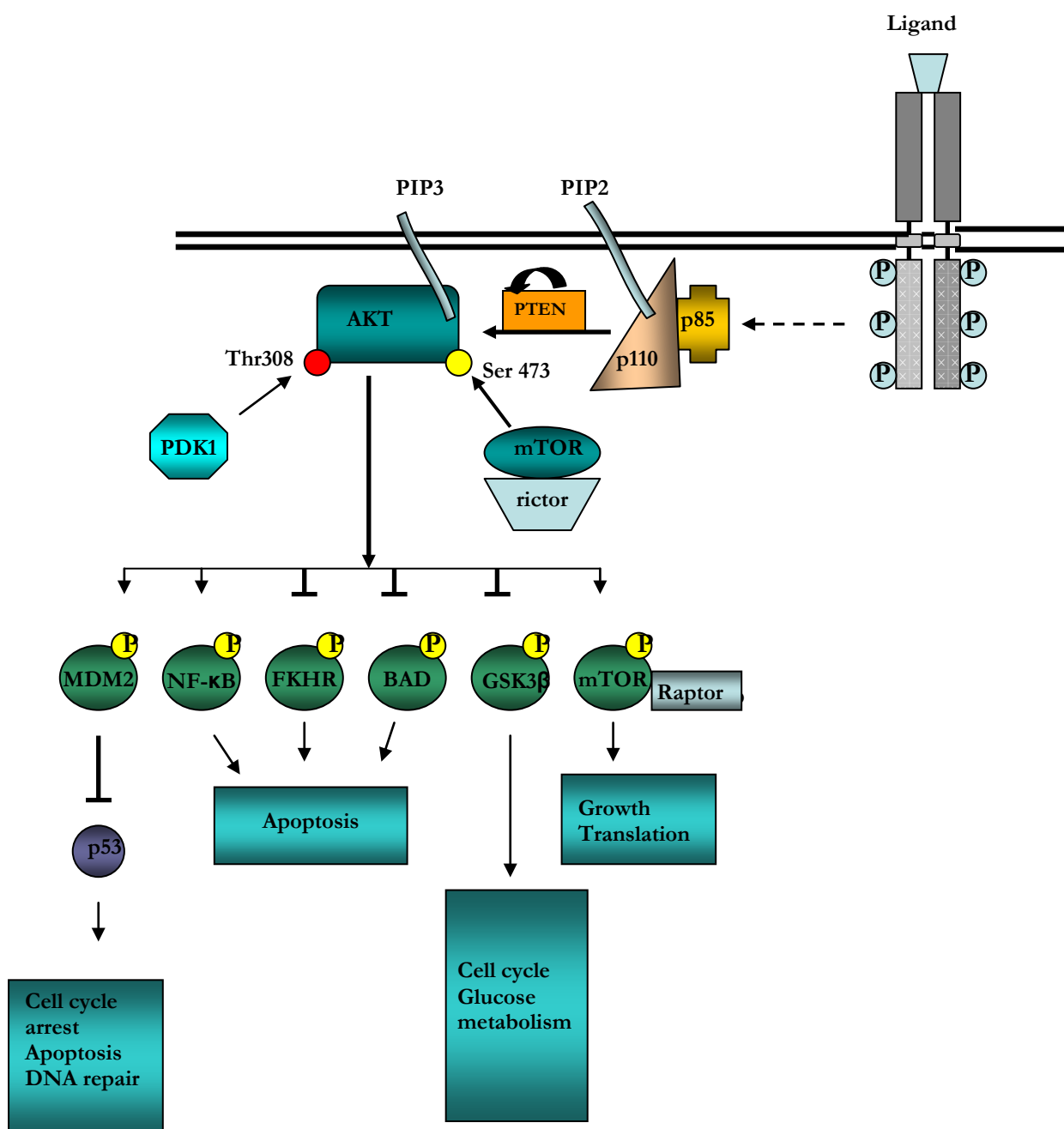


Figure 1.4 PI3K/AKT signaling

Activation of RTKs by growth factors leads to activation of PI3K and then AKT. AKT activation leads to multiple effects such as growth, prevention of apoptosis and DNA repair.

FKHR, (Forkhead homolog 1 rhabdomyosarcoma forkhead); GSK3 (glycogen synthase kinase 3); NF- κ B (nuclear factor- κ B); BAD (Bcl-2-associated death promoter); MDM2 (murine double minute oncogene); PTEN (phosphatase and tensin homolog gene on chromosome 10) PIP2 (phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P2); PIP3 (phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P3).

mTOR nucleates two separate complexes- a rapamycin, nutrient and growth factor sensitive multiprotein complex called mTORC1 (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002) and a rapamycin and nutrient 'insensitive', growth factor sensitive multiprotein complex called mTORC2 (Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004). mTORC2 does not bind rapamycin, proving that rapamycin does not block all of mTOR's functions. However, recent data suggest that prolonged treatment with rapamycin may affect mTORC2 assembly and Akt signaling but only in certain cell types (Sarbassov et al., 2006).

mTORC1 (Figure 1.5B) consists of mTOR, RAPTOR (regulatory associated protein of mTOR), LST8 homolog (mLST8) (also known as G β L) and PRAS40 (proline rich AKT substrate 40kDa) (Hara et al., 2002; Kim et al., 2002). RAPTOR functions as a scaffold for recruiting mTORC1 substrates (Hara et al., 2002; Kim et al., 2002; Schalm et al., 2003). PRAS40 negatively regulates mTOR by binding to the mTOR kinase domain, its interaction with mTOR is induced under conditions that inhibit mTOR signaling, such as nutrient or serum deprivation (Vander Haar et al., 2007). The function of mLST8 is presently not known.

The mTORC2 (Figure 1.5C) complex consists of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated protein kinase interacting protein 1

(mSIN1), Protein observed with RICTOR (PROTOR-1 protein), mLST8 and proline rich protein 5 (PPR5) (Frias et al., 2006; Jacinto et al., 2004; Pearce et al., 2007; Sarbassov et al., 2004).

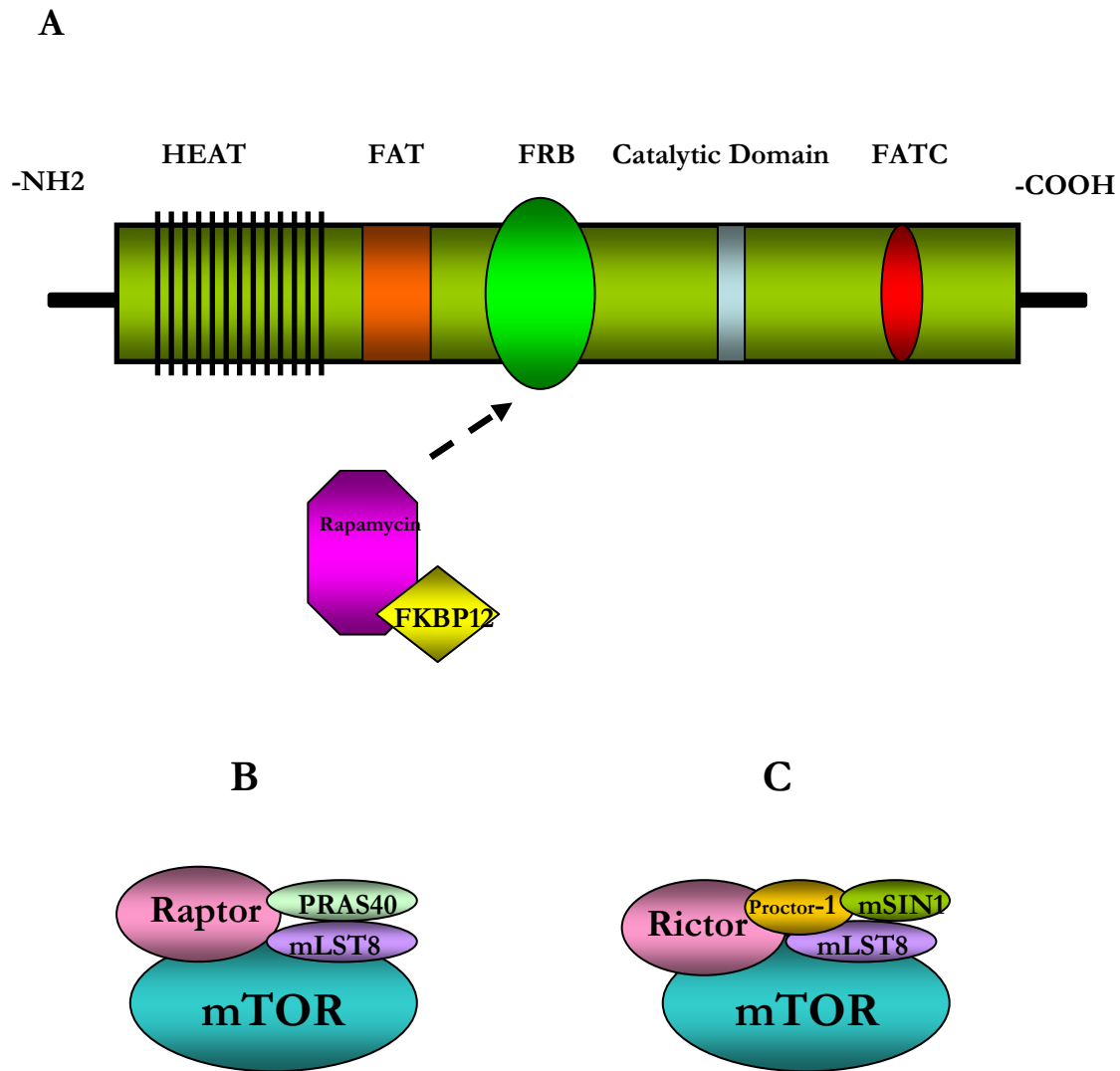


Figure 1.5 Structure of mTOR and mTOR complexes

(A) Functional regions of mTOR. The domain structure consists of about 20 tandem amino acid sequences called HEAT repeats (for Huntingtin, EF3, A subunit of PP2A) at the NH₂ terminal end, followed by the conserved FAT domain, FRB (FKBP12-rapamycin binding), the catalytic domain and the FATC domain (FAT domain close to the C-terminus).

(B) mTORC1 complex consists of mTOR, mLST8, Raptor (regulatory associated protein of mTOR), PRAS40

(C) mTORC2 complex consists of mTOR, mLST8, Rictor (rapamycin-insensitive companion of mTOR), Proctor-1, mSIN1.

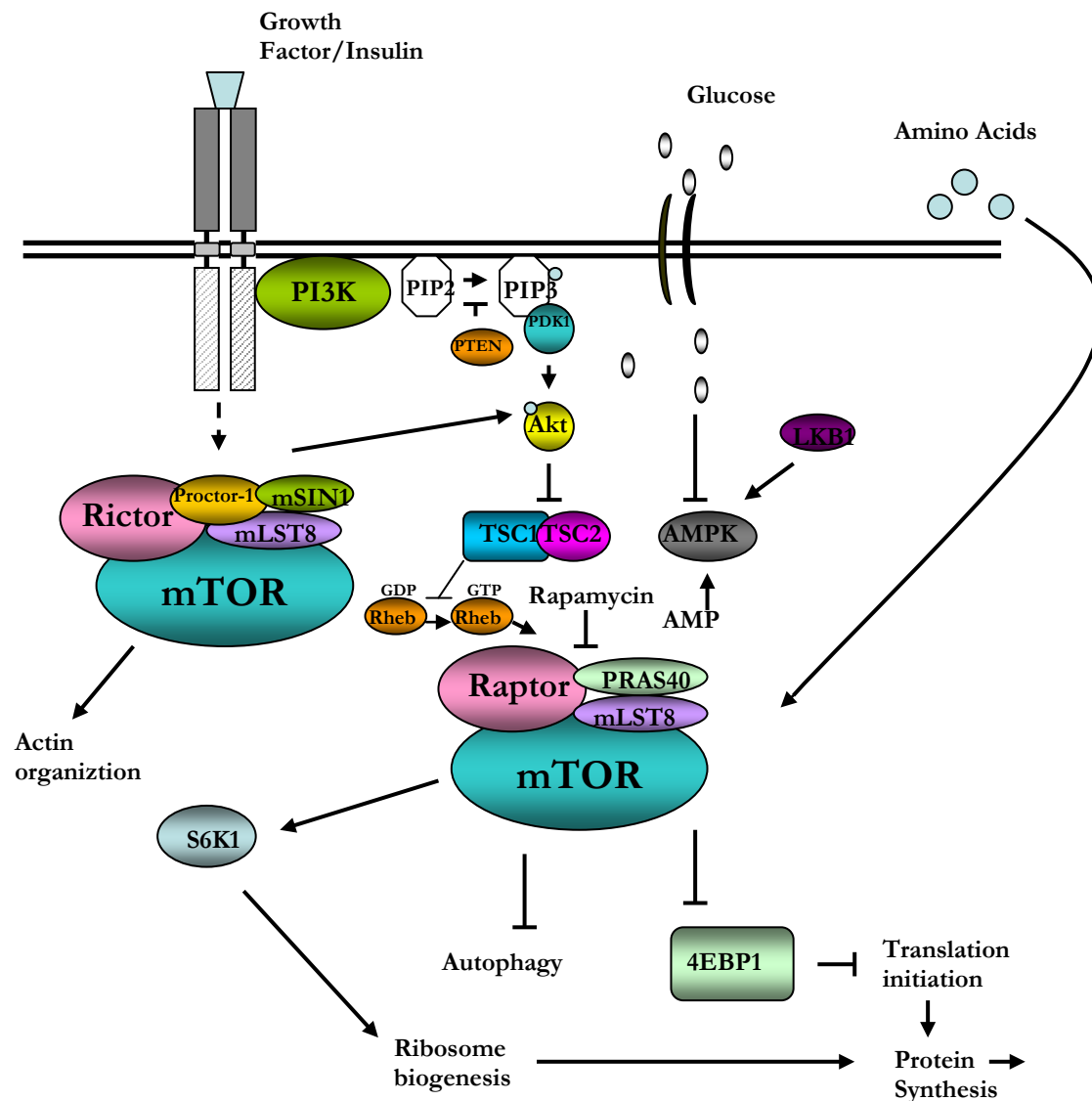


Figure 1.6 mTOR signaling

Diagram illustrating the mTOR signaling network. Growth factors/insulin initiates signaling leading to activation of PI3K. The lipid products of PI3K, PIP3 recruit both PDK1 and Akt to the plasma membrane. Akt is subsequently phosphorylated on T308 by PDK1 and by mTORC2 leading to full activation. Akt then phosphorylates numerous targets to promote growth and survival, including the TSC1/2 complex. By phosphorylating TSC2, Akt inactivates TSC2s GAP activity for the small G protein Rheb. GTP-bound Rheb is a potent activator of mTORC1. mTORC1 phosphorylates many targets, including the translation control proteins S6K1 and 4EBP1.

mTOR activation/inactivation

The mTORC1 pathway is activated by growth factors such as EGF, PDGF, IGF-I and FGFs (Bjornsti & Houghton, 2004), nutrients and mitogens (Figure 1.6).

Growth factors activate mTORC1 via the activation of PI3K and its downstream effector-Akt (Gingras et al., 1998). Akt achieves this by directly phosphorylating the tuberous sclerosis complex 2 (TSC2) (Inoki et al., 2002; Manning et al., 2002).

TSC1/2 is a heterodimer and consists of TSC1- hamartin and TSC2- tuberin (Gao et al., 2002). Akt phosphorylates TSC2 (Inoki et al., 2002) which leads to inhibition of TSC2's GTPase-activity (GAP). TSC2's heterodimerization with TSC1 allows it to exert a GAP activity toward the small GTPase called Rheb (Ras homolog enriched in brain). Rheb is a positive regulator of mTORC1. The active, GTP-bound form of Rheb binds directly to and stimulates mTORC1 (Long et al., 2005a). TSC acts as a signal-modulated suppressor of Rheb by driving Rheb into the inactive, GDP-bound state. Thus Akt stimulates the accumulation of GTP-bound Rheb, which in turn promotes the activation of mTORC1 by disrupting the TSC complex through phosphorylation of the TSC2 subunit (Crino et al., 2006).

Mutations in either TSC1 or TSC2 lead to the hamartomatous syndrome tuberous sclerosis, a cancer prone syndrome characterized by hamartomas in the brain, heart, kidney and skin (Brugarolas & Kaelin, 2004). An increase risk of renal cell carcinoma is also observed. This was the first documented molecular link between mTOR and cancer.

The best described downstream effectors of mTORC1 are S6K1 (Ribosomal p70 S6 kinase 1) and the eukaryotic initiation factor 4E Binding Protein 1 (4E-BP1). Phosphorylation of these targets by mTORC1 is mediated by sequences present on both 4EBP1 and S6K1 called TOS (target of rapamycin signaling) motifs (Schalm & Blenis, 2002). The protein raptor (a component of mTORC1), allows for the phosphorylation of 4EBP1 by binding to

the TOS domain of 4EBP1 and bringing it close to mTOR (Hara et al., 2002; Kim et al., 2002; Nojima et al., 2003; Schalm et al., 2003). Phosphorylation of 4EBP1 results in the release of eukaryotic translation initiation factor 4E (eIF4E) from 4EBP1 (Mader et al., 1995). eIF4E then binds to eIF4G and with other translation initiation factors translates RNA transcripts containing a 5' m⁷GTP cap (Pause et al., 1994). These RNA transcripts include cell cycle regulating proteins like ornithine decarboxylase 1 (ODC1) and cyclin D1. Other important proteins translated include c-MYC and hypoxia inducible factor 1 α (HIF1 α) (Hashemolhosseini et al., 1998; Mayerhofer et al., 2002; Rosenwald et al., 1995; Shantz & Pegg, 1994; Zhong et al., 2000).

Activation of S6K1 is via phosphorylation by mTOR. How this occurs is not clear. Direct phosphorylation of S6K1 has been reported (Burnett et al., 1998) however another mechanism has been suggested in which mTOR kinase activity represses the activity of a serine/threonine phosphatase (protein phosphatase 2A;PP2A) physically associated with S6K1 (Peterson et al., 1999).

S6K1 promotes protein synthesis by phosphorylating the tumour suppressor programmed cell death 4 protein (PDCD4) and targeting it for degradation (Dorrello et al., 2006). PDCD4 prevents protein translation by binding and preventing the eIF4A helicase from unwinding secondary structure in the 5' untranslated region of mRNA. Ribosomal S6 is the best characterized substrate of S6K1. Phosphorylation of this protein leads to the initiation of mRNA translation with a 5'-terminal oligopyrimidine (5'-TOP), which includes mRNAs that encode ribosomal proteins and elongation factors (Brown et al., 1995; Burnett et al., 1998; Seufferlein & Rozengurt, 1996b).

Other mTORC1 substrates include CLIP-170, LIPIN and STAT3 but their roles in mTORC1 signaling are less clear (Choi et al., 2002; Huffman et al., 2002; Yokogami et al., 2000).

Details of mTORC2 regulation are less clear than mTORC1. Growth factors stimulate mTORC2 activity, which leads to direct phosphorylation of Akt and negative regulation of FOXO1A (Sarbasov et al., 2005). Akt is phosphorylated at S473 by mTORC2 which is necessary for full activation (Figure 1.6). Another function of Akt is in regulation of the actin cytoskeleton and protein kinase C (Bjornsti & Houghton, 2004; Guertin & Sabatini, 2007; Jacinto & Hall, 2003; Schmelzle & Hall, 2000; Wullschleger et al., 2006). However further work is needed to clarify this role.

A decrease in the concentration of amino acids in a cell rapidly inactivates mTOR. Exactly how this is achieved is not clear but it does seem to be mediated by Rheb and not TSC (Nobukuni et al., 2005; Smith et al., 2005). Increased expression of Rheb in mammalian cells leads to a failure of mTOR activity to be inhibited in response to amino acid deprivation (Long et al., 2005b). TSC does not seem to regulate Rheb in response to amino acid deprivation (Smith et al., 2005).

Another downregulator of mTOR is low cellular energy levels (Inoki et al., 2003). Changes in energy levels are sensed by a change in the concentration of intracellular adenosine monophosphate (AMP) (resulting from the cleavage of ATP). Elevated AMP levels lead to the association of AMP with AMP-dependent protein kinase (AMPK). This association converts the enzyme into a substrate for phosphorylation by the kinase serine/threonine kinase 11 (STK11)/LKB1. This phosphorylation leads to activation of AMPK. Activated AMPK then directly phosphorylates TSC2 and activates the GAP function of TSC2. The activation of TSC2 results in an increase in the population of the inactive GDP-bound form

of Rheb and leads to downregulation of mTOR activity (Shaw et al., 2004; Woods et al., 2003).

Hypoxia leads to rapid inactivity of mTOR, as measured by autophosphorylation and phosphorylation of 4E-BP1 and S6K1. Again the mechanism is not known but may involve Rheb and TSC2 (Arsham et al., 2003).

This information shows that the protein Rheb acts as a gatekeeper to mTOR activity, sensing the cellular environment for conditions favorable to growth.

mTOR and cancer

mTOR is an attractive anti-cancer target since in many malignancies distinct mechanisms can result in constitutive activation of the PI3K/AKT/mTOR pathway.

PTEN, the phosphatase that negatively regulates PI3K, is mutated, silenced or deleted in many cancers (Table 1.4). The result is an upregulation or constitutive activation of Akt and hence mTOR. As previously mentioned, activating mutations in Akt2 or gene amplification of Akt2, are also frequently seen in malignancy (Table 1.4).

Mutations in TSC proteins lead to the cancer prone tuberous sclerosis syndrome. Inactivating mutations in the kinase LKB1 are associated with Peutz-Jeghers cancer prone syndrome (Park et al., 1998). Inactivation of LKB1 leads to inhibition of the ability of AMPK to activate TSC2, which would happen in low energy conditions. Since TSC2 inhibits Rheb, the result is that LKB1 mutations lead to a higher level of mTOR activity under low energy conditions.

There are no known mutations of mTOR (Rosner et al., 2008) however, recently it has been shown that increased mTOR staining is linked to adverse prognosis in breast, biliary cancers and gastric cancers (Herberger et al., 2007; Murayama et al., 2009; Zhou et al., 2004).

Downstream of mTOR, cancer related changes are seen in some malignancies. For example, S6K1 is overexpressed in certain tumour cell lines (Pardo et al., 2001) and in the early stages of transformation in ovarian surface epithelium associated with BRAC1 mutations (Wong et al., 2001). In certain breast cancers S6K1 is also amplified (Couch et al., 1999).

The gene coding eIF4E is altered in many cancers. Amplification is seen in head and neck, ductal breast, and thyroid cancers (Haydon et al., 2000; Sorrells et al., 1999; Wang et al., 2001). The levels of eIF4E are raised in some colon cancers and also increased in some bladder and breast cancers that have a poor prognosis (Berkel et al., 2001; Crew et al., 2000; Li et al., 2002; Rosenwald et al., 1999).

mTOR and lung cancer

Since the focus of this thesis is lung cancer, the discussion will now turn to mTOR and lung cancer.

Many lines of evidence point to the importance of deregulated PI3K/Akt/mTOR signaling in lung cancer. This pathway was found to mediate the effects of RTKs, such as c-Met, EGFR, c-Kit and IGF-IR on proliferation and survival in SCLC and NSCLC (Arcaro et al., 2002; Krystal et al., 2002; Mukohara et al., 2003; Pisick et al., 2004; Tsao et al., 2005). In primary lung carcinomas, overexpression of p85 α and p110 α subunits of PI3K has been reported (Lin et al., 2001). Amplification of PIK3CA was also observed in many lung cancers and in pre-invasive bronchial lesions (Massion et al., 2004).

As previously mentioned, PTEN mutations are not common in SCLC (only in 15%)(Burbee et al., 2001; Yokomizo et al., 1998) but more common in NSCLC (Steck et al., 1997).

Regarding Akt, increased phosphorylation was reported in pre-malignant (metaplastic) and malignant human bronchial epithelial cells as compared to normal bronchial cells and

responses to a new pro-apoptotic drug were higher in those with greater Akt activation levels (Chun et al., 2003). Other studies showed that phosphorylation or overexpression of Akt correlated with poor prognosis in NSCLC patients with stage I disease or with primary tumours (David et al., 2004; Tsurutani et al., 2007).

Data from our group (Seckl laboratory) have shown that mTOR is overexpressed in SCLC cell lines as compared to primary normal lung epithelial cells (Arcaro et al., 2002; Marinov, 2009) and over expressed in SCLC patient samples as compared to normal lung tissue (Pardo et al., 2006).

Activation of PI3K pathway has been shown to increase resistance to chemotherapy and radiotherapy (Brognard et al., 2001). Inhibition of PI3K led to increased sensitivity of lung cancer cells to radiotherapy (Gupta et al., 2004) and chemotherapy (Krystal et al., 2002). Subsequently RAD001 (Everolimus), an mTOR inhibitor/rapalogue sensitized NSCLC cells to cisplatin chemotherapy (DNA damaging agent), by down-regulation of p21, which is essential for DNA repair (Beuvink et al., 2005).

Similar studies in SCLC showed that another mTOR inhibitor CCI-779 (Temsirolimus) sensitized cisplatin-resistant SCLC to the effects of cisplatin (Wu et al., 2005). Again at the cell line level rapamycin can inhibit growth in some lung cancer cell lines and produce cell cycle arrest (Boffa et al., 2004; Sun et al., 2005).

Human and murine preneoplastic lung lesions induced by tobacco exposure are characterized by increased activation of the Akt/mammalian target of rapamycin (mTOR) pathway, suggesting a role for this pathway in lung cancer development. It was found that when mice were exposed to the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, those given rapamycin, led to markedly reduced development and

growth of tumors suggesting that tobacco carcinogen-induced lung tumors in mice are dependent upon mTOR (Granville et al., 2007).

Multiple NSCLC cell lines when treated with mTOR inhibitors leads to a decrease in phosphorylation of 4E-BP1 and S6K1 and paradoxically to an increase in phosphorylation of Akt (Boffa et al., 2004; Buck et al., 2006; Sun et al., 2005). This is likely to be due to an inhibition of a normally occurring feedback loop. The underlying mechanism for this are that mTOR inhibitors/rapalogues activate AKT through IGF-1R (Wan et al., 2007). Akt phosphorylation may lead to an increase in tumour survival (O'Reilly et al., 2006; Shi et al., 2005; Wan et al., 2007).

Unfortunately, CCI-779 did not improve patient survival but did improve progression free survival, when administered as a single agent as maintenance therapy in a Phase II clinical trial for SCLC patients after previous chemotherapy (Pandya et al., 2005). This trial did not utilize a placebo control arm instead used 2 different doses of mTOR inhibitors.

In the FGF growth factor family, the role of FGF-2 has been elucidated in SCLC and it has been found to promote proliferation and chemoresistance in these cells (Pardo et al., 2002; Pardo et al., 2001). These effects were found to be consistent with and dependent on activation of MEK/ERK signaling in these cells and a novel cross-talk between MEK/ERK and ribosomal S6 kinase 2 (S6K2). The FGF-2 induced survival against the chemotherapeutic agent etoposide in SCLC cells was found to be regulated by increased translation of the anti-apoptotic proteins Bcl-XL, Bcl-2 and IAPs (Pardo et al., 2003). It was also shown that FGF-2 treatment of SCLC cells results in the formation of a signaling complex comprising PKC ϵ , B-Raf and S6K2, which promotes growth factor prosurvival effects (Pardo et al., 2006).

S6K2 is a serine/threonine kinase identified as a homologue of S6K1. S6K1 and S6K2 show different cellular localization as well as divergent amino acid sequences in the non-catalytic N- and C-terminal domains, suggesting that their cellular functions and/or regulation may not be identical (Lee-Fruman et al., 1999). Other differences include from animal knockout studies show no phenotype for S6K2^{-/-} but small animals in the S6K1^{-/-}. (Pende et al., 2004) and that unlike S6K2, MEK/ERK signaling is not required for S6K1 activation by several growth factors including FGF-2 (Pardo et al., 2001). Nevertheless, some investigators have found that S6K1 and S6K2 are regulated by mTOR although others have found that there is a differential effect of mTOR inhibitors/rapalogues on S6K1 and S6K2 activity. Work from the Seckl laboratory has shown that at low rapamycin concentrations, growth factor-induced S6K1 activation is selectively inhibited without effect on S6K2. In contrast, at 10-fold higher concentrations of rapamycin, there is a partial inhibition of S6K2 activation, which correlates, with inhibition of MEK/ERK signaling (Pardo et al., 2001) (Figure 1.7).

Both mTOR and its downstream target S6K1 are known to be involved in growth factor induced proliferative signaling and S6K2 was recently implicated in FGF-2-induced chemoresistance (Pardo et al., 2006). The potential for rapamycin or similar drugs to block FGF-2-induced chemoresistance in lung cancer at high as opposed to low concentrations needs investigation and could be relevant in the development of new therapeutic strategies.

This information clearly indicates that targeting mTOR is a promising therapeutic approach in lung cancer.

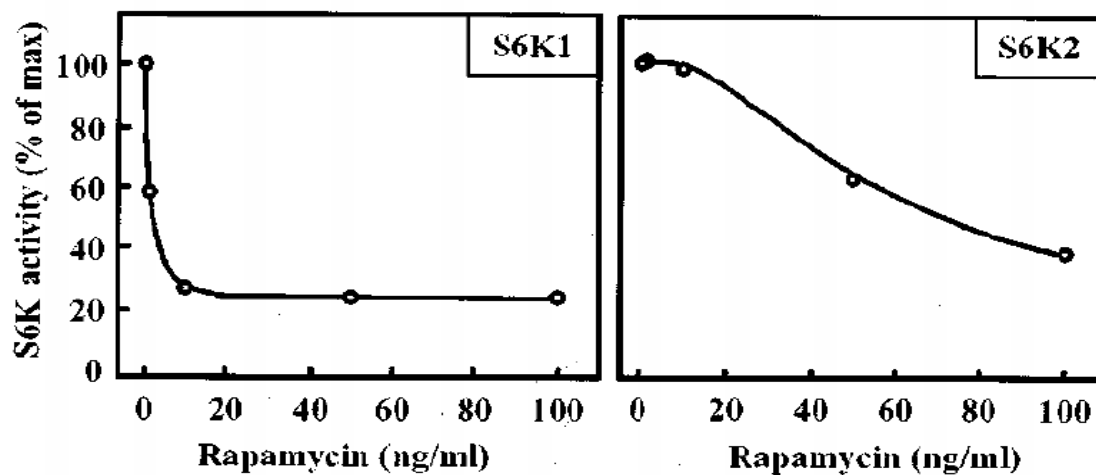
The discussion will now focus on the drugs which inhibit mTOR.

mTOR inhibitors

As mentioned rapamycin is a natural antibiotic, a macrocyclic lactone, produced by *Streptomyces hygroscopicus*, a soil bacterium native to Easter Island (Rapa Nui). It is the original mTOR inhibitor.

Rapamycin/Sirolimus forms a ternary complex with FKBP12 and mTOR (Figure 1.8). This complex then leads to inhibition of mTOR signaling by binding to FRB region of mTOR. Structural studies indicate that there are relatively few contacts between the proteins (Choi et al., 1996) This suggests that when FKBP12 binds to rapamycin, this may place rapamycin into a position to allow for interaction with mTOR. Other mTOR inhibitors have been developed; they all are the result of minor modifications to the structure of rapamycin, making them more soluble and stable. They are known as 'rapalogues'. They are CCI-779/Temsirolimus (Wyeth), RAD001/Everolimus (Novartis), and AP23573/Deforolimus (Ariad). The differences are made by modifications of the C40 hydroxyl position of rapamycin. Most have substituted this group for an ester or ether. AP23573 substitutes a phosphonate for the C40 hydroxyl. CCI-779 is a pro-drug; metabolized to rapamycin in the body. Despite these relatively new agents, novel mTOR inhibitors have been developed which bind to the ATP site and therefore target mTORC1 and mTORC2 (Feldman et al., 2009). These molecules are pyrazolopyrimidines and also inhibit other members of the PI3K family, including mTOR (Apsel et al., 2008). Two of these molecules, PP242 and PP30 are potent selective and ATP-competitive inhibitors of mTOR. These molecules have been named TORKinibs for TOR kinase domain inhibitors. Recent data suggest that these agents are more active than rapamycin with respect to inhibiting proliferation and are active in situations where cells are resistant to rapamycin (Feldman et al., 2009).

A



B

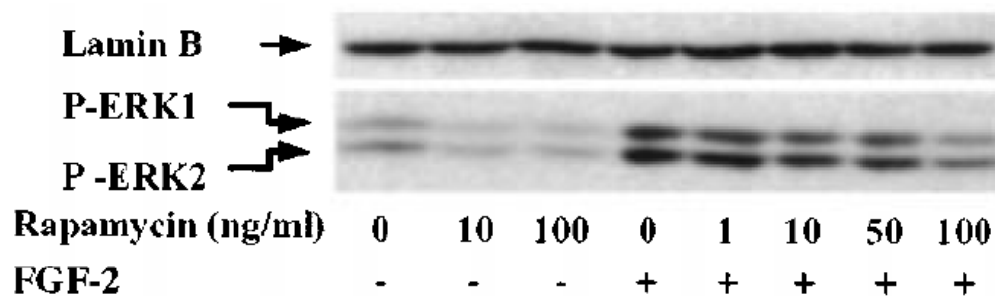


Figure 1.7 S6K1 and S6K2 are regulated differently with respect to mTOR

(A) The effect of increasing doses of Rapamycin on S6K1 and S6K2 activation by FGF-2 as assessed by kinase assay.

(B) The effect of increasing doses of Rapamycin on FGF-2 induced ERK1/2 phosphorylation in H510 SCLC line.

From Pardo et al 2002

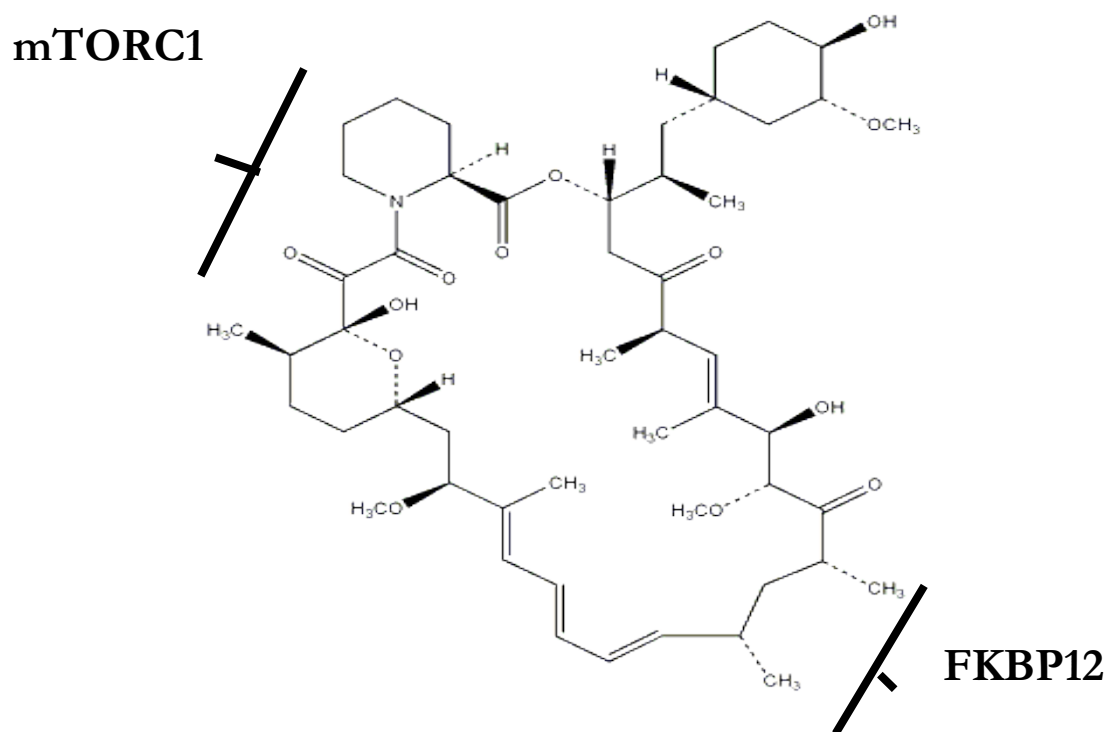


Figure 1.8 Structure of rapamycin

Chemical structure of rapamycin, in the conformation known to bind FKBP12 and the FKBP12 rapamycin binding domain of mTORC1 complex.

Taken from Molecular Targeting in Oncology, 2008, Kaufman et al.

Effects of mTOR inhibition

Cell cycle arrest (G1 arrest) is a major effect of blocking mTOR, be that in yeast by deletion of TOR1 or by using rapamycin in mammalian cells (Cafferkey et al., 1994; Fingar et al., 2004). mTOR inhibitors prevent phosphorylation of 4EBP1; which then inhibits ⁷mGTP cap-dependent translation, particularly of proteins essential for cell cycle progression such as c-MYC, ODC and cyclin D. Not only is the translation of these proteins blocked but the level of p27 kip1; an inhibitor of CDK2 is increased. CDK2 is required for cell cycle progression to S-phase. How this happens is not clear (Barata et al., 2001). The cell cycle effects are not the same in all cells, and are probably dependent on cell type and genetic changes in the individual cells.

Treatment of certain cell types with rapamycin leads to apoptosis. Two proteins have been implicated namely c-MYC and Akt. c-MYC translation is regulated in part by cap-dependent mechanisms, thus it is dependent on eIF4E. c-MYC has both anti and pro apoptotic effects (Secombe et al., 2004). Akt phosphorylates the pro-apoptotic protein BAD (BCL-2 associated death promoter)(Datta et al., 1997).

Under serum free conditions, rapamycin treatment causes apoptosis in tumour cell lines with mutated p53. Overexpression of wild type p53 or p21 protects these cells from rapamycin induced apoptosis (Huang et al., 2001). Apoptosis, from treatment with rapamycin on p53 mutant cells is a result of stress-dependent activation of the c-Jun N-terminal kinase (JNK) pathway. Activation of JNK pathways is dependent on the presence of 4EBP1 and its ability to bind eIF4E when mTOR is inhibited by rapamycin (Huang et al., 2003).

The mTOR pathway is involved in angiogenesis through regulation of HIF α . HIF α is a primary activator of VEGF (Jiang et al., 2001; Laughner et al., 2001). Treatment of endothelial cells with rapamycin reduces production of VEGF (Guba et al., 2002).

mTOR in clinical trials

Once the original identification of the anti-cancer effects of rapamycin were established, rapamycin and later analogues have been tested on many tumour cell lines and xenograft tumour models (Douros & Suffness, 1981; Eng et al., 1984; Houchens et al., 1983). Proliferation was inhibited in many cell lines including rhabdomyosarcoma, glioblastoma, small cell lung carcinoma and renal cell carcinoma (Dilling et al., 1994; Hosoi et al., 1998; Seufferlein & Rozengurt, 1996b).

Starting with Temsirolimus, which has been extensively used in clinical trials and has recently been approved for use in the US for advanced renal cell carcinoma (Hudes et al., 2007).

A phase I clinical study looked at daily and weekly intravenous use. There were significant toxicities with daily use such as hypocalcaemia, vomiting, thrombocytopenia and hepatic transaminase increases. Weekly schedules did not reveal any grade 3 toxicities (Dancey, 2002). Based on the results from phase I clinical trials phase II studies were set up with advanced stage renal cell carcinoma, refractory mantle cell lymphoma and metastatic breast cancer.

A phase II trial in renal cell carcinoma showed an increase in survival (4 months) (Atkins et al., 2004). In mantle cell lymphoma a small increase in time to progression was observed as compared to placebo (Witzig et al., 2005). Advanced breast cancer patients responded reasonably well- overall response rates were around 9% (Chan et al., 2005).

Data from these trials and others using RAD001 indicate that the drugs are well tolerated and safe (Fouladi et al., 2007; J. C. Yao, 2007).

A phase III clinical trial showed that as compared with interferon alfa, temsirolimus improved overall survival significantly among patients with metastatic renal-cell carcinoma with a poor prognosis (Hudes et al., 2007), showing proof of principle that this class of drug

can improve survival in cancer patients. Many other phase II/III trial are on going in many cancer types (Strimpakos et al., 2008).

However good these results are, the overall picture at the clinical trial level is somewhat disappointing with single agent mTOR inhibitors showing promise in only a few types of malignancy. It is not clear how to combine mTOR inhibitors with existing therapies. Biomarkers are needed to identify which patients may respond to these drugs. The new mTOR kinase inhibitors may provide more impressive results.

Clearly the mTOR pathway and mechanism of action of mTOR inhibitors are not completely understood.

I.5 The Thesis

Rapamycin and the clinically relevant orally bioavailable derivative RAD001 and CCI-779 are inhibitors of mTOR. It has previously been shown that low concentrations of rapamycin selectively inhibit mTOR/S6K1 mediated proliferation induced by FGF-2 (Pardo et al., 2001). In preliminary experiments, it was shown that low concentrations of rapamycin or RAD001 blocks proliferation induced by multiple growth factors in H510 and H69 SCLC cells in vitro. Moreover, orally administered RAD001 blocks H69 xenograft growth in vivo. Thus, inhibition of mTOR may provide a useful therapeutic strategy for SCLC by blocking tumour proliferation.

Furthermore as mentioned, clinical data suggests that the mTOR inhibitor-CCI-779, when used as maintenance therapy after initial chemotherapy for SCLC prolongs the time to relapse (Pandya et al., 2005). Intriguingly, rapamycin may also block prosurvival signals in SCLC cells. As mentioned it has also previously been shown that higher concentrations of rapamycin block MEK/ERK and S6K2 signaling which is necessary for pro-survival/chemoresistance effects induced by FGF-2 in SCLC cells (Pardo et al., 2001).

At least two explanations for this observation can be envisaged:

- i) the drug is hitting a second target or
- ii) High concentrations of drug cause an additional conformational change in FKBP12/mTOR resulting in these secondary effects.

Importantly, the original data with rapamycin suggest that at higher, but clinically achievable concentrations, this compound blocks chemoresistance induced by FGF-2 in SCLC cells. Can the more clinically relevant RAD001 do the same? If so, one might expect that adding an mTOR inhibitor such as RAD001 to chemotherapy should result in additive and/or

synergistic effects. Since it is possible that sequencing of the drugs may effect whether additive/synergistic as opposed to antagonistic effects are seen, it will also be determined whether giving RAD001 before or after cisplatin or etoposide makes a difference to cell death.

In this study to further investigate these findings both in SCLC and NSCLC cell lines and tissue biopsies, we will determine the following:

- What is the expression of mTOR in lung cancer biopsy specimens and how does this relate to clinical outcome?
- Do high concentrations of RAD001 like rapamycin also block FGF-2-induced MEK/ERK/S6K2 activation and chemoresistance and is this dependent on mTOR or another target?
- Assessment of the combination of mTOR inhibitors with cytotoxic drugs in vitro.

The information derived from these studies should provide new mechanistic information and help to underpin phase I/II trials of mTOR inhibitors in lung cancer.

CHAPTER II

Materials and Methods

II.1 Materials

Cell lines 11.1.1

Cell Line Source	HCC 78 Non-small cell lung carcinoma (Adenocarcinoma) pleural effusion	HCC 95 Non-small cell lung carcinoma (Squamous), pleural effusion	MCF-7 Breast Adenocarcinoma, pleural effusion	
Gender, Age Karyotype data	M, 65 years hypotetraploid karyotype	M,65 years	F, 69 years Modal chromosome number=82 Range =66 to 87, tetraploid	
Morphology Growth Medium	Adherent epithelial cells DMEM with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin,2mM Glutamine	Adherent epithelial cells DMEM with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin,2mM Glutamine	Adherent epithelial cells DMEM with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin,2mM Glutamine	
Temperature, Atmosphere Subcultivation	37°C, 10% CO2 1:3 to 1:5, 2-3 times a week	37°C, 10% CO2 1:3 to 1:5, 2-3 times a week	37°C, 10% CO2 1:3 to 1:5, 2-3 times a week	
Cell Line Source	P2G Type II pneumocytes, immortalised from human lung resection specimen lung tissue	H69 Small cell lung carcinoma, pleural effusion	H510 Small cell lung carcinoma, pleural effusion	H524 Small cell lung carcinoma, lymph node
Gender, Age Karyotype data	Not available Chromosome number=46	M, 55 years Modal chromosome number=76-78 Range=40 to 87, Aneuploid cell line	M, 56 years Modal chromosome number=54, hypotriploid cell line	M, 63 years Modal chromosome=51-55, hypotriploid cell line
Morphology	Adherent epithelial cells	Suspension cells, grown in aggregates	Suspension cells, grown in aggregates	Suspension cells, grown in aggregates
Growth Medium	DCCM-1 with 10% newborn calf serum, 100units/ml penicillin,100µg/ml streptomycin, 2mM Glutamine	RPMI1640 with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin, 2mM Glutamine	RPMI1640 with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin, 2mM Glutamine	RPMI1640 with 10%FCS, 100units/ml penicillin,100µg/ml streptomycin, 2mM Glutamine
Temperature, Atmosphere Subcultivation	37°C, 5% CO2 1:2, once a week	37°C, 5% CO2 1:2, once a week	37°C, 5% CO2 1:2, once a week	37°C, 5% CO2 1:2, once a week

Three SCLC cell lines (H510, H524, and H69), two NSCLC cell lines (HCC78, HCC95), a breast cancer cell line (MCF-7) and one type II lung pneumocyte (P2G) were used in this study. They have all been well characterized and detailed information is in Table 2.1. The cells were grown in complete media and were changed to conditioned media prior to experiments.

Condition media

SCLC cell lines: Every week, a proportion of cells were passaged into serum free Roswell Park Memorial Institute 1640 (RPMI) media containing glutamine/penicillin/streptomycin and supplemented with insulin, transferrin, sodium selenite (NaSe) and bovine serum albumin (BSA) also referred to as SITA in the following concentrations:

	<u>Concentration in medium</u>	<u>Stock solution</u>
Insulin	5 μ g/ml	2.5 mg/ml in dH ₂ O (4°C)
Transferrin	10 μ g/ml	50mg/ml in PBS (-20°C)
NaSe	30nM	100 μ M in PBS (-20°C)
BSA	0.25% (w/v)	125 mg/ml in dH ₂ O (-20°C)

The cells were grown in this media for 4-7 days prior to experiments.

For NSCLC cells the growth media was changed to serum free Dulbecco's Modified Eagle's Medium (DMEM) containing glutamine/penicillin/streptomycin the day before experiments.

II.1.2 Antibodies

Antibody and working dilution	Supplier	Source	Working Antibody Dilution
ERK1/2	Santa-Cruz Biotechnology, Germany	Rabbit	1:1500
AKT	Cell Signaling, USA	Mouse	1:1000
Biphospho-ERK1/2	Sigma-Aldrich Company Ltd	Mouse	1:1000
Phospho- AKT (Ser473) (IHC specific)	Cell Signaling, USA	Rabbit	1:50
FKBP12	Cell Signaling , USA	Rabbit	1:500
mTOR	Cell Signaling , USA	Rabbit	1:1000
Phospho-mTOR (Ser 2448)	Cell Signaling , USA	Rabbit	1:1000
Phospho-mTOR (Ser2448) (IHC specific)	Cell Signaling, USA	Rabbit	1:1000
Phospho- S6K1 (T389)	Cell Signaling , USA	Mouse	1:1000
LaminB	Santa-Cruz Biotechnology, Germany	Goat	1:500

Table 2.2 List of antibodies

II.1.3 Reagents

Human recombinant FGF-2 (50µg/ml stock in phosphate buffer solution (PBS) containing 0.1% BSA, stored at -80°C), SCF (10µg/ml stock in dH₂O, stored at -80°C) and ADH (30µM stock in dH₂O, stored at -80°C) were from Merck Biosciences Ltd (UK). Phorbol ester (PDB) (400µM stock in Dimethyl sulfoxide (DMSO), stored at -20°C) was purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Etoposide (100mM stock in DMSO, stored at -20°C) and cisplatin (100mM stock in DMSO, stored at -20°C) were also purchased from Merck Biosciences Ltd (UK). RAD001 (Everolimus) (100µM stock in DMSO stored at -20°C) was a gift from Novartis International AG (Basel, Switzerland). Okadaic acid powder (10mg stored at -20°C) was purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Enhanced chemo-luminescence (ECL) reagents were purchased from GE Healthcare (UK)/Millipore Ltd (UK)/Perbio Science Ltd (UK). RPMI 1640 and Dulbecco's Modified Eagle's Medium (DMEM) were from Sigma-Aldrich Company Ltd (Dorset, UK) and foetal calf serum (FCS) from First Link (Birmingham, UK). mTOR rabbit blocking peptide was purchased from Cell Signaling Technology Inc (Danvers, USA).

Polymer-Horseradish peroxidase (HRP) immunohistochemistry (IHC) detection kit was purchased from Biogenex Laboratories Inc (San Remon, USA). Diaminobenzidine tablets were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). On target plus siRNA oligonucleotides for mTOR/FKBP12, DharmaFECT D4 transfection reagent and 5XsiRNA buffer were purchased from Dharmacon RNAi technologies, Thermo Scientific (Lafayette, USA).

II.1.4 Buffers

Sodium Dodecyl Sulphate- Polyacrylamide Gel electrophoresis (SDS-PAGE)

1.5M Tris (pH 8.8)

182g Tris-base dissolved in
800ml ddH₂O
pH to 8.8 with HCL
made up to 1l with ddH₂O

1.0M Tris (pH 6.8)

121g Tris-base dissolved in
800ml ddH₂O
pH to 6.8 with HCL
made up to 1l with ddH₂O

10% SDS

50g SDS
dissolved in
500ml ddH₂O

10% APS

2g APS dissolved in
20ml ddH₂O stored
At 4°C

2x SDS Loading Buffer

100mM Tris-HCL (pH 6.8)
4% SDS
0.2% Bromophenol blue
20% Glycerol
200mM DTT

Running buffer

250mM Glycine
25mM Tris
0.1% SDS

5x stock: 94g Glycine
15g Tris, 5g SDS
dissolved in 1l ddH₂O

Transfer buffer

39mM Glycine
48mM Tris
0.037% SDS
20% methanol

10x stock: 29g Glycine,
58g Tris, 3.7g SDS
Dissolved in 1l ddH₂O
20% methanol was added prior
to use.

WESTERN BLOTTING

5X TBS

750mM NaCl
100mM Tris (pH 7.5)

1x: 60ml 5M NaCl
40ml 1M Tris (pH 7.5)
Up to 2L with ddH₂O

Blocking buffer

1x TBS containing 0.1%
Tween (TBST)
3% Marvel (or BSA)

Washing buffer

1 x TBST

II.2 METHODS

II.2.1 SDS-PAGE

Gels were made with a 4% stacking gel and a 8-12% acrylamide resolving gel (according to the method of Laemmli (Laemmli, 1970)). The samples were boiled at 100°C in 2X sample buffer (3% SDS, 5% glycerol, 2% β -mercaptoethanol, 10mM Tris HCL, pH 6.8) for 5 minutes prior to either storage at -20°C or electrophoresis. Electrophoresis was performed at maximal 20mA per gel until the bromophenol blue reached the bottom of the gel. The gels were then prepared for transfer onto Immobilon Polyvinylidene Fluoride (PVDF) membranes.

II.2.2 Western Blotting

The proteins from samples run on acrylamide gels were subsequently transferred onto PVDF membranes using a wet-blotting apparatus. Transfers were run at 400mA for 2 hours at 4°C. The membranes were then in blocking buffer containing milk (5%) and 0.05% Tween 20 for 1 hour at room temperature, and probed with the appropriate antibodies diluted in Tris buffered saline-Tween (TBST) with 3% milk or BSA. Dilutions were made according to the datasheets for the individual antibodies and probing was performed overnight at 4 °C. The membranes were then washed three times for 15 minutes each before incubating with the suitable HRP-conjugated antibodies in 3% milk in TBST for 45 minutes. Three more 15 minute washes were then performed and the membranes were subjected to ECL according to the manufacturer's instructions (Millipore). The proteins on the membranes were revealed using X-ray film.

II.2.3 Growth factor stimulation assays

SCLC cells: Cells were grown in serum free media (SFM) for 4-5 days. They were subsequently centrifuged at 1000rpm for 5 minutes and washed in RPMI 3 times, aliquoted in 1.5ml eppendorf tubes at a concentration of 2×10^6 cells/ml and left in the incubator (37°C , $5\%\text{CO}_2$) for 1 hour. When relevant they were treated with RAD001, at the concentrations for each experiment for 1 hour and then stimulated with FGF-2, SCF, ADH or PDB at the concentrations indicated for each experiment in the results section. At the appropriate time points, the samples were transferred on ice, centrifuged at 4000 rpm for 1 minute and washed with 1ml cold PBS. The cells were centrifuged for a further 1 minute at 4000rpm and then lysed in 2x SDS sample buffer (70 μ l for the pellets of 2×10^6 cells). Lysates were analyzed by SDS-PAGE and immunoblotted with the appropriate phospho-specific antibodies.

NSCLC and MCF-7 cells: Cells were plated out to $\sim 80\%$ confluence in 6-well plates and left overnight to adhere to the surface of the plates. The cells were subsequently placed in 0.5% FCS/DMEM overnight. They were then placed in DMEM the next morning for 1 hour. Where relevant, they were pre-treated with RAD001 (indicated concentrations) for 1 hour prior to addition of FGF-2 (1ng/ml) or PDB (400nm) for the appropriate times.

The cells were centrifuged for a further 1 minute at 4000 rpm and then lysed in 100 μ l 2x SDS sample buffer. Lysates were analysed by SDS-PAGE and immunoblotted with the appropriate phospho-specific antibodies.

II.2.4 Phospho-signal stability studies

SCLC and NSCLC cells in growth media were lysed in 70µl of 2x SDS sample buffer at the time points indicated in the experiments. Lysates were analysed by SDS-PAGE and immunoblotted with the appropriate antibodies.

For the study of formalin fixed phospho-signals, cells (from growth media)/patient NSCLC tissue samples (straight from the operating theatre) were fixed in formalin at the time points indicated in the experiments for four hours. Formalin fixed cells were then processed for immunostaining described later.

II.2.5 Cell death/chemoresistance assays

For cell death assays, H510 cells that were grown in SFM for 3-5 days were resuspended in fresh SFM and aliquoted in 24-well plates at a density of 5×10^4 cells/ml. They were then pre-treated, where appropriate, with RAD001 (mTOR inhibitor) at the concentrations indicated for 1 hour and with FGF-2 (1ng/ml) for 4 hours prior to a single addition of etoposide (0.1µM). Every condition was set up in triplicate and the cells were counted after 4 days using a haemocytometer.

HCC95 and HCC78 cells were treated similarly; however 100nM PDB and 10µM etoposide was used in the HCC78 cell line and 1ng/ml FGF-2 and 20µM etoposide in the HCC95 cells.

II.2.6 Immunohistochemistry Studies

Patients

A consecutive series of 134 patients with primary operable NSCLC (Stage IA-IIIB) were investigated from July 1987 to March 2002 at Perugia University Hospital, Italy. At surgery,

an average of 9 lymph nodes (range 1-22) was sampled per patient. Surgical specimens were routinely processed and classified according to the World Health Organisation criteria (WHO, 2007). Histopathological data on tumour type, grade, lymph node status, stage and angio-invasion together with clinical data including age, sex, performance status, time to relapse and overall survival were routinely recorded prospectively in a database as previously published (Cagini et al., 2000). The study was approved by the local institution review board. Normal lung tissue and SCLC patient tissue microarrays were purchased from Biomax, Inc (Rockville, USA), code BN04011 and BS04111 respectively.

Tissue microarray construction

NSCLC tissue microarrays (TMA) were prepared as previously described (Kononen et al., 1998). In the tissue technique, a hollow needle is used to remove tissue cores of 1 mm in diameter from the most representative areas of tumours in paraffin-embedded tissues. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide and then analyzed as per below. Each microarray block can be cut into 100 – 500 sections, which can be subjected to independent tests. Each case was sampled three times.

Immunostaining and Immunohistochemistry scoring for mTOR

mTOR immunoreactivity was evaluated on TMA sections using the mTOR rabbit monoclonal antibody in a modification of the antigen retrieval technique of Shi and colleagues (Shi et al., 1991). Briefly, the sections were rehydrated in graded alcohols, heated in a microwave oven at 900W for 20 min in Citrate buffer at pH6. They were cooled at room

temperature before immunostaining. mTOR monoclonal antibody was used at 1:50 dilution for 1 hour at room temperature and then processed with Polymer-HRP Kit with Diaminobenzidine development and Mayer haematoxylin counterstaining. An mTOR positive SCLC cell line (H510) cyto block was used as a positive external control. Negative controls were obtained by omitting the primary antibody, using competing peptide used to raise the primary antibody and by staining H510 cells in which mTOR had been selectively knocked down by RNAi.

A semi quantitative immunohistochemical score (IHS) was used including assessment of both the intensity of staining and the percentage of positive cells. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. IHS and similar semi quantitative scoring systems have been successfully used for other TMA evaluations (Herberger et al., 2007).

Each core was scored individually. Three cores were evaluated from each tumour and the mean of the 3 readings was calculated. If one core was uninformative the score applied was the mean of the remaining two cores. All immunohistochemical staining was independently evaluated by two pathologists without knowledge of the clinical data. The interobserver discrepancy was limited to 3% of cases. These cases were reviewed at the double head microscope and an agreement was reached.

Statistical analyses

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows (Version 15) (SPSS Inc, Chicago, IL, USA) and the R Project version 7.0. Percentages were rounded to the nearest whole integer. The Kaplan-Meier method was used to determine survival (Bland & Altman, 1998). The overall survival was defined as the time

(in months) from the date of the primary treatment to the time of death. To define the optimum mTOR IHS a Martingale graph plotting excess deaths against mTOR value for lung cancer was determined (Therneau T, 1990). The optimal cutoff is identified as the point where the curve cuts the x axis and the strength of the estimates cutoff is indicated by the slope of the increase in excess deaths with increasing mTOR value was performed (Therneau T, 1990). We assessed the univariate effect of each variable on survival by log rank test (this statistical test has no hazard ratio) for categorical predictors or the univariate Cox model for continuous predictors. Variables which were significant univariately (stage, mTOR and angioinvasion) were candidates for a multivariate Cox model. Selection of predictors in this final model of 0.05 for entry into the model and $p=0.1$ for removal of insignificant predictors. These are the default cutoffs in SPSS 15. The fit of the multivariate and univariate Cox models was confirmed by the supremum test of Martingale-based residuals.

II.2.7 Drug Combination Analysis

Growth Inhibition Assay

Cell counting using a haemocytometer was used to evaluate the individual growth inhibitory activities of RAD001 and the cytotoxic chemotherapy drugs etoposide and cisplatin in SCLC cell lines. SCLC cells were seeded at 1,500 per well in 24-well plates; at 24 h after seeding, cells were treated with varying concentrations of RAD001, cisplatin or etoposide as indicated in the results. After 4 days of treatments, growth inhibition was measured by cell counting. The experiments were performed in triplicate. Results were plotted on graphs by using CalcuSyn software (Biosoft, Missouri, USA) and used to determine respective inhibitory concentration (IC) $IC_{12.5}$, IC_{25} , IC_{50} , IC_{100} , and IC_{200} for combination studies.

Drug combination analysis by the isobologram/combination index method

To determine synergy, additivity or antagonism of combinations of these drugs in SCLC cell lines; the isobologram/combination index method was used which relies upon the calculation of the fixed dose combination that cause a given effect, like 12.5%, 50% or 100% growth inhibition (Chou, 2006). For each fixed dose combination, the combination index (CI) was calculated and plotted on a graph using Calcosyn software. For example, the dose of RAD001 and etoposide that causes 50% (IC_{50}) of killing in H510 SCLC cells were combined together in the SCLC cells to determine synergy, additivity or antagonism of the combination depending on the interaction index, defined as = 1, <1, or >1, respectively.

SCLC cells in SFM were seeded in 12 well plates, five fixed drug ratios above and below the IC_{50} , as mentioned were explored by incubating the drug combinations with cells for 4 days and then determining the degree of cytotoxicity by cell counting with trypan blue exclusion was used to assess cell viability. The CI values obtained from all experiments (all repeated in triplicate) within a cell line and sequence were pooled, and the mean and the variance calculated.

II.2.8 RNA interference (siRNA)

MCF-7 breast cancer cells were plated out to ~80% confluence overnight in 6-well plates. The next morning the growth media was aspirated off and the cells were washed with DMEM. 800 μ l neat DMEM was then added to each well.

Meanwhile pooled RNAi oligonucleotide (20 μ M) (Pool consists of 4 deconvoluted oligonucleotides each at 20 μ M concentration) (For individual sequences for RNAi oligonucleotides for mTOR and FKBP12 see Table 2.3) and a pool of non-specific targeting sequences were diluted in DMEM to a final concentration of 75nM per well. Dharmafect D4

(transfection reagent) diluted in DMEM (2µl of D4 to 98µl DMEM per oligonucleotide) was incubated at room temperature for 5 minutes. Dharmafect D4 was subsequently added to the pooled diluted oligonucleotide and incubated for 20 minutes at room temperature. The siRNA/D4 complex was added to the cells and incubated at 37°C for 5 hours. Subsequently 500µl of 3x media (DMEM/30% FCS/3% glutamine/3% Pencillin/3% streptomycin) was added per condition and incubated at 37°C for 48 hours.

Cells were then washed in DMEM and then 2ml of DMEM was added. Where relevant, they were pre-treated with RAD001 (indicated concentrations) for 1 hour prior to addition of FGF-2 (1ng/ml) for the appropriate times.

The cells were centrifuged for a further 1 minute at 4000 rpm and then lysed in 100µl 2x SDS sample buffer. Lysates were analysed by SDS-PAGE and immunoblotted with the appropriate specific antibodies.

Gene	Target Sequence
mTOR	ACATATGT [*] TTAAAAAT [*] TCTG; ATTGGCACAAAAAT [*] TAT [*] TC; GGTGTCTAGACATGGCTAC; ACTTTGTGCAT [*] TAGTTGA
FKBP12	GCCGGCATAAAGCACT [*] TTT;TGAGAAGGGGCTGAGGGAGG; AGGAGATGCTGAAGCTGCG; CTACAAACAGCACCT [*] TCAA

Table 2.3 RNAi target sequences for individual oligonucleotides for mTOR and FKBP12

CHAPTER III

mTOR is overexpressed in lung cancer

III.1 Introduction

Lung cancer is the biggest cancer killer accounting for more deaths than breast, prostate and colon cancers combined (Parkin et al., 2005). Non-small cell lung cancer is responsible for 80% of cases. Most affected patients present with inoperable locally advanced (Stage IIIB) or metastatic (Stage IV) disease which is nearly always incurable. The best chance of achieving long term survival is in early stage disease when complete surgical resection is possible. However, even in resected stage IA patients 30% succumb from their disease within 5 years (Tsuchiya et al., 2007).

To further improve the outcome of early stage (IA-IIIA) resected patients, several studies have examined the benefits of post-operative adjuvant chemo- and or radio-therapy. Current data indicate that only stage II-IIIA patients gain additional benefit from adjuvant chemotherapy (Arriagada et al., 2004; Douillard et al., 2006; Winton et al., 2005). However, stage IA and probably stage IB patients as well as those without lymph node involvement fail to demonstrate a survival advantage with this treatment (Douillard et al., 2006; Wakelee et al., 2007).

Consequently, there has been considerable interest in defining new biomarkers which could accurately identify, for example, the 30% of stage IA patients who relapse and die from lung cancer following surgery. Such individuals could then be targeted for novel therapies.

Vascular or lymphatic vessel invasion have both been suggested as potential markers for poor outcome in stage IA patients but results have been controversial and inconsistent (Hung et al., 2007; Macchiarini et al., 1993; Shields, 1983; Tsuchiya et al., 2007). Other predictive factors such as sex, performance status and sublobar resections have also given variable results (Dehing-Oberije et al., 2008). More recently there has been considerable interest in new molecular biomarkers such as the ERCC1 but its role in predicting outcome

in early stage resected patients is promising but not yet clear (Olaussen et al., 2006; Simon et al., 2005; Zheng et al., 2007). Genomic strategies have also showed promising results (Potti et al., 2006). As previously mentioned mTOR is overexpressed in lung cancer cells compared to 'normal' lung epithelial cells (Marinov, 2009).

SCLC makes up ~20% of cases. Initially it is highly responsive to chemotherapy and radiotherapy treatment, however relapse is common and the overall 5-year survival is less than 5%. Clearly novel strategies are required to improve this statistic. In recent years, as previously mentioned a rising number of biological dysfunctions involved in SCLC pathogenesis were reported, including neuro-endocrine regulatory peptides, overexpression of Myc family oncogenes and extracellular matrix proteins, and genetic abnormalities in the tumour suppressor genes p53 and pRB (Sethi et al., 1999; Wistuba et al., 2001). Moreover it has been shown that polypeptide growth factors such as SCF and FGF-2 induce a variety of responses in human SCLC cells, including growth and proliferation, chemoresistance and motility (Heasley, 2001; Pardo et al., 2002; Pardo et al., 2001). Furthermore it has been found that the ribosomal S6K1 and 2 are highly overexpressed in SCLC cells compared to normal human type II pneumocytes, and that mTOR transduces mitogen-induced proliferation in SCLC (Pardo et al., 2001). Therefore targeting these pathways and in particular with mTOR inhibitors may lead to the development of novel treatment for SCLC in patients.

The aim of this part of the study was to determine if mTOR was overexpressed in SCLC and NSCLC patient samples and if possible to determine if this was linked to prognosis. If so these patients may benefit from an mTOR inhibitor which would be determined by further clinical trials.

III.2 RESULTS

II.2.1 mTOR is overexpressed in NSCLC patient specimens and is prognostically linked to survival

The mean age of the population was 65.6 years (range 31-86) and most (88%) were male (Table 3.1). All NSCLC tumour types with stages IA to IIB were present in the series and at the time of primary diagnosis, 100 (74%) patients were lymph node-negative and 34 (26%) had positive lymph nodes. All the patients had a Karnofsky performance status of at least 90%. The median follow up was 52.9 months (range 1-202 months) and a total of 91 (68%) patients died in the study period. Importantly, the overall survival of this group of patients stratified by stage (Figure 3.3) is in keeping with other published series (Demmy & Curtis, 1999; Jemal et al., 2006).

We next examined mTOR staining in the tissue arrays established from the resected tumours. Immunostaining of mTOR was mostly cytoplasmic (Figure 3.1) and was found in 0-100% of the tumour cells with a varying intensity (grades 1-3). To define the optimum mTOR IHS a Martingale graph plotting excess deaths against mTOR value for lung cancer was performed (Figure 3.2) (Therneau T, 1990). The optimal cutoff is identified as the point where the curve cuts the x axis and the strength of the estimates cutoff is indicated by the slope of the increase in excess deaths with increasing mTOR value for lung cancer survival was performed. A similar approach has been used in the past to establish a 10% staining cut-off for Her2/neu in breast cancer (Zarbo & Hammond, 2003). The best cut-off for mTOR staining in NSCLC was provided at an IHS of 30 (Figure 3.2) where 73 (55%) of the patients stained positive for mTOR (Table 3.1). Table 3.2 shows that as expected, stage ($p = 0.04$ by log rank; Hazard Ratio (HR) = 1.76, 95% confidence interval (CI) = 0.992-3.12), lymph node status ($p=0.049$ by log rank, HR = 1.59, 95% CI = 1.0-2.56)

Patient Characteristics	No. of cases (%)
Age	
≥65	57 (43)
<65	77 (57)
Gender	
Male	117 (88)
Female	17 (12)
Stage	
1a	34 (24)
1b	57 (41)
2a	13 (9)
2b	30 (26)
Lymph node	
Positive	34 (25)
Negative	100 (75)
Histology	
Adenocarcinoma	41 (30)
Squamous	56 (42)
Large cell	26 (19)
Broncho-alveolar	9 (7)
Mixed	1 (1)
Neuroendocrine	1 (1)
Surgical intervention	
Lobectomy	112 (84)
Pneumonectomy	22 (16)
Grade	
1	8 (6)
2	43 (31)
3	82 (60)
4	1 (3)
Dead	
Yes	91 (68)
No	43 (32)
mTOR staining	
+ve	73 (55)
-ve	61 (45)
Angioinvasion	
+ve	27 (28)
-ve	69 (72)

Table 3.1 Clinico-pathological characteristics of 134 patients with early NSCLC

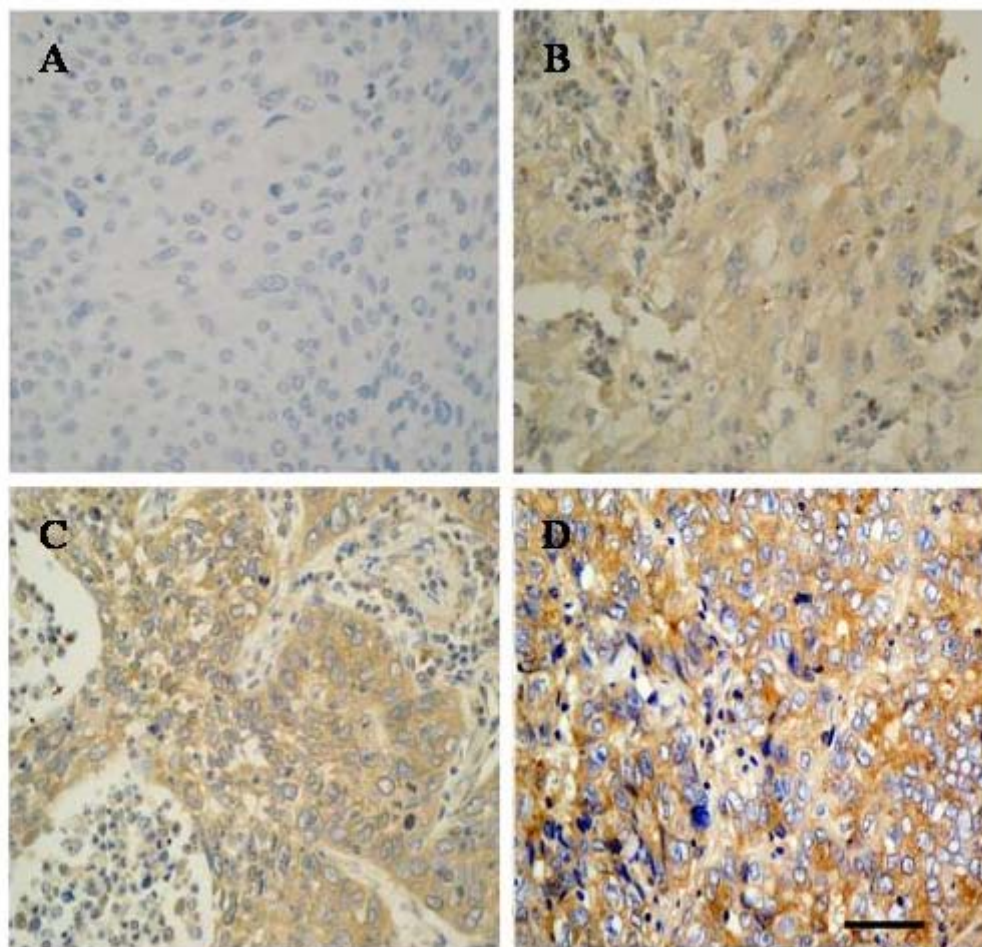


Figure 3.1 mTOR is overexpressed in NSCLC patients.

Panel A example of negative mTOR staining in NSCLC, Panel B weak mTOR staining, Panel C moderate mTOR staining and panel D strong mTOR staining. Bar, 100 μ m
Sections were stained with mTOR antibody at a 1:50 dilution prior to visualisation by HRP as described in the materials and methods. The images shown are representative of 10 independent control experiments performed prior to performing our tissue array work.

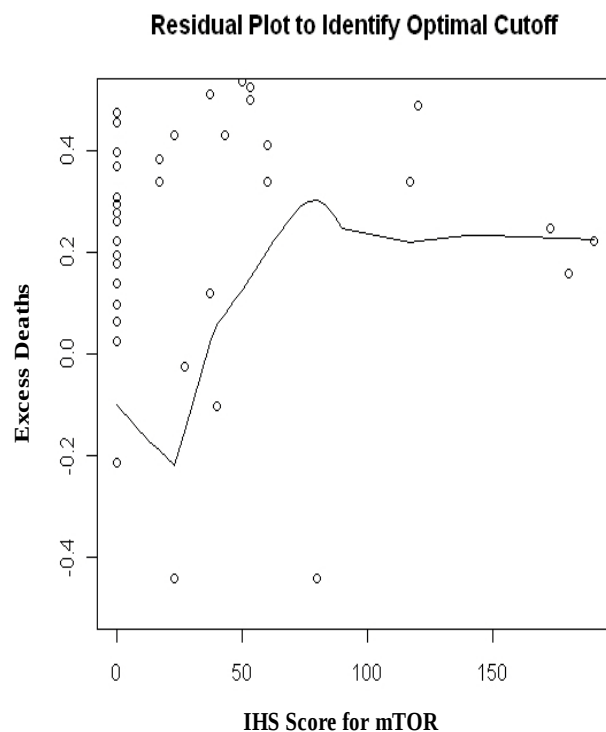


Figure 3.2 Martingale residual plot for lung cancer survival in 134 NSCLC patients

Martingale graph plotting excess deaths against mTOR value for lung cancer was determined to define the optimum mTOR IHS. The optimal cutoff is identified as the point where the curve cuts the x axis and the strength of the estimates cutoff is indicated by the slope of the increase in excess deaths with increasing mTOR value was performed

Patient Characteristics	Univariate analysis P value	HR	95% CI	Multivariate analysis P value	HR	95%CI
Age	0.445	0.990	0.77-1.28	NA		
Gender	0.44	0.87	0.68-1.13	NA		
Stage	0.04			NS		
1b v 1a		0.57	0.37-0.86			
2a v 1a		1.00	0.72 - 1.39			
2b v 1a		1.33	0.79 -2.24			
Lymph node	0.049	1.59	1.0 -2.56	NA		
Histology	0.767			NA		
Large cell v squamous		1.31	0.55-3.09			
Adeno v squamous		1.24	0.49-3.15			
Alveolar v squamous		1.02	0.41-2.50			
Surgical intervention	0.125	0.656	0.38-1.13	NA		
Lobectomy						
Pneumonectomy						
Grade	0.320			NA		
2 v 1		0.57	0.07-4.77			
3 v 1		0.40	0.05-3.01			
4 v 1		0.62	0.09-4.53			
mTOR staining	0.007	1.77	1.17 - 2.73	0.046	1.66	1.01-2.74
Angioinvasion	0.017	1.91	1.12 - 3.31	0.016	1.95	1.13 – 3.35

N.B Univariate analysis performed using log rank test therefore no hazard ratio listed (Except for age) NA= Not applicable, NS=Not significant

Table 3.2 Univariate and multivariate analysis of patient characteristics for survival

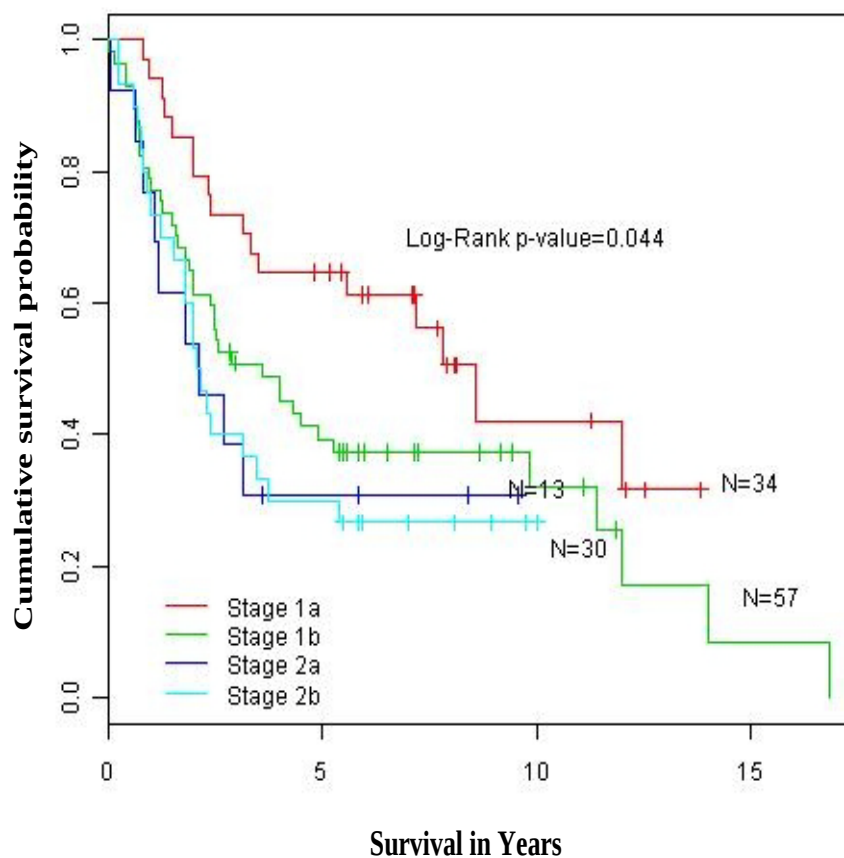


Figure 3.3 Kaplan-Meier survival plot of survival of 134 NSCLC patients for mTOR positive and mTOR negative by stage.

Kaplan-Meier curves were performed using SPSS software. Survival was defined as the time (in months) from the date of primary treatment to time of death.

and angio-invasion ($p=0.017$; HR = 1.91, 95% CI = 1.12-3.31) were significantly associated with poor survival in agreement with previously published findings (Hung et al., 2007; Shields, 1983).

Strikingly, Table 3.2 and Figure 3.4 demonstrate that mTOR staining was also significantly associated with poor survival ($p=0.007$ (log rank), 95% CI=1.16-2.71). Moreover, only mTOR positive staining ($p=0.046$ (log rank), 95% CI 1.01-2.74) and angio-invasion ($p=0.016$ (log rank), 95% CI=1.13-3.35) remained significant as predictors of overall survival on multivariate analysis.

We next investigated whether mTOR staining might predict poor survival in patients who were lymph-node negative as this group currently has no clear prognostic factor. Figure 3.5 shows that the survival of lymph-node negative patients whose primary tumours were mTOR positive was significantly lower ($p=0.016$ (log rank) 95% CI=1.11-3.05). In contrast, angio-invasion and other factors were non-predictive (data not shown). These results suggest that the survival of patients with stage IA disease who are all lymph-node negative might be most influenced by mTOR staining. Indeed, Figure 3.6 demonstrates that mTOR staining was a highly significant prognostic marker for this group of patients. The median survival was 2.1 years in the mTOR positive versus 5.6 years in the mTOR negative patients ($p=0.007$, HR=4.38, 95% CI=1.62-11.83). Taken together, a positive mTOR IHS of >30 appears to predict poor outcome in stage IA and lymph-node negative early stage NSCLC patients

III.2.2 mTOR is overexpressed in SCLC patient specimens

The expression of mTOR using immunohistochemistry in a total of 30 normal lung tissue samples and 30 SCLC patients was determined (Figure 3.7). 50% of the SCLC specimens

were positive for mTOR, whereas mTOR expression was not detectable in the normal lung specimens. This data indicates that up-regulation of mTOR may contribute to the abnormal growth and survival properties of SCLC. Patient clinical data was not available; so prognostic links to mTOR expression could not be determined.

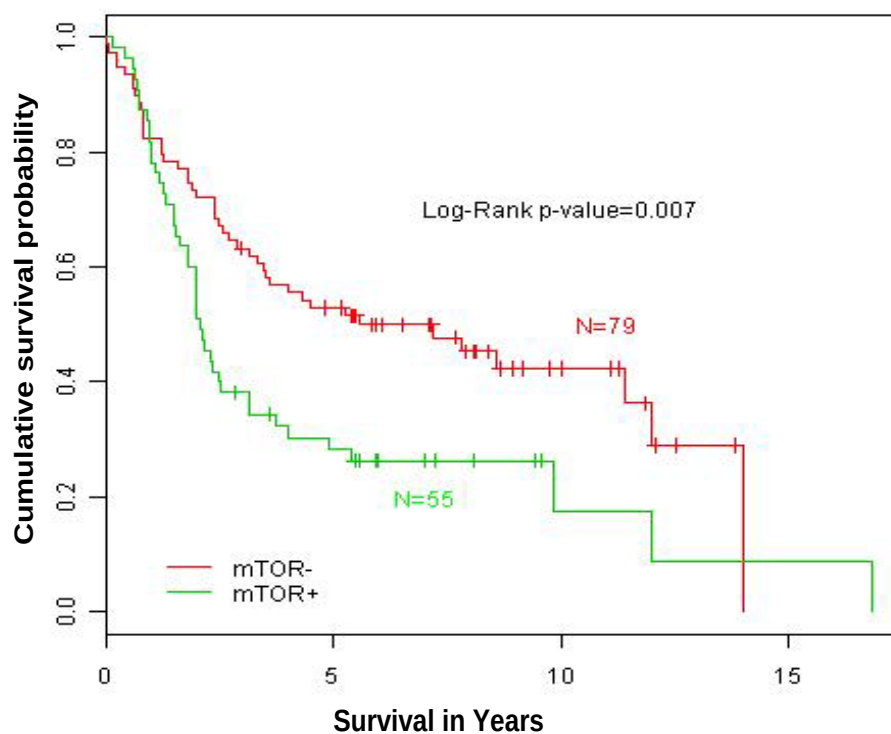


Figure 3.4 Kaplan-Meier survival plot of survival of 134 NSCLC patients for mTOR positive and mTOR negative.

Kaplan-Meier curves were performed using SPSS software. Survival was defined as the time (in months) from the date of primary treatment to time of death.

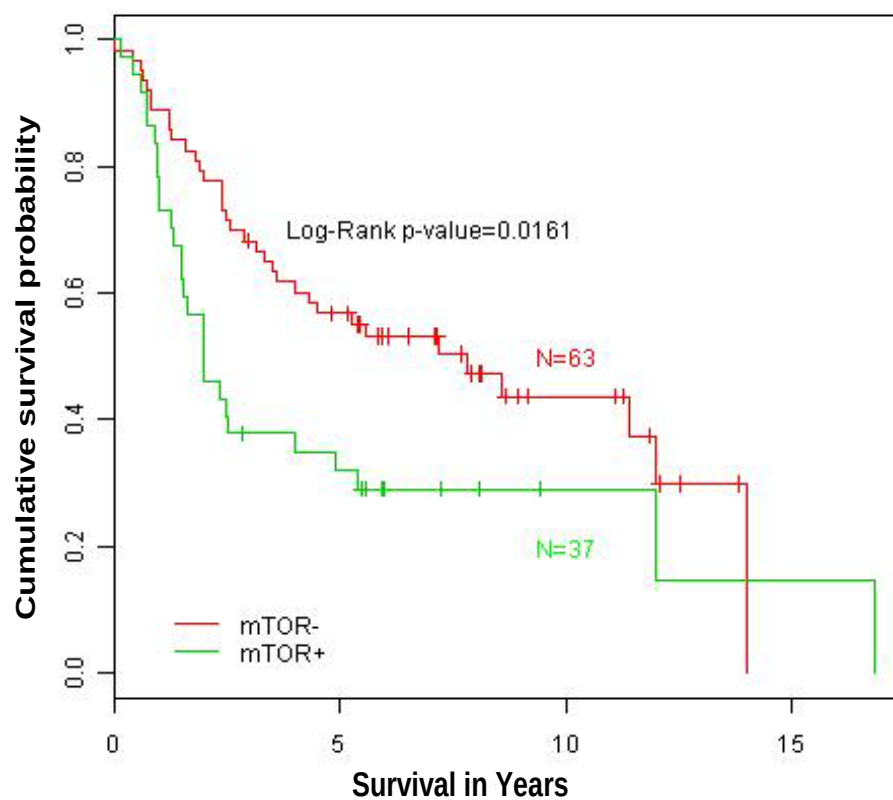


Figure 3.5 Kaplan-Meier plot demonstrating the influence of mTOR staining on LN negative NSCLC patient survival

Kaplan-Meier curves were performed using SPSS software. Survival was defined as the time (in months) from the date of primary treatment to time of death.

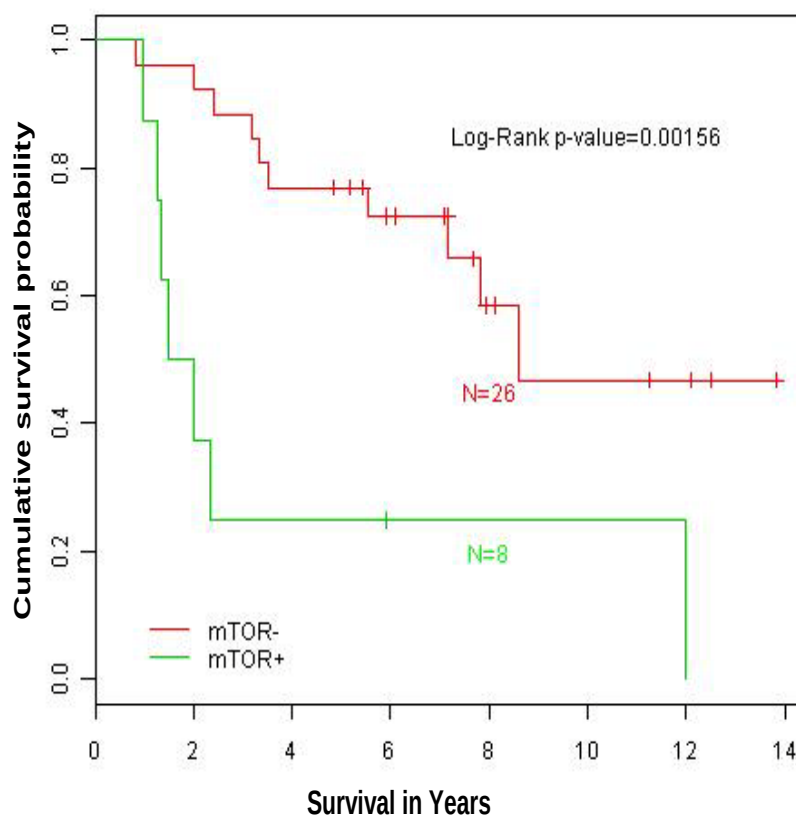


Figure 3.6 Kaplan-Meier survival plot of survival of stage 1A NSCLC patients for mTOR positive and mTOR negative

Kaplan-Meier curves were performed using SPSS software. Survival was defined as the time (in months) from the date of primary treatment to time of death.

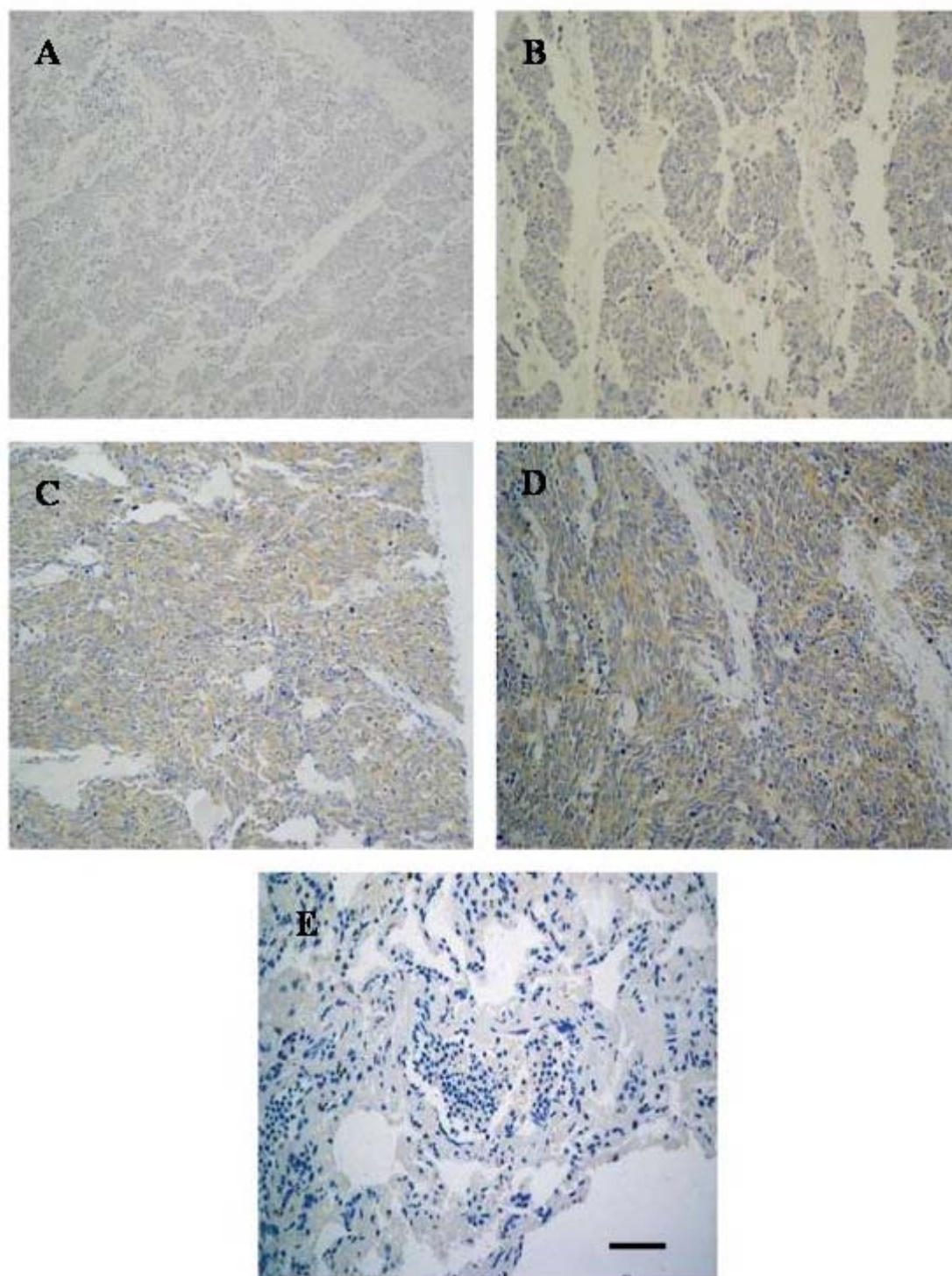


Figure 3.7 mTOR is overexpressed in SCLC patients.

Panel A example of negative mTOR staining in SCLC, Panel B weak mTOR staining, Panel C moderate mTOR staining and panel D strong mTOR staining, Panel E normal lung. Bar 100 μ m

Sections were stained with mTOR antibody at a 1:50 dilution prior to visualisation by HRP as described in materials and methods. The images shown are representative of 10 independent control experiments performed.

III.3 Discussion

Lung carcinoma is the commonest cancer killer and the best hope of cure lies currently with surgical resection of early stage disease. For NSCLC patients with resected stages IIA-IIIa, clinical trials have demonstrated additional survival benefits with adjuvant chemotherapy. However for stage IA patients and probably IB and those without lymph node involvement, additional chemotherapy does not appear to improve survival (Pignon et al., 2008). This might be because novel treatments are required and/or because the existing treatments only aid a small percentage of patients and most stage IA/B patients are cured. Whatever the explanation, it seems likely that outcome might be improved if we could more precisely identify the subset of patients destined to do badly following surgery.

Here we show that mTOR immunohistochemical staining predicts poor outcome in early stage NSCLC. More specifically, it appears to identify poor outcome in lymph node negative and stage IA patients where previously other markers such as angio-invasion have failed to consistently yield prognostic benefit. Obviously, the data presented here just represents a single patient cohort and it would be important to know whether this could be reproduced in larger independent series. Potti et al in 2006 using a multigene-expression profile predicted recurrence for patients significantly better than clinical prognostic factors in early NSCLC (Potti et al., 2006). Recently, immunohistochemical staining for L-type amino acid transporter 1 (LAT 1) has also been shown to provide prognostic information in resected NSCLC within a Japanese patient population (Kaira et al., 2008). The expression/activity of this transporter appears to be closely linked to mTOR (Fuchs & Bode, 2005). It is therefore tempting to speculate that the expression of these two molecules in clinical material may be linked and that our findings may not be isolated to one patient cohort. In addition, it is

noteworthy that others have recently shown increased mTOR staining is linked to adverse prognosis in breast and biliary cancers (Herberger et al., 2007; Zhou et al., 2004). However a paper published recently showed the opposite- i.e. high expression of mTOR is linked to better outcomes in early NSCLC (Anagnostou et al., 2009). This would seem counter-intuitive with respect to known mTOR biology and to mTOR expression and prognosis in other cancers mentioned above. Possible reasons for these differences include differing immunohistochemical techniques and having more women in the cohort tested in the more recent study.

Criticism of tissue microarray technology must be acknowledged such as the small cores sampled may not be representative of the whole tumor, particularly in heterogeneous cancers such as prostate adenocarcinoma and non-small cell lung cancer and so validation studies against whole tissue sections could have been performed.

The phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway is frequently deregulated in cancers and represents an attractive target for anticancer therapy. Sirolimus and its analogues Temsirolimus, Everolimus, and Deforolimus are specific small molecule inhibitors of mTOR and some of these agents are now being examined as novel therapies for lung cancer. Our results suggest that early stage NSCLC patients who are mTOR positive might benefit from an mTOR inhibitor and/or chemotherapy. However, it is recognised that these findings are preliminary and a further validation study is now urgently required.

The SCLC patient findings with respect to mTOR expression suggest that mTOR is overexpressed in about 50% of cases. The clinical significance of this observation is not yet clear and will be determined by future studies examining how this correlates with survival/response to therapy. Nevertheless, clinical trials using mTOR inhibitors in

unselected SCLC patients have already started and have shown some promise with 2 different doses of CCI-779 (Pandya et al., 2005).

CHAPTER IV

**Phospho-signals are not stable when
growth media/blood supply are removed
from lung cancer cells/tumours**

IV.1 Introduction

Phosphorylation of proteins on serine, tyrosine or threonine residues are key events modulating the activity or cellular localization of many key signaling molecules in cells (Hunter, 1987). Therefore, phosphoproteins have been frequently used as markers of signaling activity in tumours. Protein phosphorylation depends on a balance between the rate of phosphorylation by specific kinases and dephosphorylation by phosphatases. Often, phosphorylation events are in fact transient as protein functions are switched on and off (Hunter, 1989).

So for phosphoproteins to be useful as a marker of signaling activity, knowledge of their stability after blood supply removal should be determined. This may prove important since many publications looking at phosphoprotein expression in tumour biopsies do not state at what time point from removal from the patient they were fixed in formalin (Herberger et al., 2007; Zhou et al., 2004). It may be the case that in these studies, it is better to stain for the total protein in question since this may be more stable and give more meaningful and consistent results. Indeed, cancer may upregulate or loose expression of signaling proteins as part of oncogene addiction. However, total protein does not necessarily reveal altered biological function.

A previous study found rapid dephosphorylation of Akt in cancer xenografts when removed from blood supply and in clinical samples (gastro-oesophageal junction tumours) phospho-Akt was only evaluable in biopsies and not in surgically resected specimens (Baker et al., 2005)

In this chapter, this question of whether phosphoproteins in particular mTOR/phospho-mTOR remain stable after removal of growth media/blood supply was examined in cell

lines, formalin fixed cell lines and patient clinical samples, considering in the previous chapter we examined the expression of total mTOR in clinical samples.

IV.2 RESULTS

IV.2.1 The strength of phospho-mTOR signals varies with time in HCC78 adenocarcinoma NSCLC cells removed from growth media but not in one NSCLC patient sample (Adenocarcinoma histology).

We wanted to determine if, at the cell line level, phosphoprotein signals vary with time from removal from growth media. We chose to look at mTOR- for obvious reasons and also it is an important component of one of the major intracellular signaling pathways. Phosphorylation of serine 2448 was selected as it is one of the sites thought to correlate with mTOR activation (Chiang & Abraham, 2005). Firstly, HCC78 NSCLC cells were removed from their growth media and left at room temperature and lysed with 2x SDS sample buffer at various time points up to 2 hours after removal from growth media. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. The results are shown in Figure 4.1A. It is clear that the phospho-mTOR signal varies with time compared to the total mTOR (which was also used as a loading control). Phospho-mTOR signal intensity peaked around 10 minutes and was lowest around 120 minutes. The total mTOR signal did not vary with intensity with time.

We then wanted to see the effect of formalin fixation on the variation of the phospho-mTOR signal with time in the same cell line. Tissue biopsies or surgical specimens are normally fixed in formalin before staining with relevant antibodies. A semi quantitative immunohistochemical score (IHS) was used including assessment of both the intensity of staining and the percentage of positive cells. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. Figure 4.1B, 4.1C and Table 4.1 shows the results. Again the phospho-mTOR signals varies with time (B) compared to the total mTOR (C). At 15

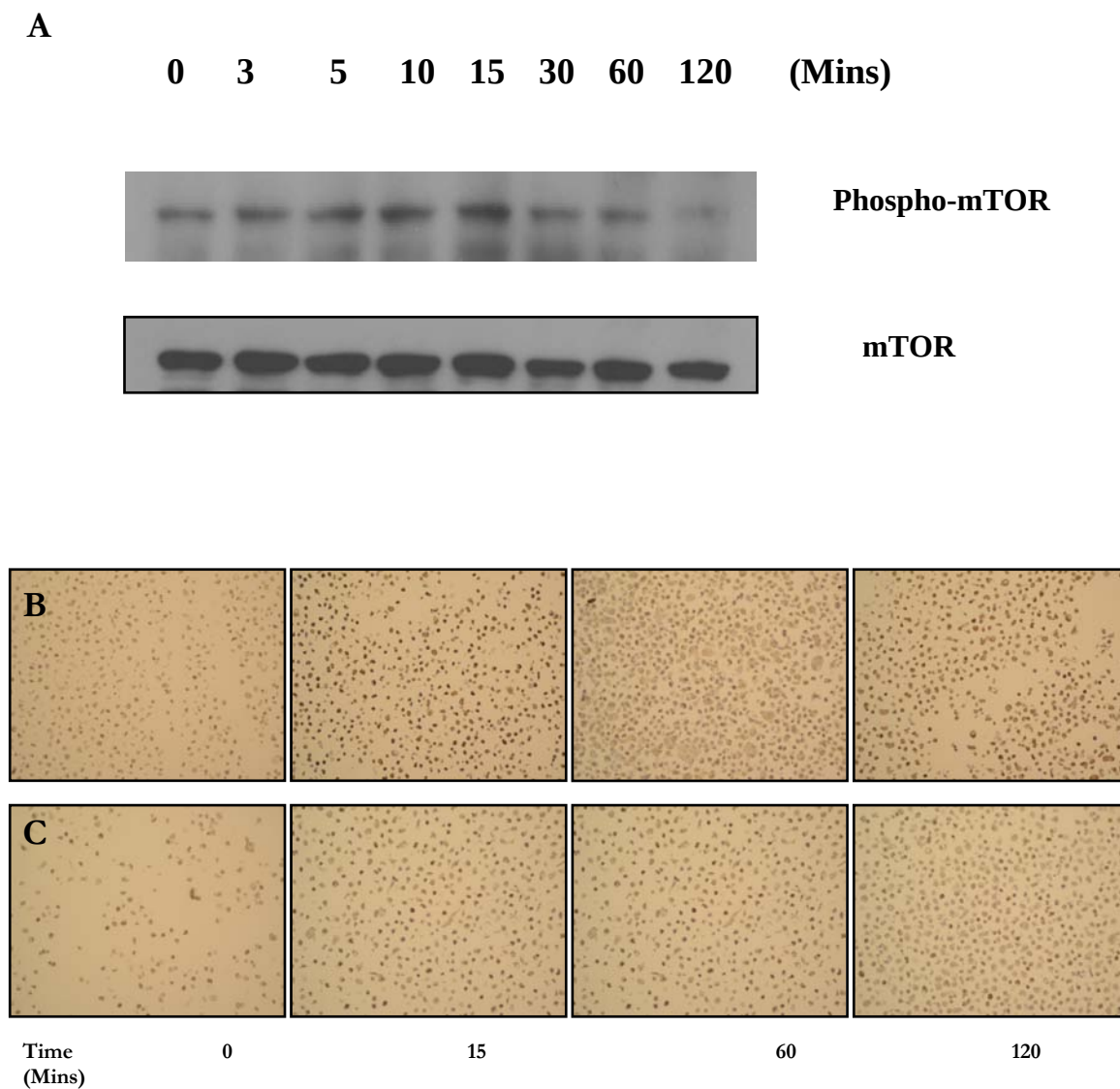


Figure 4.1 Phospho-mTOR signals varies with time in HCC78 NSCLC cells.

(A) HCC 78 NSCLC cells were removed from 10% FCS+media and lysed at the following time points- 0, 3, 5, 10, 15, 30, 60 and 120 minutes. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. HCC78 cells were removed from 10% FCS+media and fixed in formalin at the following time points 0, 15, 60 and 120 minutes and then the sections were stained with (B) phospho-mTOR (Ser2448) and (C) total mTOR antibodies at a 1:50 dilution prior to visualisation by HRP as described in the materials and methods. The images shown are representative of 10 independent control experiments performed. Results are representative of 3 repeat experiments.

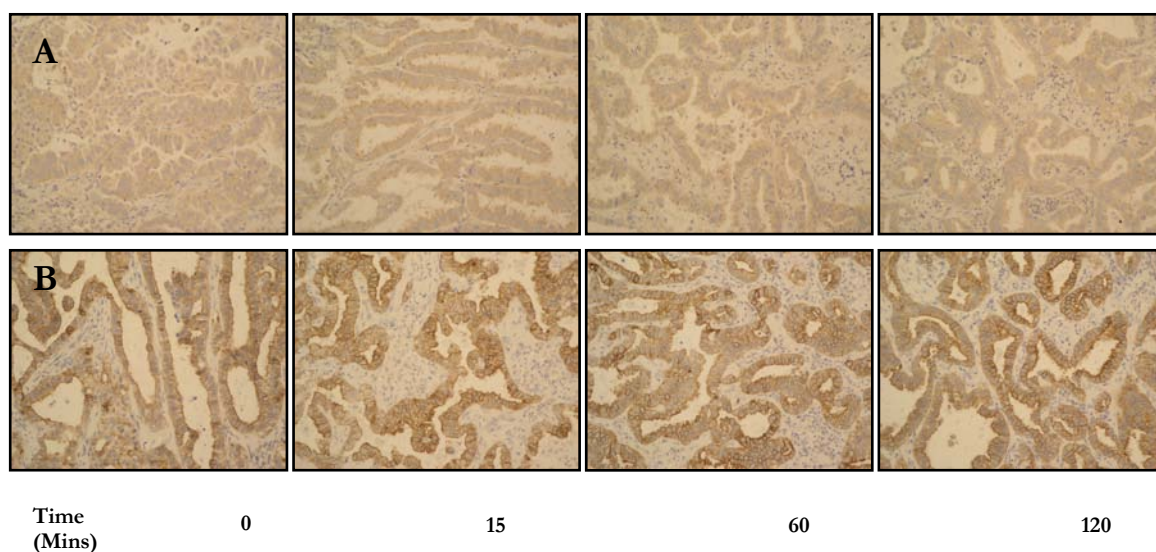


Figure 4.2 Phospho-mTOR signals do not vary in time in a patient's lung cancer tissue sample (Adenocarcinoma)

NSCLC (adenocarcinoma) patient tissue stained for (A) mTOR and (B) phospho-mTOR. Patient sample was surgically removed and fixed in formalin at the time points indicated. mTOR antibodies at a 1:50 dilution prior to visualisation by HRP as described in materials and methods. Result is from one experiment.

minutes the phospho-mTOR signal is more intense than at 0 and 60 minutes (IHS of 100 and 150, respectively). The obvious next question is to see if these results are consistent in lung cancer patient surgical specimens. We obtained a single sample of a NSCLC-adenocarcinoma, the same histological sub-type as the HCC78 NSCLC cell line. I was called prior to the elective surgical removal of a lung cancer by the thoracic surgeon at the Hammersmith Hospital. I received the surgical specimen minutes after it was removed by the surgeon just outside the operating theatre and then fixed parts of the specimen in formalin at fixed time points up to 120 minutes post resection before staining with relevant antibodies. Figure 4.2A/B and Table 4.1 show the results. The results from one patient did not show any variation in (A) phospho-mTOR / (B) total mTOR IHS score with time up to 120 minutes.

IV.2.2 The strength of phospho-mTOR signals varies with time in HCC95 squamous NSCLC cells removed from growth media and in one NSCLC patient sample (squamous histology)

We wanted to verify our findings from the previous section in another NSCLC cell line and another NSCLC patient sample of the same phenotype as the cell line. HCC95 NSCLC cells were removed from their growth media and left at room temperature and lysed with 2x SDS sample buffer at various time points up to 2 hours after removal from growth media. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. The results are shown in Figure 4.3A. It is clear that the (A) phospho-mTOR signal varies with time compared to the (B) total mTOR (which was also used as a loading control). Phospho-mTOR signal intensity peaked around 0-15 minutes and then fades out. The total mTOR signal did not vary in intensity with time.

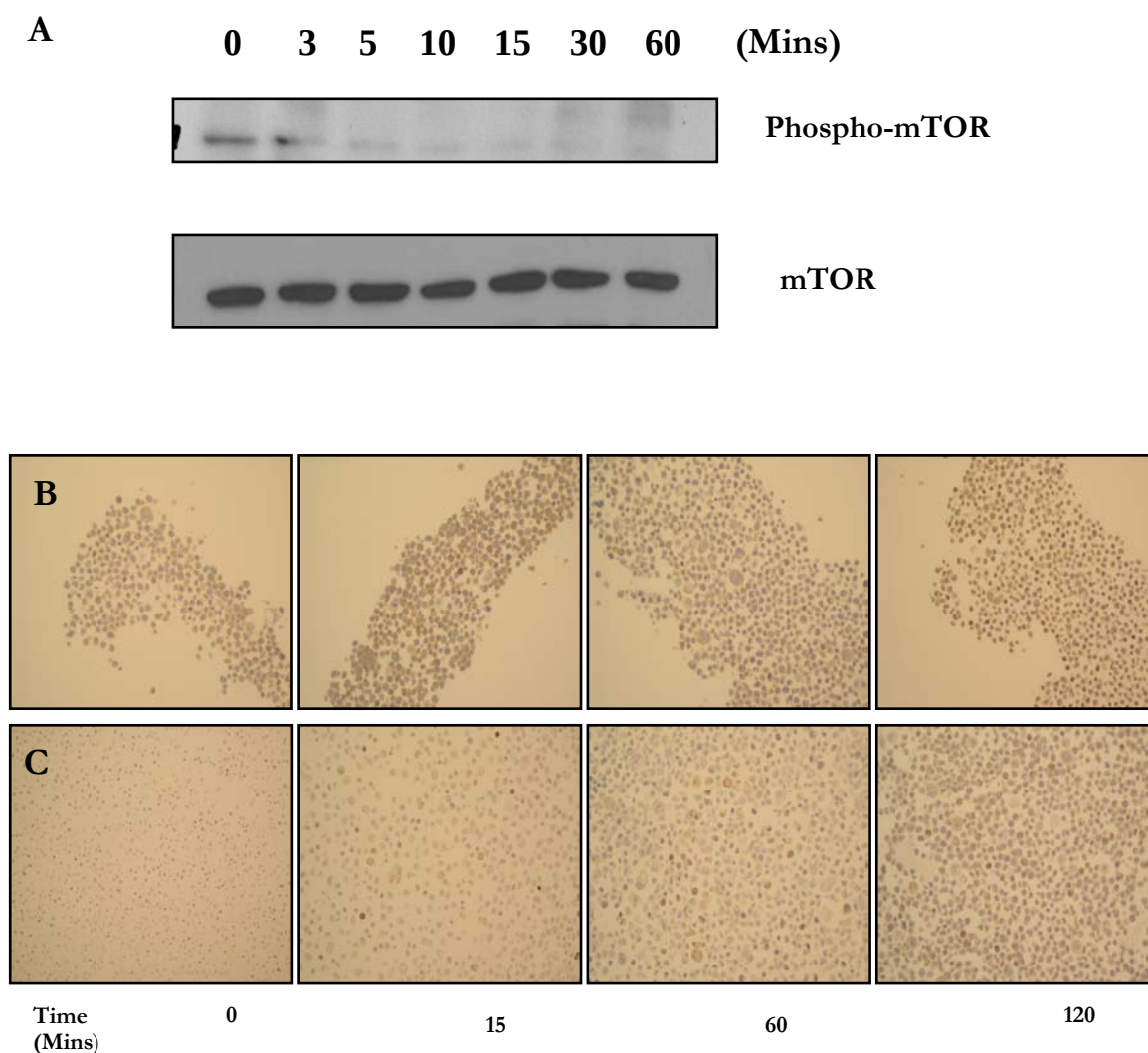


Figure 4.3 Phospho-mTOR signals varies with time in HCC95 NSCLC cells.

(A) HCC95 NSCLC cells were removed from 10% FCS+media and lysed at the following time points- 0, 3, 5, 10, 15, 30, 60 and 120 minutes. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. HCC95 cells were removed from 10% FCS+media and fixed in formalin at the following time points 0, 15, 60 and 120 minutes and then the sections were stained with (C) phospho-mTOR (Ser2448) and (B) total mTOR antibodies at a 1:50 dilution prior to visualisation by HRP as described in the materials and methods. The images shown are representative of 10 independent control experiments performed. Results are representative of 3 repeat experiments.

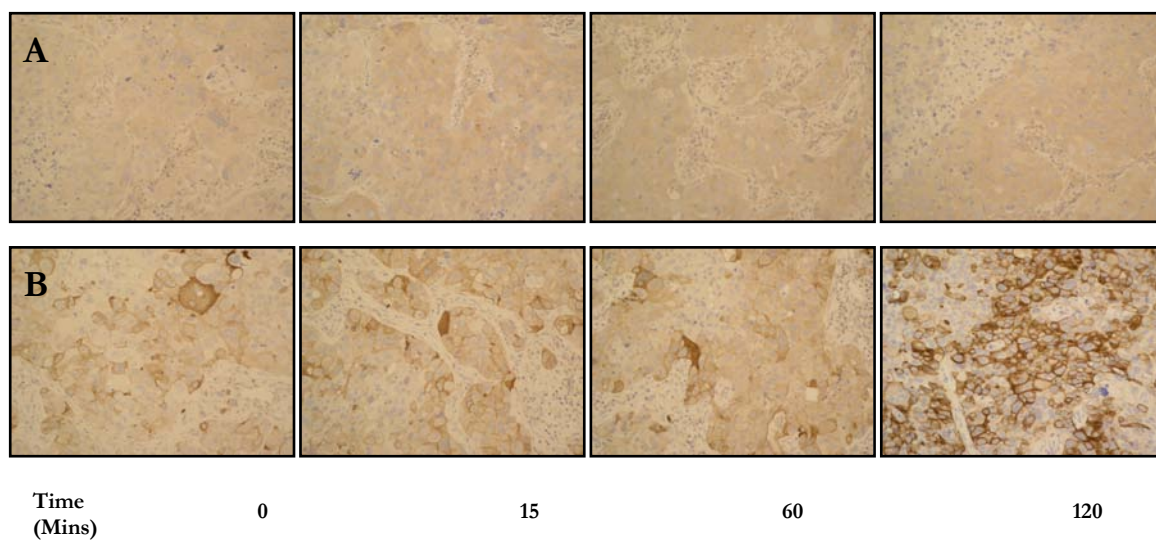


Figure 4.4 Phospho-mTOR signals varies in time in a patient's lung cancer tissue sample (Squamous)

NSCLC (Squamous) patient tissue stained for (A) mTOR and (B) phospho-mTOR. Patient sample was surgically removed and fixed in formalin at the time points indicated. mTOR antibodies at a 1:50 dilution prior to visualisation by HRP as described in materials and methods. Result is from one experiment.

We then wanted again to see the effect on formalin fixation on the variation of the phospho-mTOR signal with time in the same cell line. Figure 4.3B, 4.3C and Table 4.1 shows the results. The phospho-mTOR signal varies with time, IHS increasing as time goes by (C), whilst the total mTOR signal/IHS stayed stable (B) for example, at 0 minutes IHS was 60 whilst at 60 minutes it was 100. We again went on to look at a patient surgical specimen obtained as previously described in the last results section. We obtained a NSCLC specimen of squamous histology; the same phenotype as the HCC95 cell line. The specimens were placed in formalin at fixed time points up to 120 minutes post resection before staining with relevant antibodies. Figure 4.4A, B and Table 4.1 show the results. The total mTOR HIS did not vary with time (A) whilst the phospho-mTOR IHS varies with time (B), after 120 minutes the signal/IHS was more intense than at 0 minutes (IHS of 80 and 150, respectively).

IV.2.3 The strength of phosphoprotein signals varies with time in fixed H510 SCLC cells removed from growth media.

After having examined the stability of phospho-mTOR in two NSCLC cell lines/surgical specimens, we moved on to a SCLC cell line- H510. H510 SCLC cells were removed from their growth media and left at room temperature and lysed with 2x SDS sample buffer at various time points up to 2 hours after removal from growth media. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. The results are shown in Figure 4.5A. The phospho-mTOR signal peaked in intensity at 3 minutes and then dropped down to almost undetectable. We then wanted again to see the effect on formalin fixation on the variation of the phospho-mTOR signal with time in the same cell line. Figure 4.5B, 4.5C and Table 4.1 shows the results. Figure 4.5B shows a stable total

mTOR signal/IHS with time and figure 4.5C shows the strength of the phospho-mTOR signal varies with time, with the greatest IHS at around 60 minutes post formalin fixation (IHS score increasing from 40 at 0 minutes to 180 at 60 minutes).

(A) H510 SCLC cells were removed from 10% FCS+media and lysed at the following time points- 0, 3, 5, 10, 15, 30, 60 and 120 minutes. Lysates were analysed by SDS-PAGE for the presence of phospho-mTOR (Ser2448) and total mTOR. H510 cells were removed from 10% FCS+media and fixed in formalin at the following time points 0, 15, 60 and 120 minutes and then the sections were stained with (C) phospho-mTOR (Ser2448) and (B) total mTOR antibodies at a 1:50 dilution prior to visualisation by HRP as described in the materials and methods. The images shown are representative of 10 independent control experiments performed. Results are representative of 3 repeat experiments.

Time points (Mins)	0	15	60	120
HCC78 mTOR	90	90	90	80
HCC78 p-mTOR	100	180	150	180
Adenocarcinoma tissue mTOR	90	80	90	90
Adenocarcinoma tissue p-mTOR	180	160	180	180
HCC95 mTOR	160	180	180	180
HCC95 p-mTOR	60	60	100	180
Squamous Tissue mTOR	40	60	40	40
Squamous Tissue p-mTOR	80	100	100	150
H510 mTOR	40	40	60	40
H510 p-mTOR	40	40	180	60

Table 4.1 Mean immunohistochemical score (IHS) of formalin fixed NSCLC cell lines and NSCLC patient tissue.

A semi quantitative immunohistochemical score (IHS) was used to assess degree of staining (the intensity of staining and the percentage of positive cells.) of mTOR and phospho-mTOR in HCC78, HCC95, H510, adenocarcinoma lung cancer patient sample and squamous cell lung cancer patient sample. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate and strong positivity, was recorded. The range of possible scores was thus 0 to 300. The results were means except in the tissue samples where these are the sole results.

IV.3 Discussion

In this chapter we looked at the issue of whether phosphorylated proteins are stable after the cells/tissue has been removed from growth media/blood supply. Phosphoproteins are frequently used to assess the activity of intracellular pathways. In particular, with respect to immunohistochemical studies looking at expression of phosphoproteins as markers of activation of intracellular signaling pathways. We show here that in 3 lung cancer cell lines – two NSCLC (adenocarcinoma and squamous phenotype) and 1 SCLC; that phospho-mTOR signal varies with time from removal from growth media. Total mTOR signals stayed stable. Total mTOR was used as a loading control and control for the stability of the phospho-mTOR protein.

We then looked at the same cell lines removed at fixed time points from growth media, fixed in formalin and then immunostained as described in the methods section. We did this since formalin fixing could alter the stability of phosphoproteins. Results were similar to those observed with cells removed from media and lysed and subjected to SDS-PAGE analysis. Phospho-mTOR signals again varied with time from removal from growth media in the 3 lung cancer cell lines examined and total mTOR stayed stable in all cell lines.

These experiments were repeated using other phospho-proteins since the stability of phosphoproteins may depend on the individual protein studied. Phospho-ERK/ERK and phospho-Akt/Akt were examined since they are important components of 2 major intracellular signaling pathways- PI3K/Akt/mTOR/S6K1 and MEK/ERK/S6K2. Results were very similar to those seen with phospho-mTOR (data not shown); with respect to stability of phospho-signals i.e. phospho-signals/IHS varied in intensity with time from removal from growth media and total protein signals remained stable.

With these results in mind we examined the stability of phospho-mTOR in two NSCLC surgical specimens. We were fortunate to have the co-operation of a thoracic surgeon who gave us access to patient specimens and gave us notice prior to elective surgery to collect tissue just after surgical removal. We obtained one adenocarcinoma and one squamous NSCLC patient samples. In our 'adenocarcinoma' patient sample no observed difference was seen in the intensity of the phospho-mTOR signal with time (Figure 4.2) but signal intensity, with respect to phospho-mTOR, did vary with time in the 'squamous' phenotype. (Total mTOR signals did not vary in intensity). Clearly this sample size is very small to make any conclusions and more samples are needed to make any judgment on the stability of phosphoproteins. These studies are difficult to organize. Firstly the numbers of patients who have surgery for early NSCLC are few and appear unpredictably. This means that it would take a long time to obtain sufficient patients for any decent size study. To obtain sufficient numbers of patients would probably involve other surgical centres but this may lead to variations in methods/timing with respect to collection and immunohistochemical analysis.

We could use tumour biopsies i.e. a small sample taken by a radiologist to make a diagnosis however, different results may have been seen in tissue biopsies since the samples are removed straight from the tumours and fixed very quickly. Surgical tumour specimens have their blood supply tied and they may stand at 37°C for many minutes. Clearly rapid processing would seem essential however timings would have to be stated when this data is presented.

From the data obtained from cell lines, it does seem in lung cancer, at least that phospho-mTOR/ERK/Akt signals do vary in intensity with time from removal from growth media. Clearly a more complete study would involve studying different cell lines/tumour types and other phosphoproteins since as mentioned previously proteins may vary in their stability. It

must also be made clear that particular phosphoproteins chosen must correlate with activation of the particular protein/signaling pathway.

If we assume that the results shown here are reproducible, in particular in patient surgical specimens, this would mean that when patient samples are used to determine activation of intracellular signaling pathways time from surgery to formalin fixation would have to be documented and taken into account with respect to any conclusions made from such studies. It could also mean that previously published reports which use these kinds of studies may be invalid unless repeated and 'fixation times' documented.

In the previous chapter we examined the expression of total mTOR in 134 early NSCLC patients; total mTOR was chosen because of the results of this chapter. However expression may not relate to altered biological function. Despite this we did show that prognosis was directly related to survival in these cases.

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CHAPTER V

Chemoresistance and mTOR in lung cancer

V.1 Introduction

Small cell lung cancer is an aggressive and mostly incurable cancer. Despite early responses to chemotherapy; the disease relapses and responds poorly to further therapy. Most patients die within a year of diagnosis. Novel therapeutic strategies are needed and will only arise from a greater understanding of the disease biology and causes of resistance to treatment.

Several growth factors have been implicated in the biology of SCLC and have been shown to trigger proliferation in SCLC cells in vitro. Among the FGF family, the role of FGF-2 has been studied extensively in SCLC. Previous work from our group has shown that FGF-2 promotes proliferation via S6K1 and this is dependent on mTOR since rapamycin blocks S6K1 activation/proliferation in all SCLC cell lines tested. Not only can FGF-2 promote proliferation but resistance to etoposide. Further investigation of the underlying mechanisms by which FGF-2 could trigger resistance to etoposide killing revealed that several anti-apoptotic proteins including Bcl-XL, Bcl-2, XIAP and cIAP1/2 were upregulated. This was achieved through enhanced translation rather than transcriptional control mechanisms. So how could FGF-2 signaling regulate the translational machinery? Recent work from the laboratory has shown that FGF-2 but not other growth factors triggers the assembly of a novel complex comprising of B-Raf, PKC ϵ and S6K2 which promotes growth factor prosurvival effects (Pardo et al., 2001; Pardo et al., 2003; Pardo et al., 2006).

It was found that at low rapamycin concentrations, growth factor-induced S6K1 activation is selectively inhibited without effect on S6K2. In contrast, at 10-fold higher concentrations of rapamycin, there is a partial inhibition of S6K2 activation, which correlates, with inhibition of MEK/ERK signaling (Figure 1.7). This may suggest that high dose mTOR inhibition with rapalogues could potentially block FGF-2 induced chemoresistance. This could be relevant in the development of new therapeutic strategies in SCLC.

This phenomenon potentially opens up a new pathway in mTOR biology. The mTORC1 complex is modulated by MAP kinases (p44/42 MAP) via phosphorylation and inactivation of the tuberous sclerosis complex (Tee et al., 2003a; Tee & Blenis, 2005; Tee et al., 2002; Tee et al., 2003b). However a connection from mTORC1 modulating activity of MEK/ERK signaling has not been reported, however a couple of papers have shown anecdotal evidence that mTOR inhibition can influence activation of MEK/ERK signaling, in the VMM18 melanoma cell line (Molhoek et al., 2005) and Rh1 sarcoma cells (Harwood et al., 2008).

Therefore, the questions that will be addressed in this chapter are: Do high concentrations of the clinically more relevant mTOR inhibitor/rapalogue RAD001 like rapamycin also block FGF-2-induced MEK/ERK/S6K2 activation in SCLC cell lines? Is this phenomenon relevant to other growth factors/chemicals that activate MEK/ERK/S6K2 signaling in SCLC cell lines? Are these observations relevant in NSCLC or other cancers? Can RAD001 block FGF-2 induced resistance to etoposide killing in lung cancer cell lines?

V.2 RESULTS

V.2.1 FGF-2 and PDB stimulation of ERK and S6 Kinase 1 in H510 SCLC cells can be blocked by RAD001

Rapamycin at high concentrations (high but still tolerable to patients) has previously been shown to impair MEK/ERK signaling induced by FGF-2 in H510 SCLC cells. Rapamycin is not very soluble nor stable so its clinical uses are limited (Easton & Houghton, 2006), the newer ‘rapalogs’ are analogues of rapamycin, they are clinically relevant and are already in use. However, it is unclear whether similar effects might be seen with other rapalogues such as RAD001, with different growth factors and with other cell lines. To gain insight into these issues, we initially investigated the effects of increasing concentrations of RAD001 on ERK phosphorylation induced by FGF-2 in H-510 cells.

H510 cells were grown in serum-free medium for 4 days prior to the experiments. RAD001 at the following doses 1, 3, 10, 50, 100ng/ml were added to the cells 1 hour prior to FGF-2 (0.1ng/ml) stimulation. The cells were lysed after 5 and 10 minutes of stimulation for subsequent analysis by western blotting for bi-phospho ERK1/2 and phospho-S6K1, respectively. These doses of RAD001 were chosen since they cover the range of doses which are tolerable to patients and are similar to the doses utilized in the ‘original’ rapamycin studies from our laboratory. The times chosen for the FGF-2 stimulations were 5 minutes- optimal to observe maximal phosphorylation of ERK1/2 (Pardo et al., 2001), a marker of activation of MEK/ERK signaling and 10 minutes for observation of phosphorylation of S6K1. Phosphorylation of S6K1 was used as a control to ensure the RAD001 was active, since low doses of this drug are known to inhibit phosphorylation of S6K1 on threonine 389. Figure 5.1A shows that even at RAD001 dose of 10ng/ml, the biphospho-ERK1/2 signal is reduced significantly. Figure 5.1B shows that the RAD001 was active, reducing

phosphorylation of S6K1 at 1ng/ml. So compared to the previously published effects of rapamycin in H510 cells (Pardo et al., 2001), RAD001 can also block FGF-2 stimulation of MEK/ERK signaling and at even lower doses.

Many types of growth factors; be they neuropeptide and polypeptides are known to be involved in SCLC biology. The next question we asked was whether other growth factors/chemical activators of MEK/ERK signaling in H510 could be blocked by RAD001 or whether this was a phenomena restricted to FGF-2.

Protein kinase C (PKC) is a known regulator of S6K2 (Valovka et al., 2003) via MEK/ERK in many cell lines including SCLC cells (Kawauchi et al., 1996; Seufferlein & Rozengurt, 1996a; Zou et al., 1996). Phorbol esters are plant derived tumour promoters (Blumberg, 1988). They can activate some PKC members (cPKCs and nPKCs (Way et al., 2000)). We assessed if phorbol ester stimulated MEK/ERK signaling could be blocked in H510 cells by RAD001. H510 cells were set up as before, and stimulated with 400nM of phorbol ester (determined by dose response experiments- data not shown) for 5 minutes before lysis. Blots were probed for bi-phospho ERK1/2 and Lamin B followed by SDS PAGE analysis. Figure 5.1C shows that high doses of RAD001 block phorbol ester stimulation of biphospho-ERK1/2. This was observed only at 100ng/ml of RAD001 as opposed to 10ng/ml RAD001 when FGF-2 was used to stimulate MEK/ERK signaling in this cell line.

V.2.2 SCF and ADH stimulation of ERK and S6 Kinase 1 in H510 SCLC cells can be blocked by pre-treatment with RAD001

Next we looked at other growth factors important in SCLC biology and known to activate MEK/ERK signaling. The transmembrane protein c-Kit is a RTK whose primary ligand is stem cell factor (SCF) and is expressed in SCLC (Naeem et al., 2002). SCF was used to activate MEK/ERK signaling in H510 SCLC cells. H510 cells were set up as before and

stimulated with SCF (3ng/ml) for 5 and 10 minutes and then lysed. Blots were probed for bi-phospho ERK1/2 or phospho-S6 Kinase 1 (Threonine 389) (depending on the experiment) and Lamin B followed by SDS PAGE analysis. Figure 5.2A shows that high dose RAD001 can block SCF activation of bi-phospho-ERK1/2. Again 100ng/ml of RAD001 blocks activation of MEK/ERK. Figure 5.2B shows that 1ng/ml of RAD001, blocks SCF activation of S6K1 consistent with active RAD001.

Vasopressin/ADH is a neuropeptide growth factor able to sustain growth of SCLC cells. This peptide exert its characteristic effects on cellular processes by binding to specific G protein-coupled receptors on the surface of their targets cells (Sethi & Rozengurt, 1991).

We wanted to see if ADH, as an example of a neuropeptide growth factor, activation of MEK/ERK signaling could be blocked by RAD001.

H510 cells were set up as before and stimulated with ADH (300 μ M) for 5 and 10 minutes. Blots were probed for bi-phospho ERK1/2 or phospho-S6 Kinase 1 (Threonine 389) (depending on the experiment) and Lamin B followed by SDS PAGE analysis. Figure 5.3A shows again that high doses of RAD001 (starting from around 50ng/ml) could block ADH induced phosphorylation of ERK1/2. Figure 5.3B shows, as a control, low dose (3ng/ml) RAD001 blocking ADH stimulated phosphorylation of S6K1.

V.2.3 Growth factor/chemical stimulation of ERK and S6 Kinase 1 in H524 and H69 SCLC cells can also be blocked by pre-treatment RAD001

So far we have shown that in H510 SCLC cells be that polypeptide, neuropeptide and tumour promoter/chemical activation of MEK/ERK signaling could be blocked by the mTOR inhibitor RAD001. Is this phenomenon isolated to this one SCLC cell line or are these observations seen in other SCLC cell lines?

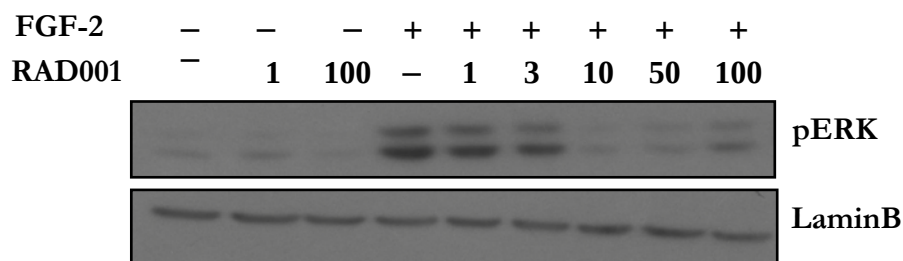
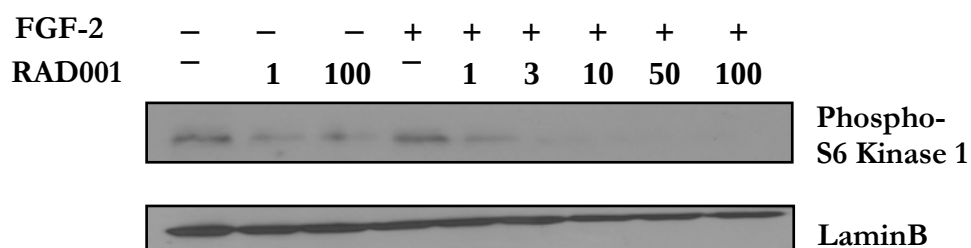
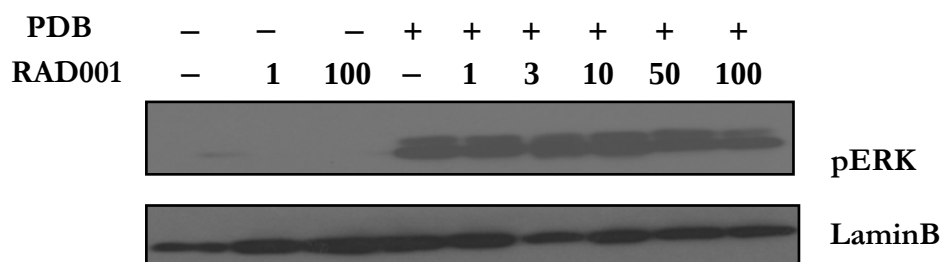
A**B****C**

Figure 5.1 FGF-2 and PDB stimulation of ERK and S6 Kinase 1 in H510 SCLC cells can be blocked by RAD001

H510 SCLC cells stimulated with FGF-2 (0.1ng/ml) for 5 minutes (A) and 10 minutes (B) and PDB (400nm) for 5 minutes (C). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biposphorylated ERK1/2 (A/C) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiments repeated at least three times, blot is representative of results.

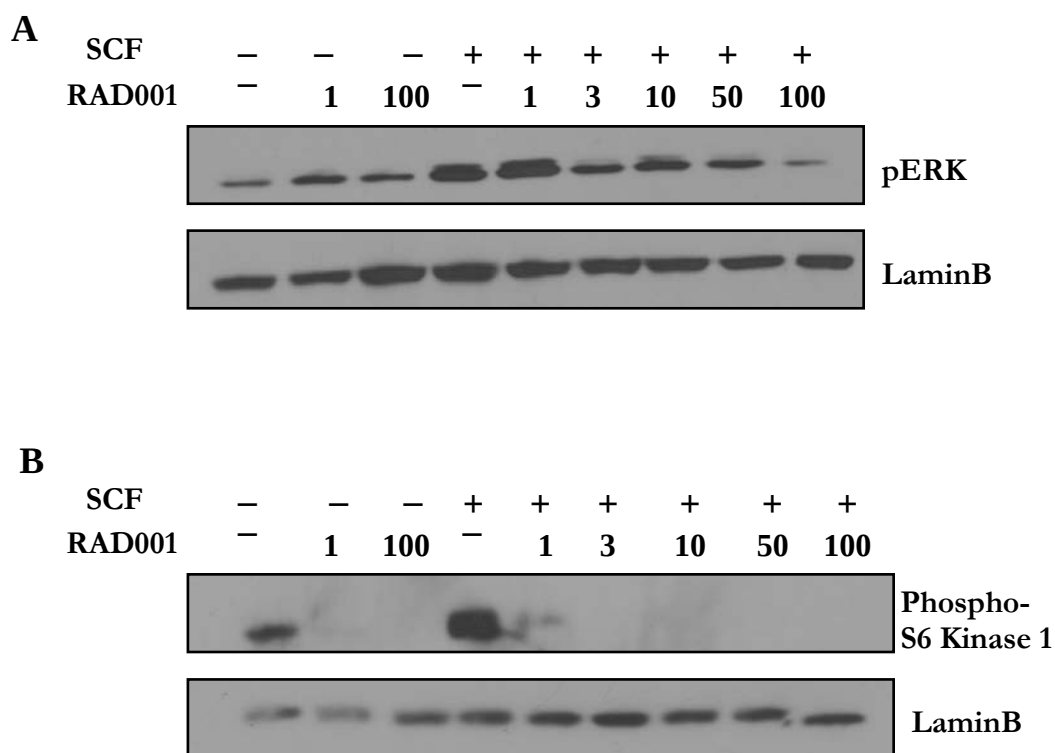


Figure 5.2 SCF stimulation of ERK and S6 Kinase 1 in H510 SCLC cells can be blocked by RAD001

H510 SCLC cells stimulated with SCF (3ng/ml) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

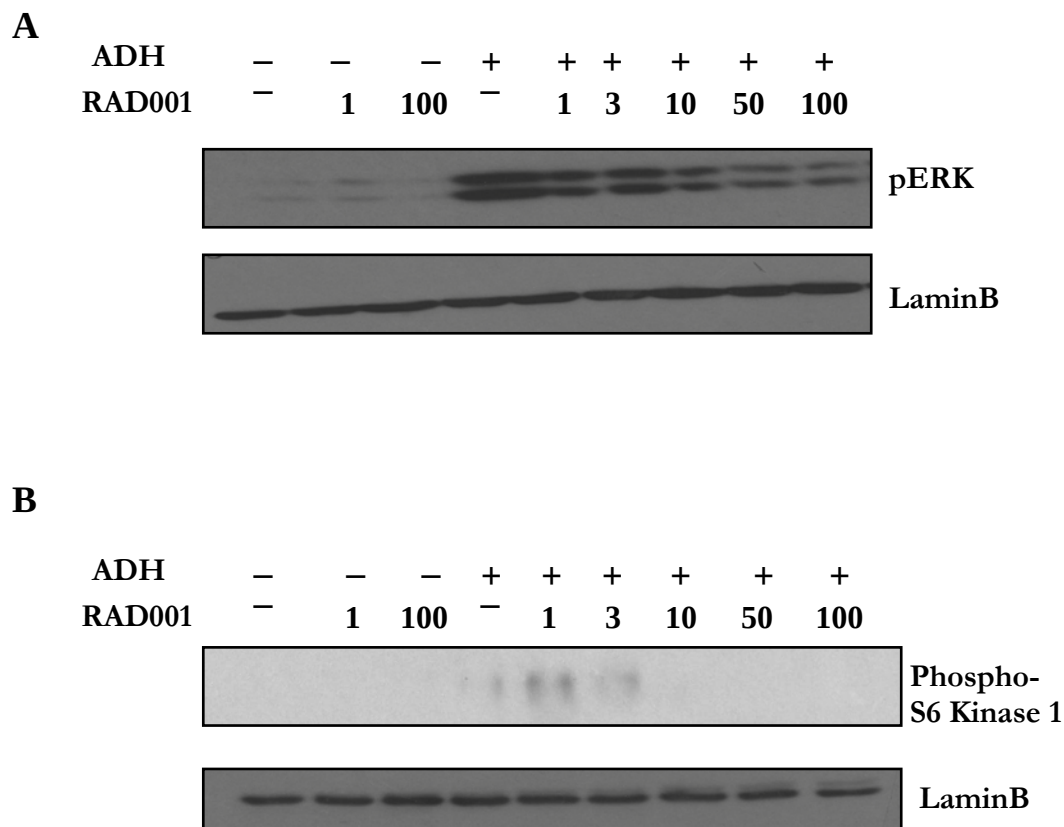


Figure 5.3 ADH stimulation of ERK and S6 Kinase 1 in H510 SCLC cells can be blocked by RAD001

H510 SCLC cells stimulated with ADH (300 μ M) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiments repeated at least three times, blot is representative of results.

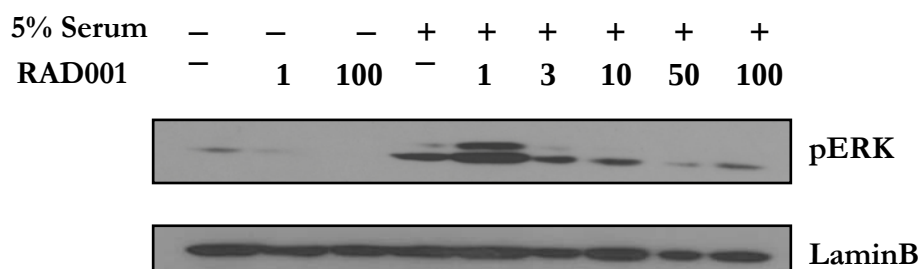
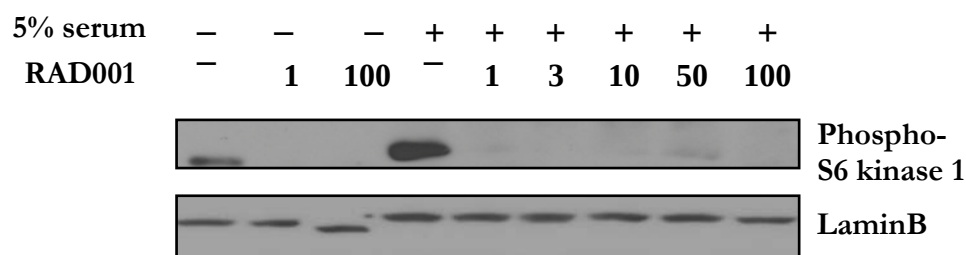
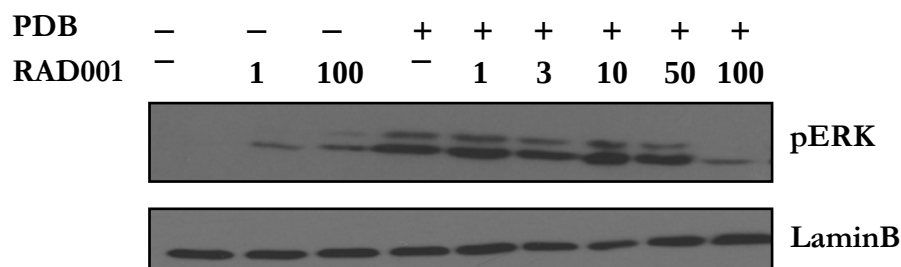
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Figure 5.4 5% serum and PDB stimulation of ERK and S6 Kinase 1 in H524 SCLC cells can be blocked by RAD001

H524 SCLC cells stimulated with fetal calf serum (5%) for 5 minutes (A) and 10 minutes (B) and PDB (400nm) for 5 minutes (C). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A/C) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

H69 and H524 SCLC cell lines were examined. FGF-2 is known to activate MEK/ERK signaling in H524 cells (unpublished data). However stimulation of biphospho-ERK by FGF-2 was difficult in this cell line, despite trying at least two different batches of FGF-2. 5% foetal calf serum (FCS) was used instead to activate MEK/ERK signaling in this particular cell line. Cells were set up as previously described for H510 cells and stimulated with 5% FCS for 5 and 10 minutes respectively. Figure 5.4A shows that again RAD001 blocked FCS induced stimulation of biphospho-ERK1/2 at doses of around 10ng/ml. Figure 5.4B shows that low dose RAD001 blocked phosphorylation of S6K1 as a control for RAD001 activity.

Phorbol ester, known to activate MEK/ERK signaling in many SCLC cell lines, was used this time in H524 SCLC cells. H524 cells were set up as for H510 cells and phorbol ester (400nM) was used to activate MEK/ERK signaling (5 minute stimulation). Figure 5.4C shows that again 100ng/ml of RAD001 could block phorbol ester stimulated biphospho-ERK1/2, consistent with previous findings described here.

Similar results are seen in Figures 5.5A and 5.6B. H524 cells were set up as for the H510 cells. SCF (3ng/ml) and ADH (300 μ M) respectively were used to activate MEK/ERK signaling for 5 and 10 minutes depending on the experiment. High doses of RAD001 blocked this activation as evidenced by a reduction in the biphospho-ERK1/2 signal at 100ng/ml. Controls (Figure 5.5C and Figures 5.6C) indicate that the RAD001 was active, as evidenced by blocking, at low dose, phosphorylation of S6K1.

The H69 SCLC cell line was examined next. Previous data from our laboratory showed that FGF-2 does not induce MEK/ERK signaling in this cell line since the FGF receptor (FGFR) is uncoupled from the MEK/ERK pathway (Pardo et al., 2001). Phorbol ester (Figure 5.7A), SCF (Figure 5.7A and B) and ADH (Figures 5.8 A and B) were used to

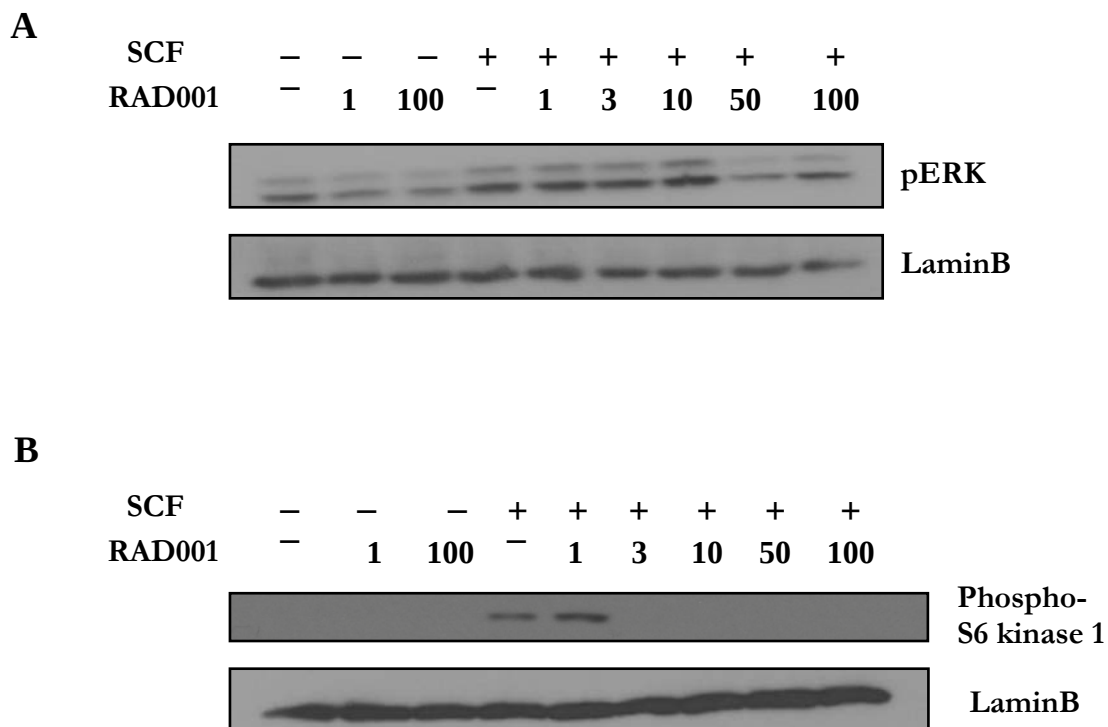


Figure 5.5 SCF stimulation of ERK and S6 Kinase 1 in H524 SCLC cells can be blocked by RAD001

H524 SCLC cells stimulated with SCF (3ng/ml) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiments repeated at least three times, blot is representative of results.

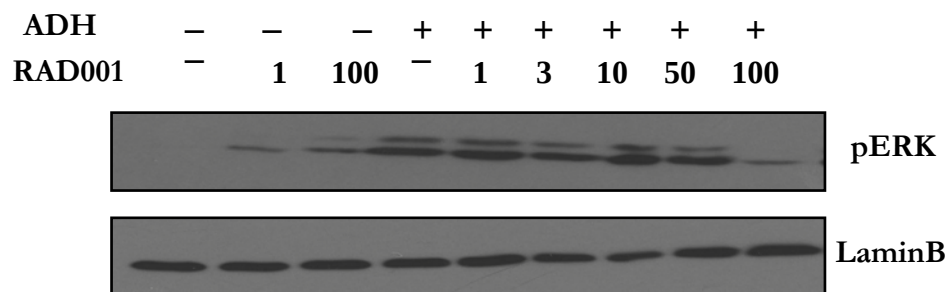
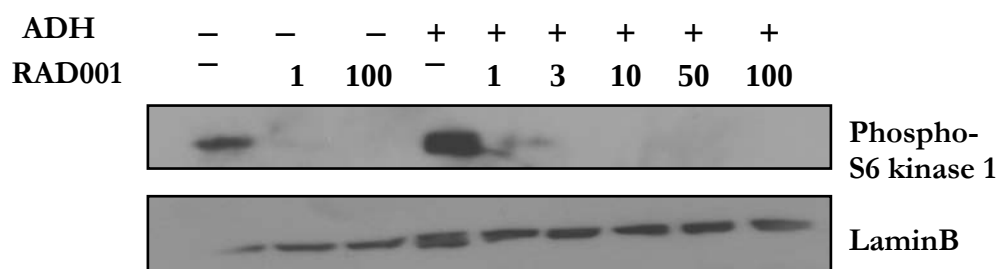
A**B**

Figure 5.6 ADH stimulation of ERK and S6 Kinase 1 in H524 SCLC cells can be blocked by RAD001

H524 SCLC cells stimulated with ADH (300 μ M) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

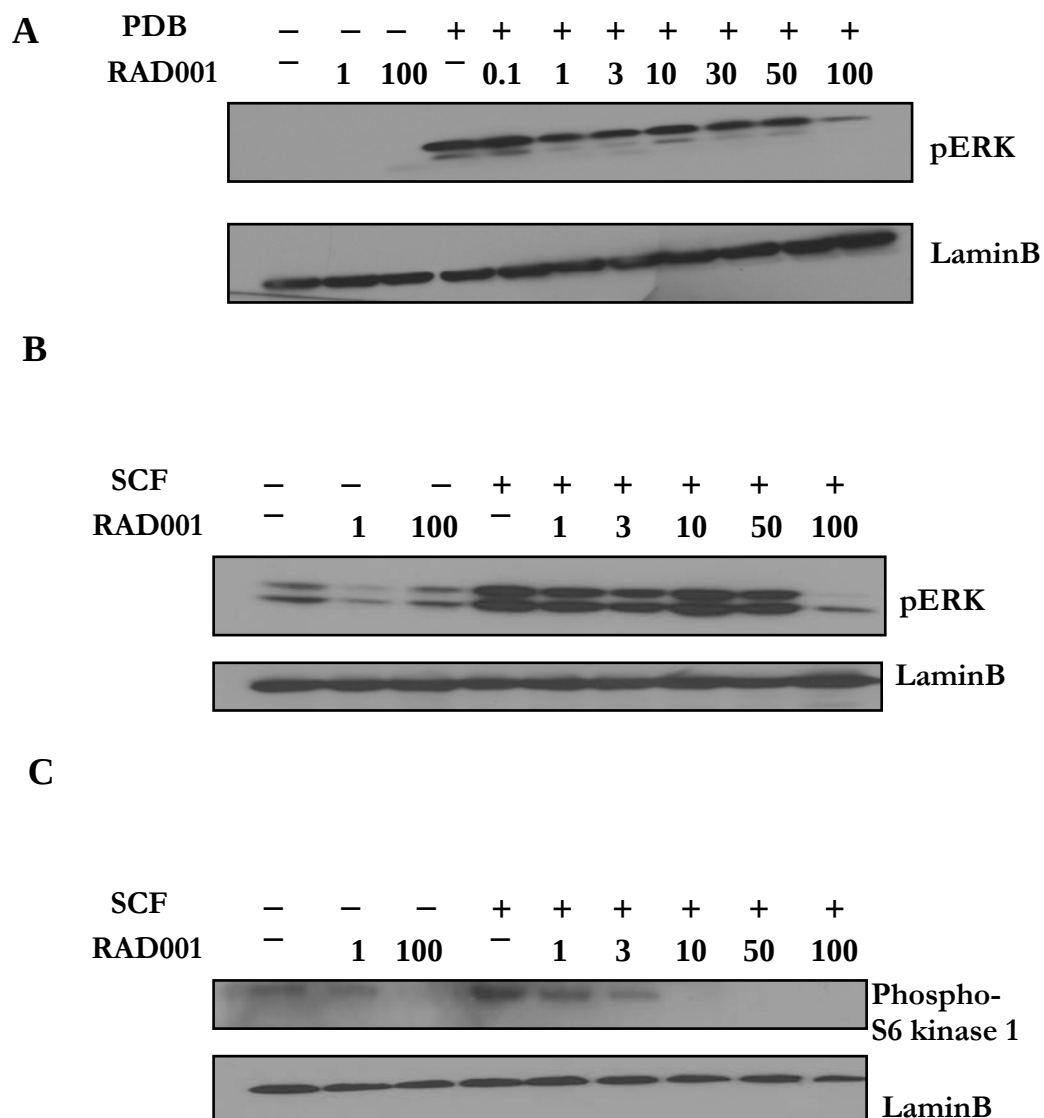


Figure 5.7 SCF and PDB stimulation of ERK and S6 Kinase 1 in H69 SCLC cells can be blocked by RAD001

H69 SCLC cells stimulated with PDB (400nm) for 5 minutes (A), SCF (3ng/ml) for 5 minutes (B) and 10 minutes (C). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A/B) or phosphorylated S6 Kinase 1 (Thr389) (C). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

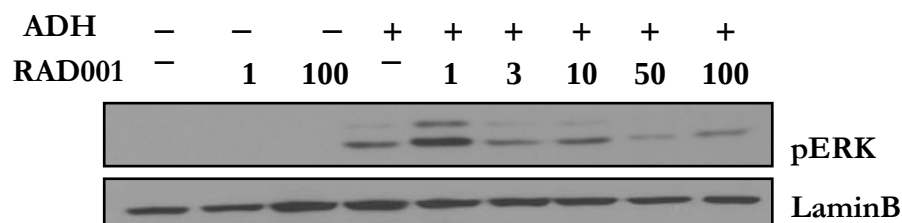
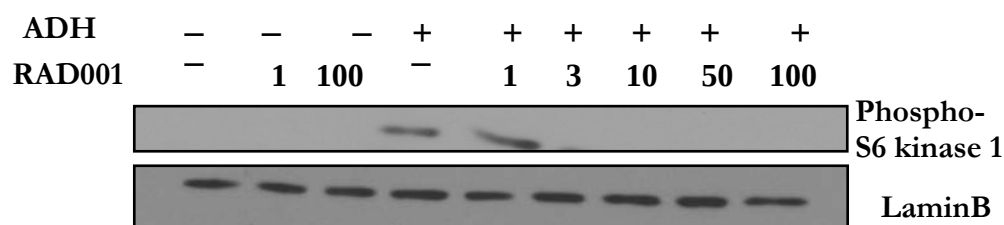
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Figure 5.8 ADH stimulation of ERK and S6 Kinase 1 in H69 SCLC cells can be blocked by RAD001

H69 SCLC cells stimulated with ADH (300 μ M) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

activate MEK/ERK signaling in H69 SCLC cells. All were set up as for the H510 cells. H69 cells were stimulated for 5 and 10 minutes depending on the experiment. RAD001 blocked bipospho-ERK1/2 activation at around 100ng/ml for each growth factor. Figures 5.7C and 5.8C showed that the RAD001 was active; 1-3ng/ml of RAD001 blocked SCF/ADH induced phosphorylation of S6K1.

V.2.4 Growth factor stimulation of ERK and S6 Kinase 1 in NSCLC cells (HCC78 and HCC95) can not be blocked by pre-treatment with RAD001

Since we have shown that high dose mTOR inhibitors can block growth factor/chemical activation of MEK/ERK signaling in 3 SCLC cell lines, we wanted to assess if the same observations were present in NSCLC cell lines. It is presently not known if FGF-2 can trigger chemoresistance in these cells and if so whether the mechanism is the same as described in H510 SCLC cells.

We first examined the HCC78 NSCLC cell line which has an adenocarcinoma phenotype-one of the two most common histological sub-types of NSCLC. A dose response assessment of FGF-2 activation of MEK/ERK signaling revealed no such activation (data not shown) so phorbol ester was used to activate MEK/ERK signaling in these cells. Cells were plated out to ~80% confluence in 6-well plates and left overnight to adhere to the surface of the plates. The cells were subsequently placed in 0.5% FCS/DMEM overnight. They were then placed in SFM the next morning for 1 hour. The cells were pre-treated with RAD001 (indicated concentrations) for 1 hour prior to addition of PDB (400nm) for 5 minutes and FGF-2 for 10 minutes. Blots were probed for bipospho ERK1/2 or phospho-S6 Kinase (Threonine 389) (depending on the experiment) and Lamin B followed by SDS PAGE analysis. Figure 5.9A shows that PDB induced phosphorylation of ERK could not be blocked by RAD001, even at relatively high doses.

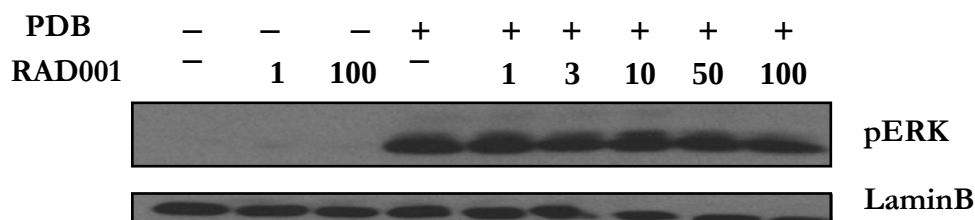
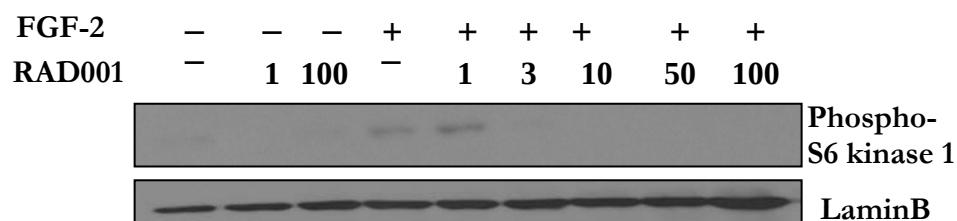
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Figure 5.9 PDB stimulation of ERK in HCC78 NSCLC can not be blocked by RAD001 however FGF-2 stimulation of S6 Kinase 1 in HCC78 NSCLC cells can be blocked by RAD001

HCC78 NSCLC cells stimulated with PDB (200nm) for 5 minutes (A) and FGF-2 (1ng/ml) for 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

Since PDB does not activate PI3K/AKT/mTOR/S6K1 signaling, FGF-2 was used to activate this pathway and figure 5.9B shows that the RAD001 was active, blocking S6K1 activation; as expected at relatively low dose of RAD001 (1ng/ml).

We assessed another NSCLC cell line- the HCC95 line which has a squamous cell phenotype. FGF-2 was found to activate MEK/ERK signaling in this cell line (data not shown) and was used in subsequent experiments at 1ng/ml dose. Cells were set up as for the HCC78 cell line. Figure 5.10A shows that FGF-2 (1ng/ml) activation of MEK/ERK signaling could not be blocked by relatively high doses of RAD001. Figure 5.1B shows FGF-2 activation of S6K1 was blocked at relatively low doses of RAD001, as expected if the RAD001 was active. Similar results were observed when EGF; an important growth factor in NSCLC biology, was exchanged for FGF-2/phorbol ester in both HCC78/95 NSCLC cell lines (data not shown).

These observations led to the question of whether higher doses of RAD001 could block growth factor activation of MEK/ERK signaling in NSCLC cell lines? It may be the case that doses needed to block MEK/ERK signaling in SCLC are lower than those needed in NSCLC cell lines. We used HCC95 NSCLC cells to try and answer this particular question.

Cells were set up as previously mentioned for HCC78 cells. Figure 5.11 shows that in HCC95 cells even using doses of up to 1000ng/ml of RAD001, which would be difficult for patients to tolerate, could not block FGF-2 induced phosphorylation of biphospho-ERK1/2.

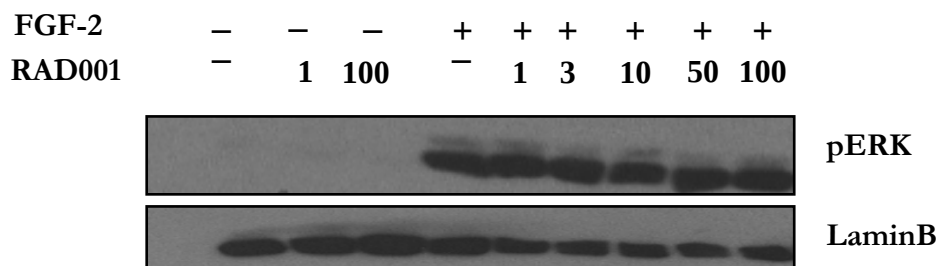
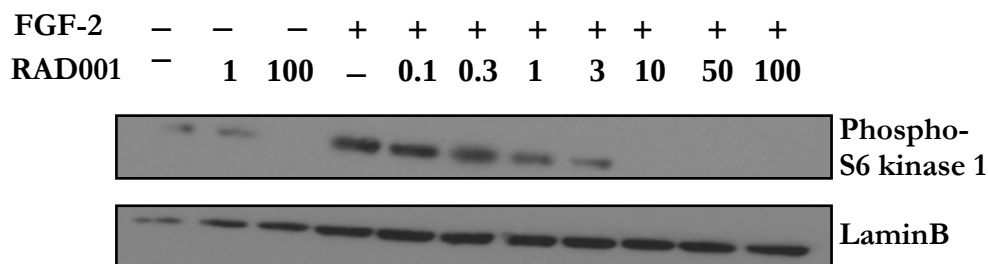
A**B**

Figure 5.10 FGF-2 stimulation of ERK in HCC95 NSCLC can not be blocked by RAD001 however FGF-2 stimulation of S6 Kinase 1 in HCC95 NSCLC cells can be blocked by RAD001

HCC95 NSCLC cells stimulated with FGF-2 (1ng/ml) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

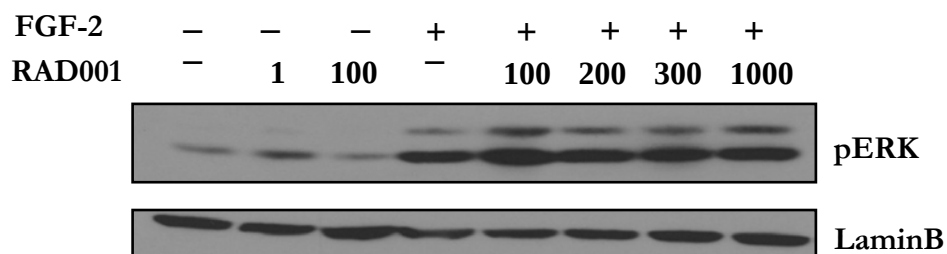


Figure 5.11 High doses of RAD001 do not block FGF-2 stimulation of ERK in HCC95 NSCLC cells.

HCC95 NSCLC cells stimulated with FGF-2 (1ng/ml) for 5 minutes. Increasing doses of RAD001 were added 1 hour before (100-1000ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2. Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

V.2.5 FGF-2 stimulation of ERK and S6 Kinase 1 in MCF-7 cells (breast cancer) can be blocked by pre-treatment with RAD001.

Since we have established that in at least two NSCLC cell lines that growth factor activation of MEK/ERK signaling could not be blocked by mTOR inhibitors, another question to ask is whether this phenomenon is only pertinent to SCLC cells. The literature indicates this observation varies depending upon the cell line. A melanoma and sarcoma cell line show this phenomenon (Harwood et al., 2008; Molhoek et al., 2005) whilst NIH3T3 (Fibroblasts) do not manifest this potential connection from mTORC1 to MAPK signaling (Chung et al., 1992).

We wished to investigate whether FGF-2 activation of MEK/ERK signaling could be blocked by mTOR inhibitors in another cell line-the MCF-7 breast cancer cell. This cell line was chosen because FGF-2 has been known to be mitogenic in this cell line (Nurcombe et al., 2000; Rahmoune et al., 1998) and FGF-2 is able to counteract the killing of agents such as etoposide (Vandermoere et al., 2005).

MCF-7 cells were plated out to ~80% confluence in 6-well plates and left overnight to adhere to the surface of the plates. The cells were subsequently placed in 0.5% FCS/DMEM overnight. They were then placed in SFM the next morning for 1 hour. The cells were pre-treated with RAD001 (indicated concentrations) for 1 hour prior to addition of FGF-2 (1ng/ml) for 5 minutes and 10 minutes depending on the experiment. Blots were probed for biphospho-ERK1/2 or phospho-S6 Kinase (Threonine 389) (depending on the experiment) and Lamin B followed by SDS PAGE analysis.

Figure 5.12A shows that 100ng/ml RAD001 could suppress FGF-2 activation of biphospho-ERK1/2 like 3 SCLC cell lines and unlike two NSCLC cell lines. Activity of RAD001 was shown by suppression of activation of S6K1 at low dose (Figure 5.12B).

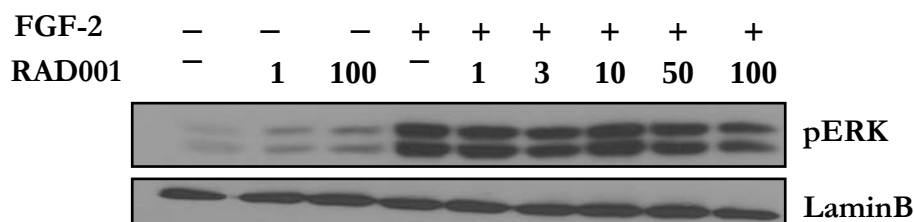
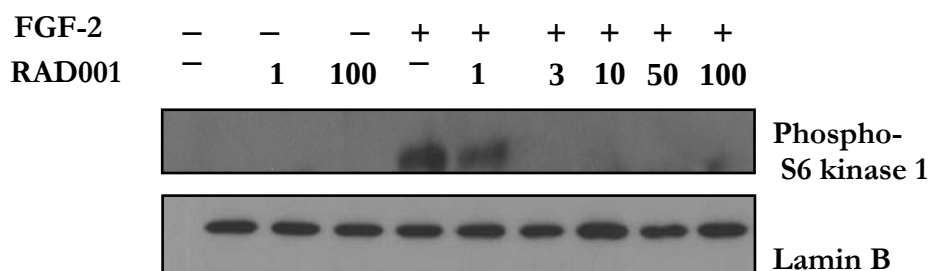
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Figure 5.12 FGF-2 stimulation of ERK and S6 Kinase 1 in MCF7 breast cancer cells can be blocked by RAD001

MCF-7 breast cancer cells stimulated with FGF-2 (1ng/ml) for 5 minutes (A) and 10 minutes (B). Increasing doses of RAD001 were added 1 hour before (1-100ng/ml). Lysates were analysed by SDS-PAGE for the presence of biphosphorylated ERK1/2 (A) or phosphorylated S6 Kinase 1 (Thr389) (B). Lamin B detection was used as a loading control. Experiment repeated at least three times, blot is representative of results.

V.2.6 Can high concentrations of rapalogues block FGF-2 and/or PDB-mediated chemoresistance in H510, HCC78 or HCC95 cells?

FGF-2 induces chemoresistance in H510 SCLC cells; these effects were found to be consistent with and dependent on activation of MEK/ERK signaling in these cells and a novel cross-talk between MEK/ERK and S6K2. The FGF-2 induced survival against the chemotherapeutic agent etoposide in SCLC cells was found to be regulated by increased translation of the anti-apoptotic proteins Bcl-XL, Bcl-2 and IAPs. High doses of RAD001 reduce FGF-2 activation of MEK/ERK signaling in H510 cells. Can RAD001 block FGF-2/PDB induced chemoresistance in NSCLC and SCLC cells?

To answer this question, H510 SCLC were treated with two different concentrations of RAD001 (1ng and 100ng/ml, doses at either end of the clinically relevant range) for 1 hour prior to addition of FGF-2 (0.1ng/ml) for 4 hours. Etoposide chemotherapy (0.1 μ M) was then added and cells were counted after 4 days. Etoposide was added with each dose of RAD001 (ER1 and ER100) and then FGF-2 to each combination of RAD001 and etoposide (EFR1 and EFR100). Both the 4 hour pre-treatment with the growth factor and the 4 day incubation, as well as the starting number of cells have been determined from previous work from the group (Pardo et al., 2001). Etoposide was chosen because it is commonly to treat lung cancer. For HCC95 similar doses of RAD001 were used. FGF-2 at 1ng/ml and etoposide at 20 μ M was utilized. For HCC78 cells again similar doses of RAD001 were used, PDB was used to activate MEK/ERK signaling at 100nM. 10 μ M etoposide was utilised. (Dose response curves were performed for etoposide in order to determine the respective concentrations that caused approximately 50% cell death after 4 days-data not shown). Experiments were repeated at least 3 times.

The results for H510 cells are shown in figure 5.13. In agreement with our previous work, etoposide induced killing was rescued by pre-treating the cells with FGF-2 (F/E). This was

unlikely to be a consequence of proliferation as FGF-2 alone did not increase the number of cells seen compared to control. RAD001 at 1ng/ml (R1) did not induce much cell killing as expected whilst with RAD001 at 100ng/ml (R100) around 65% cell killing was observed. Low dose RAD001 and etoposide did not have any synergistic/additive effects (ER1) whilst higher dose RAD001 and etoposide did (ER100) have some additive effects on cell killing. FGF-2 and RAD001 at low dose together had no effects when combined (FR1), whilst FGF-2 did not rescue H510 cells from higher dose RAD001 killing (FR100). Next we wanted to see if RAD001 could block FGF-2 induced resistance to etoposide. Low dose and high dose RAD001 appear to prevent FGF-2 induced resistance to etoposide (EFR1 and EFR100) as evidenced by comparing the columns E/F with EFR1 and EFR100. The level of cell killing is higher in the two conditions with RAD001.

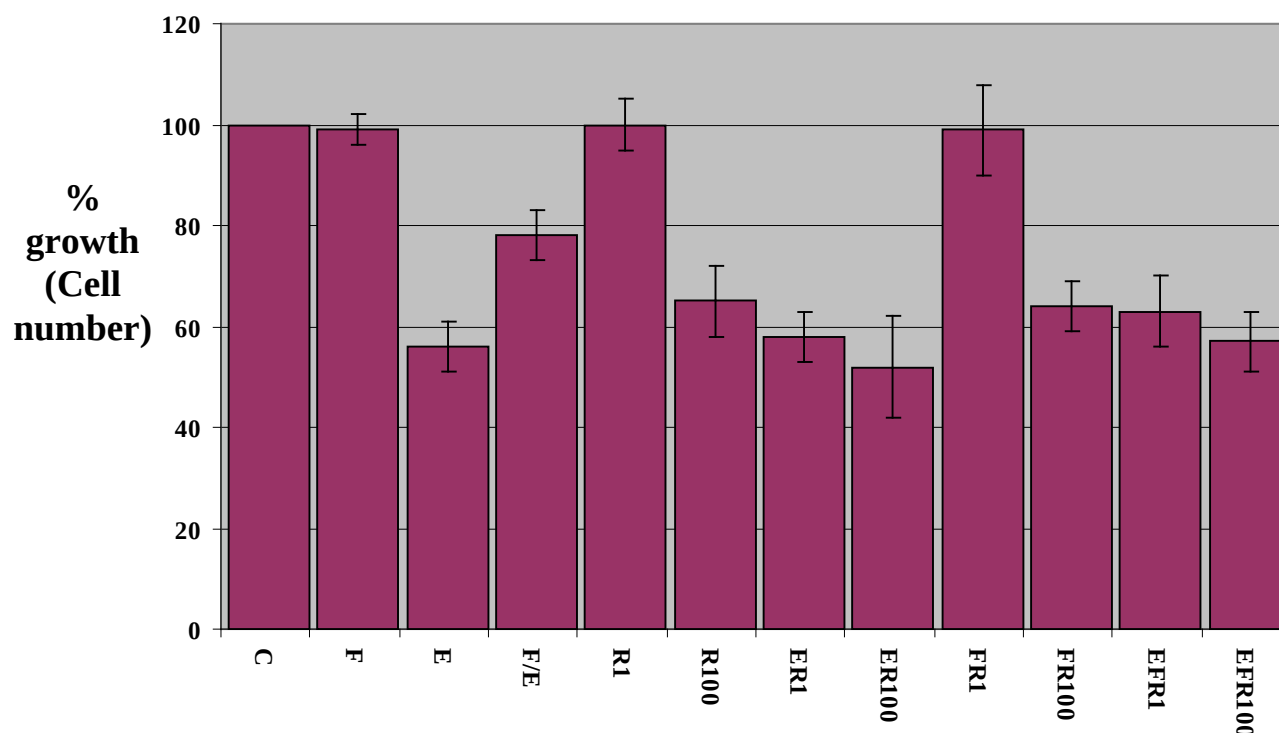


Figure 5.13 RAD001 blocks FGF-2 induced rescue from etoposide cell death in H510 SCLC cells.

Cell survival study H510 SCLC cells. Cells were grown in serum free medium in the absence (C) or presence of either 1 ng/ml FGF-2 (F), 0.1 μ M etoposide (E), FGF-2 plus etoposide (F/E), RAD001 at 1 or 100 ng/ml (R1 or R100), etoposide and RAD001 at 1 or 100 ng/ml (E/R1 or E/R100). The addition of FGF-2 to etoposide and Rad001 at 100 ng/ml and 1 ng/ml, (EFR1 and EFR100).

The FGF-2 was added 4 hours before the etoposide and in all conditions where RAD001 was used; this was added 1 hour before all other agents. Cell counting with trypan blue exclusion was used to assess cell viability 4 days later. The results shown are the mean of three independent experiments with each condition performed in triplicate $\pm$ the SEM.

Next we looked at the NSCLC cell line HCC95. Similar conditions were set up as for the H510 cell line. Figure 5.14 shows the results. FGF-2 appeared to rescue the cells from etoposide induced killing (F/E). Again this was unlikely to be a consequence of proliferation as FGF-2 alone did not increase the number of cells seen compared to control. RAD001 at 1ng/ml (R1) did not induce much cell killing as expected whilst with RAD001 at 100ng/ml (R100) around 60% cell killing was observed. Low dose RAD001 and etoposide did not have any synergistic/additive effects (ER1) whilst higher dose RAD001 and etoposide did (ER100) have some small additive effects on cell killing. FGF-2 and RAD001 at low dose together had no effects when combined (FR1), whilst FGF-2 did not rescue HCC95 cells from higher dose RAD001 killing (FR100). Next we wanted to see if RAD001 could block FGF-2 induced resistance to etoposide. Low dose and high dose RAD001 did not appear to prevent FGF-2 induced resistance to etoposide (EFR1 and EFR100) as evidenced by comparing the columns E/F with EFR1 and EFR100. The level of cell killing is lower in the two conditions with RAD001.

We then moved on to investigate if RAD001 could block chemoresistance in another NSCLC cell line- HCC78. Figure 5.15 shows the results. As mentioned FGF-2 does not activate MEK/ERK signaling in HCC78 cells so PDB was used to activate this pathway. PDB appeared to rescue the cells from etoposide induced killing (P/E). Again this was unlikely to be a consequence of proliferation as PDB alone did not increase the number of cells seen compared to control. RAD001 at 1ng/ml (R1) did not induce much cell killing as expected whilst with RAD001 at 100ng/ml (R100) around 70% cell killing was observed. Low dose and high dose RAD001 with etoposide did not have any synergistic/additive effects (ER1/ER100). PDB and RAD001 at low dose together had no effects when combined (PR1), whilst PDB did rescue HCC78 cells from high dose RAD001 cell killing.

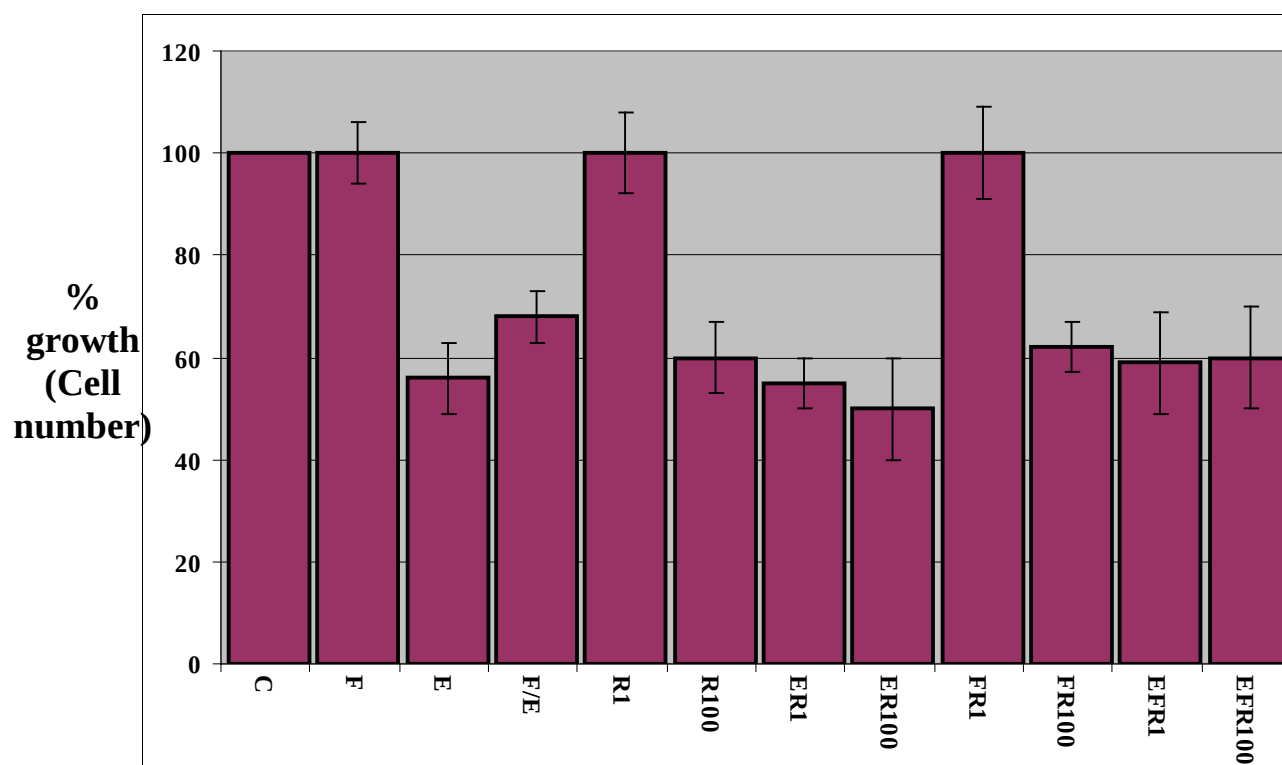


Figure 5.14 RAD001 does not block FGF-2 induced rescue from etoposide cell death in HCC95 NSCLC cells.

Cell survival study HCC95 NSCLC cells. Cells were grown in serum free medium in the absence (C) or presence of either 1 ng/ml FGF-2 (F), 20 μ M etoposide (E), FGF-2 plus etoposide (F/E), RAD001 at 1 or 100ng/ml (R1 or R100), etoposide and RAD001 at 1 or 100ng/ml (E/R1 or E/R100). The addition of FGF-2 to etoposide and RAD001 at 100ng/ml and 1ng/ml (EFR1 and EFR100).

The FGF-2 was added 4 hours before the etoposide and in all conditions where RAD001 was used; this was added 1 hour before all other agents. Cell counting with trypan blue exclusion was used to assess cell viability 4 days later. The results shown are the mean of three independent experiments with each condition performed in triplicate +/- the SEM.

Finally, we wanted to see if RAD001 could block PDB induced chemoresistance to etoposide in this cell line. Low dose and high dose RAD001 did not appear to prevent PDB induced resistance to etoposide (EFR1 and EFR100) as evidenced by comparing the columns E/F with EFR1 and EFR100. The level of cell killing is lower in the two conditions with RAD001. Low dose RAD001 did not appear to prevent PDB induced chemoresistance to etoposide (EPR1) whilst higher doses of RAD001 did appear to prevent PDB induced chemoresistance (EPR100).

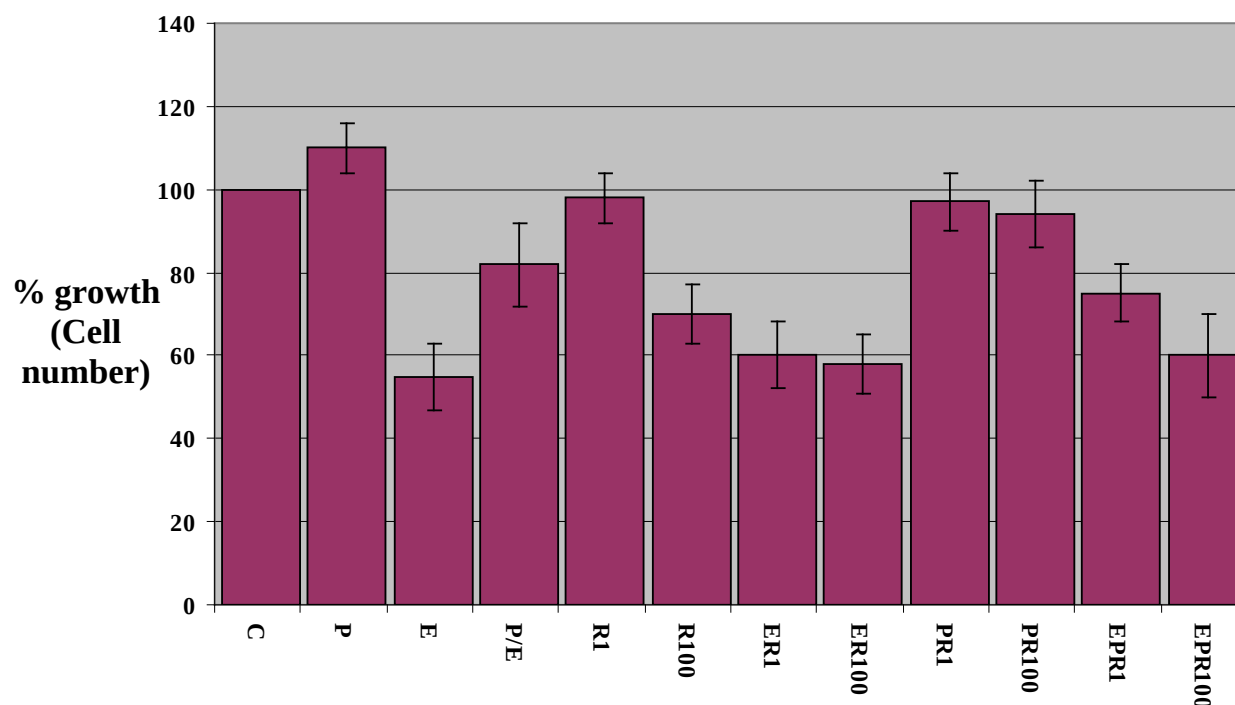


Figure 5.15 RAD001 does block PDB induced rescue from etoposide cell death in HCC78 NSCLC cells.

Cell survival study HCC78 NSCLC cells. Cells were grown in serum free medium in the absence (C) or presence of either 100nM PDB (P), 10 μ M etoposide (E), PDB plus etoposide (F/E), RAD001 at 1 or 100ng/ ml (R1 or R100), etoposide and RAD001 at 1 or 100ng/ml (E/R1 or E/R100). The addition of PDB to etoposide and RAD001 at 100ng/ml and 1ng/ml (EPR1 and EPR100).

The PDB was added 4 hours before the etoposide and in all conditions where RAD001 was used; this was added 1 hour before all other agents. Cell counting with trypan blue exclusion was used to assess cell viability 4 days later. The results shown are the mean of three independent experiments with each condition performed in triplicate +/- the SEM.

V.2.8 Discussion

These results show that pre-treatment with high dose RAD001 can block growth factor- be that polypeptide or neuropeptide, phorbol ester and serum induced phosphorylation of biphospho-ERK1/2 in 3 SCLC cell lines. These results demonstrate that the findings from our laboratory using high dose rapamycin in H510 cells to block FGF-2 induction of biphospho-ERK1/2 are applicable to other more modern clinically relevant mTOR inhibitors/rapalogues. This seems to be consistent in SCLC cell lines despite the growth factor/chemical used to activate MEK/ERK signaling. Since stimulation of this pathway can lead to activation of a novel translational mechanism to prevent etoposide induced apoptosis in H510 cells, it would seem reasonable to suggest that mTOR inhibitors could block this resistance to chemotherapy killing. This appears to be the case in H510 cells when using high doses and low doses of RAD001 (100ng/ml) in a 4 day cell survival assay. The degree of blocking of chemoresistance was not large nor was it quantified statistically, however a trend could be seen. The observation that low dose RAD001 blocks FGF-2 rescue of etoposide killing is somewhat surprising since in experiments using rapamycin low doses could not rescue this killing (Pardo et al., 2002). It may be that the extra hydroxyl ethyl group on RAD001 makes it more potent than rapamycin. This clearly needs further investigation. We could have seen if low doses of RAD001 could block S6K2 activation, for example to see if this is the mechanism.

This observation lends weight to a potential novel therapeutic strategy in SCLC of using mTOR inhibitors to help overcome cytotoxic chemotherapy resistance.

We demonstrated that FGF-2/PDB/EGF activation of MEK/ERK in 2 NSCLC cell lines, representing the two most common histological subtypes, could not be blocked by RAD001.

The reason for this was not clear. Drug metabolism could be different in NSCLC however RAD001 did block phosphorylation of S6K1. This would be another subject to investigate further. Despite these observations, FGF-2 and PDB could induce chemoresistance to etoposide chemotherapy over 4 days in HCC95 and HCC78 NSCLC cells, respectively. RAD001 could not prevent FGF-2 induced chemoresistance in HCC95 cells, but could do so in PDB induced chemoresistance in HCC78 cells. The mechanism for this chemoresistance in these NSCLC may not involve activation of MEK/ERK signaling and is not known presently. There are many putative chemoresistance mechanisms in NSCLC including DNA repair capacity after exposure of chemotherapy, expression of multi drug resistance associated-proteins and mutations in the EGFR gene (Berger et al., 1997; Hirsch & Witta, 2005; Zamble & Lippard, 1995).

Breast cancer cells (MCF-7), in whom FGF-2 can activate MEK/ERK signaling; RAD001 blocked biphospho-ERK1/2 at high dose. Again, confirming that this phenomenon is seen in some but not all cancer cell lines.

In summary these results show that in SCLC cell lines; high dose RAD001 does block growth factor/chemical activation of MEK/ERK signaling unlike in NSCLC cell lines. High doses of RAD001 also blocked FGF-2 induced chemoresistance to etoposide induced apoptosis in a 4 day assay in H510 SCLC cells; this was not the case in HCC95 NSCLC cells. However phorbol ester induced resistance to etoposide killing was blocked by RAD001 over 4 days in HCC78 NSCLC cells despite RAD001 not being able to block MEK/ERK activation in a short stimulation assay. Phorbol esters activate the second messengers-classical protein kinase C (cPKC) and non-classical PKCs. They are involved in cell proliferation and survival (Musashi et al., 2000; Wert & Palfrey, 2000). Substrates include

MAP kinase, RAF kinase and certain receptor tyrosine kinases (Mellor & Parker, 1998), so the mechanism of this phorbol ester chemoresistance to chemotherapy is not clear.

The question still remains- what is the mechanism of high dose RAD001 blocking FGF-2 activation of MEK/ERK signaling?

CHAPTER VI

mTOR and MEK/ERK signaling

VI.1 Introduction

From the previous chapters results we have seen that at in 3 SCLC cell lines and 1 breast cancer cell line that pre-treatment with mTOR inhibitors/rapalogues can impair activation of MAPks and in two NSCLC cell lines it cannot. It is documented that the extracellular signal regulating protein kinases ERK1/2 can phosphorylate and hence negatively regulate TSC leading to increased signaling by mTORC1 (Chiang & Abraham, 2007) however the opposite – mTORC1 regulating ERK1/2 and MEK/ERK signaling has not been well documented. If mTORC1 can regulate MEK/ERK signaling it would signify a new signal transduction pathway for mTOR. However there may be other mechanisms involved including high dose mTOR inhibitors/rapalogues could cause conformational changes in the FKBP12/mTOR complex resulting in secondary effects (drug saturation effects) or that mTOR inhibitors/rapalogues, at relatively high doses are hitting another target.

Another potential mechanism that could explain our observations is that mTOR inhibition activates a serine/threonine protein phosphatase that dephosphorylates regulatory sites. Research from 1999 showed that mTOR controls 4E-BP1 and S6 kinase 1 phosphorylation indirectly by restraining a phosphatase which was found to be protein phosphatase 2A (PP2) (Peterson et al., 1999).

Protein phosphatase 2 (PP2) (previously PP2A), a heterotrimeric protein phosphatase, is a ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions. PP2 consists of a core enzyme comprising the structural A and catalytic C subunits, and a regulatory B subunit. When the PP2 catalytic C subunit associates with the A and B subunits; several species of holoenzymes are produced with distinct functions and characteristics. The A subunit, a member of the HEAT repeat protein family (huntington-elongation-A subunit-TOR), is the scaffold required for the formation of the

heterotrimeric complex. When the A subunit binds it alters the enzymatic activity of the catalytic subunit, even if the B subunit is absent. While C and A subunit sequences show sequence conservation throughout eukaryotes, regulatory B subunits are more heterogeneous and are believed to play key roles in controlling the localization and specific activity of different holoenzymes. Multicellular eukaryotes express four classes of variable regulatory subunits: B (PR55), B' (B56 or PR61), B'' (PR72), and B''' (PR93/PR110), with at least 16 members in these subfamilies. In addition, accessory proteins and posttranslational modifications (such as methylation) control PP2 subunit associations and activities. Among the targets of PP2 are proteins of oncogenic signaling cascades, such as Raf, MEK, and Akt (Ory et al., 2003; Xing et al., 2006; Xu et al., 2006).

In fact last year a group from the United States found that mTORC1 signaling could regulate growth factor activation of p44/42 MAPKs through PP2 (Harwood et al., 2008). This group essentially addressed the same issue we investigate here. The cells that were used were the Rh1 human Ewing sarcoma cell line. They found that high concentrations of rapamycin induced inhibition of EGF induced phosphorylation of p44/42(Thr²⁰²) and this was reversed by low concentrations of okadaic acid. Okadaic acid at concentrations ~100nM or less is a relatively specific inhibitor of PP2A/PP2 (Favre et al., 1997; Honkanen & Golden, 2002).

In this chapter we will be asking whether mTOR or the intracellular receptor for rapalogues-FKBP12 is important for high dose mTOR inhibitors/rapalogues blocking FGF-2 activation of ERK1/2.

VI.2 RESULTS

VI.2.1 RNAi to FKBP12 does not seem to prevent high dose RAD001 blocking FGF-2 stimulated phosphorylation of ERK in MCF-7 breast cancer cells.

To assess whether the intracellular ‘receptor’ for rapamycin- FKBP12 is important in the mechanism of how high dose mTOR inhibitors/rapalogues block growth factor induced phosphorylation of MAPks, RNA interference (siRNAi) to FKBP12 was performed in MCF-7 breast cancer cells to down regulate FKBP12. Since RNAi in general to H510 SCLC has been fraught with difficulties- cells dying after transfection and low transfection rates (unpublished data from Seckl group), MCF-7 breast cancer cells were chosen to investigate this mechanism since these cells are easily transfected with RNAi oligonucleotides and importantly manifest high dose mTOR inhibitor/rapalogues blocking FGF-2 induced MEK/ERK signaling.

The specificity and optimum concentration for ‘knock-down’ of FKBP12 in MCF-7 cells was determined. We performed dose responses to determine the minimum amount of pooled siRNA required to achieve silencing and compared to a non-specific set of targeting sequences. We then deconvoluted the pooled siRNA into 4 individual siRNAs and observed that 3 out of the 4 targeting sequences achieved very similar knockdown of FKBP12 protein (data not shown). These concentrations were used in subsequent experiments. Cells (where relevant) were transfected with FKBP12 oligonucleotides for 48 hour prior to experiments as described.

Figure 6.1 shows the results of experiments using FGF-2 (for 5 minutes) to activate phosphorylation of biphospho-ERK1/2. Blots were probed for biphospho-ERK1/2, mTOR, FKBP12 and Lamin B followed by SDS-PAGE analysis. On the left hand side of the panel, where cells were not transfected, 100ng/ml RAD001 reduced the degree of FGF-

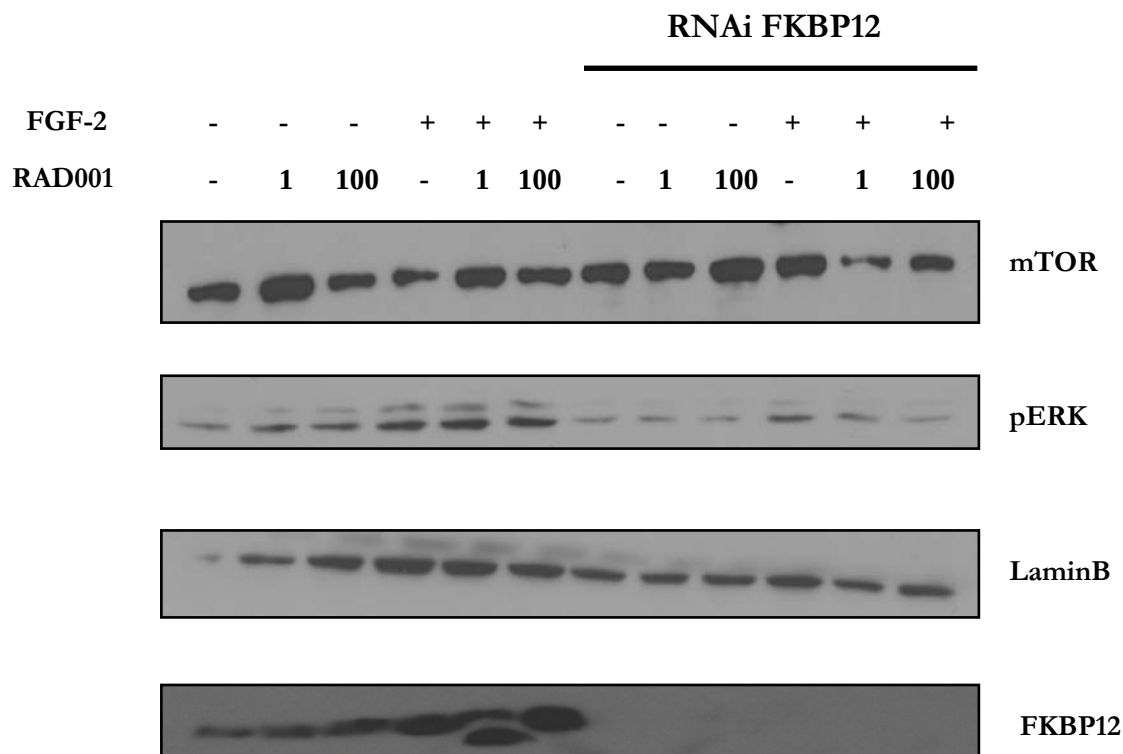


Figure 6.1 RNAi to FKBP12 does not prevent high dose RAD001 blocking FGF-2 stimulated phosphorylation of ERK1/2 in MCF-7 cells.

MCF-7 cells were transfected with pooled siRNA targeting FKBP12 48 hours before incubation for 1 hour without (-) or with RAD001 at low (1ng/ml) or high (100ng/ml) concentrations. The cells were then exposed (+) or not (-) to 1ng/ml FGF-2. Lysates were analysed by SDS-PAGE for the presence of biphospho-ERK1/2. Lamin B detection was used as a loading control. Results are representative of 3 repeat experiments.

2 induced phosphorylation of ERK1/2 as expected. On the right hand side of the panel, cells were prior transfected with RNAi to FKBP12; again 100ng/ml RAD001 reduced phosphorylation of ERK1/2. It appears that FKBP12 is not involved in this mechanism. mTOR total protein levels did not appear to be affected by siRNAi to FKBP12 which appeared to be silenced. We cannot exclude the possibility that there was some FKBP12 still present and that the RAD001 at high concentrations may still be acting through this protein to impair mTOR function and so MEK/ERK signaling.

VI.2.2 RNAi to FKBP12 does not prevent RAD001 blocking FGF-2 stimulated phosphorylation of S6 Kinase 1 in MCF-7 cells.

RAD001 blocks FGF-2 induced activation of S6K1 in H510 SCLC cells; this should be prevented by using RNAi knock down to FKBP12 since mTOR inhibitors/rapalogs firstly bind to FKBP12 before together targeting the FRB domain of mTOR. To confirm this, MCF-7 cells were set up as previously described. Blots were probed for phospho-S6 Kinase 1 (Threonine 389), mTOR, FKBP12 and Lamin B followed by SDS PAGE analysis. Figure 6.2 shows the results. On the left side of the panel, as expected in non-transfected cells when FGF-2 is used to activate S6K1 (10 minute stimulation), this is blocked by 1ng/ml of RAD001. However, this was also the case when these cells were transfected with RNAi to FKBP12 (right panel). This would seem counter intuitive, however the original film used to develop this experiment showed complete knock down of FKBP12, however when film exposure time was increased- as can be seen here, FKBP12, though reduced in expression, is still present. So these silencing experiments cannot exclude the possibility that FKBP12 mediates the high dose effects of rapalogues on MEK/Erk signaling.

A consistent problem seen when MCF-7 cells are transfected with siRNAi oligonucleotides to FKBP12 is that the controls i.e. no other condition apart from RNAi transfection greatly

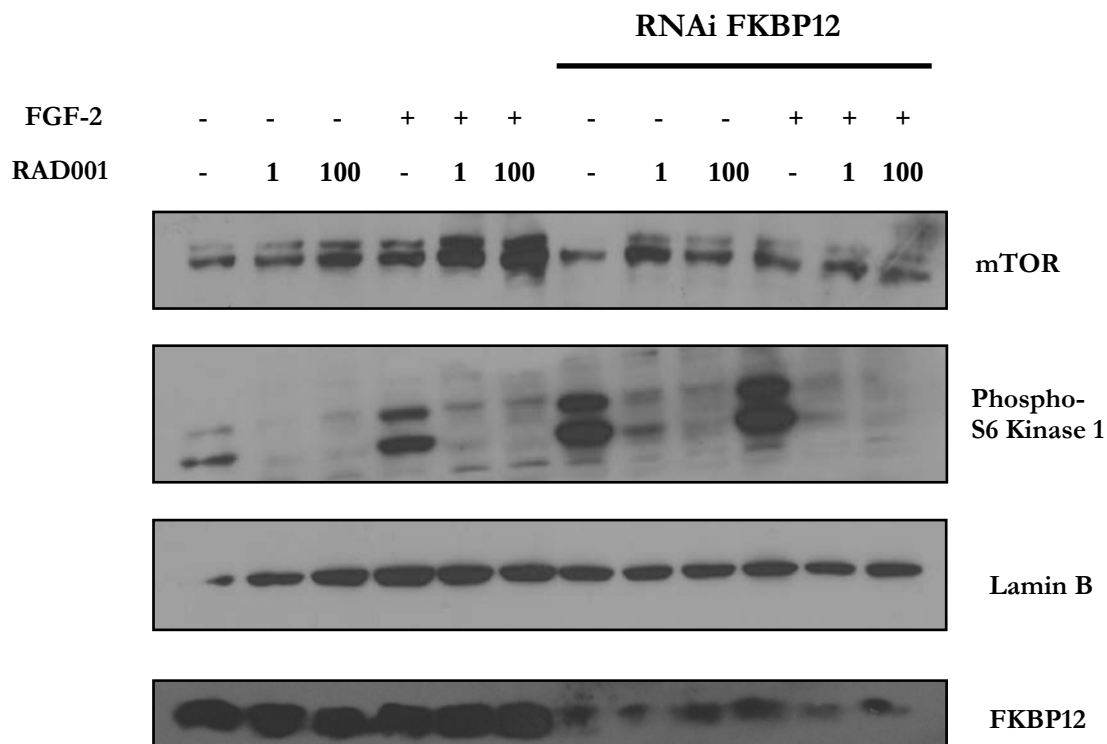


Figure 6.2 RNAi to FKBP12 does not prevent RAD001 blocking FGF-2 stimulated phosphorylation of S6 Kinase 1 in MCF-7 cells.

MCF-7 cells were transfected with pooled siRNA targeting FKBP12 48 hours before incubation for 1 hour without (-) or with RAD001 at low (1ng/ml) or high (100ng/ml) concentrations. The cells were then exposed (+) or not (-) to 1ng/ml FGF-2. Lysates were analysed by SDS-PAGE for the presence of phosphorylated S6 Kinase 1 (Thr389). Lamin B detection was used as a loading control. Results are representative of 3 repeat experiments.

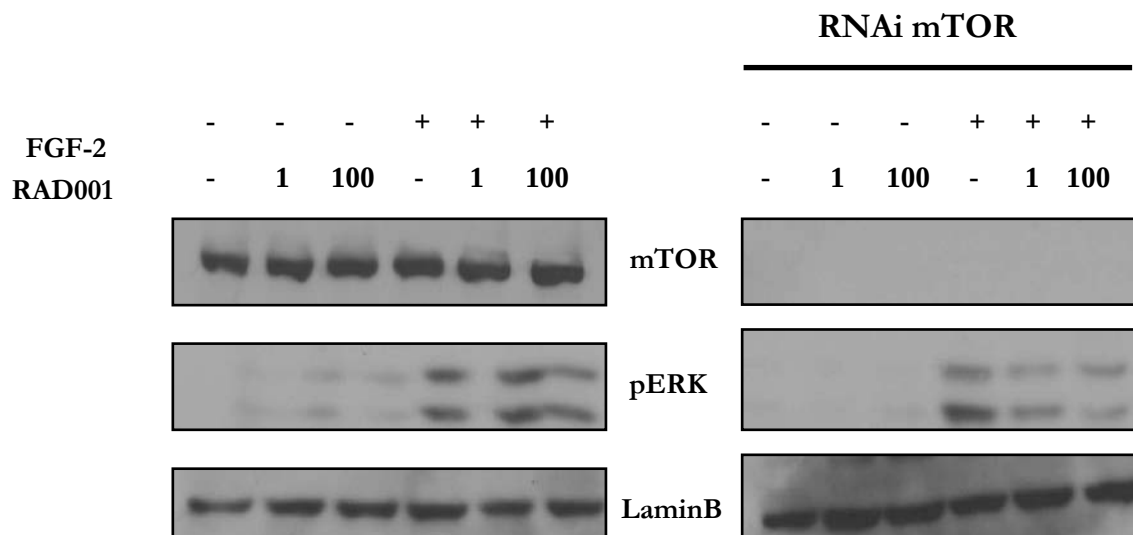


Figure 6.3 RNAi to mTOR does not prevent high dose RAD001 blocking FGF-2 stimulated phosphorylation of ERK1/2 in MCF-7 cells.

MCF-7 cells transfected with pooled siRNAi targeting mTOR 48 hours before incubation for 1 hour without (-) or with RAD001 at low (1ng/ml) or high (100ng/ml) concentrations. The cells were then exposed (+) or not (-) to 1ng/ml FGF-2. Lysates were analysed by SDS-PAGE for the presence of biphospho-ERK1/2. Lamin B detection was used as a loading control. Results are representative of 3 repeat experiments.

expressed phosphorylated ERK1/2. This was observed in all experiments including those involving specificity and dose-response of FKBP12 siRNAi oligonucleotides.

VI.2.3 RNAi to mTOR does not prevent high dose RAD001 blocking FGF-2 stimulated phosphorylation of ERK1/2 in MCF-7 cells.

The next step was to determine if knock down to mTOR itself can prevent FGF-2 induced phosphorylation of biphospho-ERK1/2. If so, that could imply that this mechanism directly involves mTOR. MCF-7 cells were set up as described before. The specificity and optimum concentration for 'knock-down' of mTOR in MCF-7 cells was determined. We performed dose responses to determine the minimum amount of pooled siRNA required to achieve silencing and compared to a non-specific set of targeting sequences. We then deconvoluted the pooled siRNA into 4 individual siRNAs and observed that 3 out of the 4 targeting sequences achieved very similar knockdown of mTOR protein. (data not shown). In the right panel, the cells were prior transfected with RNAi oligonucleotides to mTOR for 48 hours. Blots were probed for biphospho-ERK1/2, mTOR and Lamin B followed by SDS PAGE analysis. Figure 6.3 (left panel) shows the non-transfected cells and as expected the reduction in the FGF-2 induction (5 minute stimulation) of phosphorylation of biphospho-ERK1/2 with 100ng/ml RAD001. In the right panel, the cells were prior transfected with siRNAi oligonucleotides to mTOR for 48 hours. FGF-2 induction of biphospho-ERK1/2 was blocked at 100ng/ml RAD001 despite knock down of mTOR, implying that this phenomenon does not involve mTOR. However, as for FKBP12, we could not exclude the possibility that the residual amount of mTOR still present after silencing might be sufficient to mediate the effects of high concentrations of rapalogues on MEK/ERK signaling.

VI.2.4 RNAi to mTOR does not prevent high dose RAD001 blocking FGF-2 phosphorylation of S6 Kinase 1 in MCF-7 cells.

RNAi knockdown of mTOR should prevent RAD001 blocking FGF-2 phosphorylation of S6K1. To see if this is the case MCF-7 cells were prepared as described. Blots were probed for phospho-S6 Kinase 1 (Threonine 389), mTOR and Lamin B followed by SDS PAGE analysis. Figure 6.4 (left panel) shows untransfected cells, where low dose RAD001 blocks FGF-2 (10 minute stimulation) phosphorylation of S6K1. The right hand panel, where the cells have been transfected with siRNAi oligonucleotides to mTOR, RAD001 again blocks FGF-2 induced phosphorylation of S6K1. Taken together, these results suggest that knockdown of either mTOR or FKBP12 was not sufficient to prevent rapalogue action either at low or high dose. Therefore, we cannot necessarily exclude mTOR or FKBP12 from mediating the inhibition of MEK/ERK signaling by high dose rapalogues.

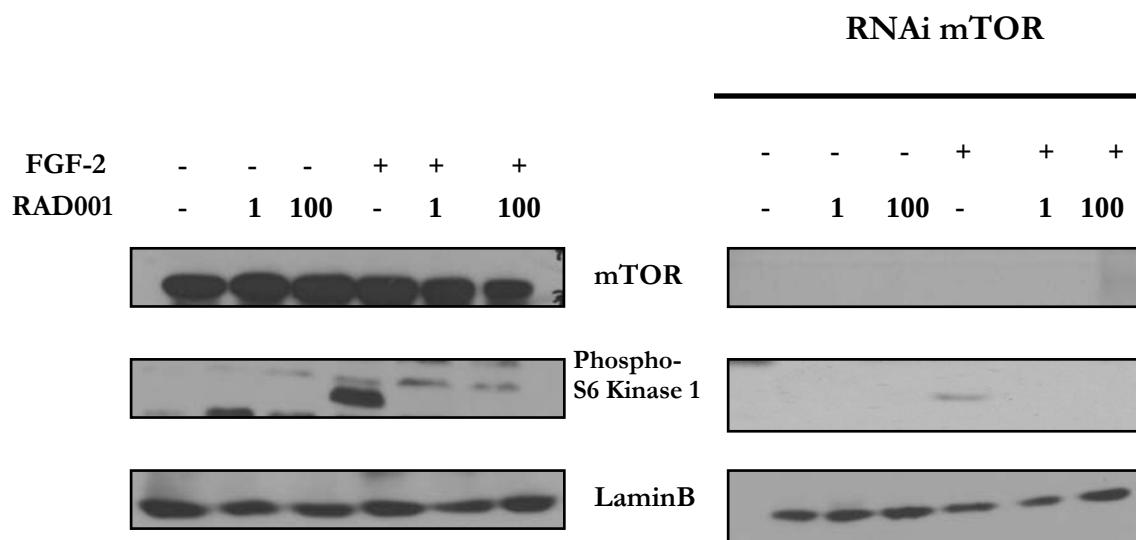


Figure 6.4 RNAi to mTOR does not prevent RAD001 blocking FGF-2 stimulated phosphorylation of S6 Kinase 1 in MCF-7 cells.

MCF-7 cells transfected with pooled siRNAi targeting mTOR 48 hours before incubation for 1 hour without (-) or with RAD001 at low (1ng/ml) or high (100ng/ml) concentrations. The cells were then exposed (+) or not (-) to 1ng/ml FGF-2. Lysates were analysed by SDS-PAGE for the presence of phosphorylated S6 Kinase 1 (Thr389). Lamin B detection was used as a loading control. Results are representative of 3 repeat experiments.

VI.2.6 Discussion

This chapter has tried to shed some light on the mechanism of how high doses of rapalogues can impair FGF-2 activation of ERK1/2 in H510 SCLC cells. FGF-2 can protect H510 SCLC cells from etoposide killing and this involves activation of MEK/ERK signaling leading to formation of a complex which upregulates certain anti-apoptotic proteins. Understanding the exact mechanism may help design novel treatments for small cell lung cancer.

We first looked at whether the intracellular receptor for rapalogues- FKBP12 has any connection with the afore mentioned phenomenon. Normally, FKBP12 combines with rapamycin and then together with the FRB domain of mTOR to block mTOR activation. If FKBP12 is knocked down by siRNA and FGF-2 induced activation of ERK1/2 is not blocked by rapalogues, then it would seem reasonable to infer that FKBP12 is involved. We showed that when FKBP12 was 'knocked down' by siRNA, RAD001 could block FGF-2 activation of ERK1/2. This result suggests that FKBP12 was not involved in rapalogues impairing FGF-2 activation of ERK1/2. Since RNAi does not completely 'knock down' protein levels, we can not rule out that low levels of FKBP12 are still present. In fact when film exposure times were increased from SDS-PAGE analysis low levels of FKBP12 were observed. Similar results were observed when we investigated whether RAD001 could block FGF-2 activation of S6K1 when FKBP12 was knocked down by RNAi. RAD001 did block S6K1 activation, which is not what we would expect if FKBP12 was completely knocked down since for rapalogues to function FKBP12 is needed. Again we could not rule out that low levels of FKBP12 were present. This makes conclusions difficult to make regarding the role of FKBP12.

We next went on to look at the role of mTOR. We showed that RNAi knock down to mTOR did not impair RAD001 blocking FGF-2 activation of ERK1/2 nor FGF-2 activation of S6K1. Conclusions were again difficult to make due to the presence of low levels of mTOR which have not been knocked down by RNAi.

An alternative to bypass this problem would be to utilize the new 'TORKinhibs' which target the ATP binding site on mTOR and hence de-activate mTOR and do not require FKBP12 to function to determine if signaling through mTOR is essential in reducing FGF-2 activation of ERK1/2. To determine whether the RAD001 inhibitory effect on MEK/ERK signaling was specifically due to RAD001 inhibition of mTORC1 signaling, a mTOR mutant could have been used which has reduced binding affinity for RAD001/rapamycin-FKBP12 complex (Ser²⁰³⁵→I1e)(Brunn et al., 1997). This mutant is then RAD001/rapamycin resistant, but still would maintain mTORC1 signaling.

Phosphorylation of biphospho-ERK1/2 is mediated by a single kinase, MEK1 (Zhou et al., 2002), it could be that RAD001 is acting not on ERK1/2 but 'above' in the signaling pathway at MEK1. Previous work from this laboratory showed that in H510 SCLC cells, FGF-2 activation of ERK1/2 could be blocked by using a MEK inhibitor, PD098059, at a dose of 25 μ M (Pardo et al., 2001). To further clarify the role of RAD001/rapamycin, we would have liked to have seen if these drugs could block activation of MEK1. In the Houghton groups work, rapamycin did not inhibit IGF-I induced phosphorylation of MEK (Harwood et al., 2008).

Following the previous experiments, it seems that mTOR and FKBP12 are not involved in high dose mTOR inhibitors blocking FGF-2 activation of MEK/ERK signaling in MCF-7 cells. The next step would be to see if the proposed mechanism of Houghton et al (Harwood et al., 2008), that mTORC1 modulates the phosphorylation of biphospho-

ERK1/2 directly or indirectly through regulation of the phosphatase PP2 (PP2A), is similar in MCF-7 cells. Okadiac acid, at relatively low doses is a relatively specific inhibitor of PP2; in preliminary experiments (data not shown) we showed that okadiac acid reversed RAD001 inhibition of FGF-2 activation of ERK1/2. At the concentrations of 10nM-300nM, biphospho-ERK was not phosphorylated in the absence of FGF-2 activation i.e. this is not a consequence of MEK1 activation by inhibiting PP2 under these conditions. This suggests that FGF-2 stimulation inactivates PP2, and this inactivation is prevented by RAD001, hence it is mTORC1 dependent. These results fit in to the premise that PP2 is involved in the rapid deactivation of the MAPK pathway (Heriche et al., 1997; Zhou et al., 2002). To clarify these results we could next use another PP2 inhibitor such as calyculin A, in similar experiments. However unlike okadiac acid, calyculin A does also inhibit protein phosphatase 1 (PP1)(Peterson et al., 1999).

It would seem phosphatase regulation may be more useful than kinase regulation, when rapid dephosphorylation is needed at multiple sites, under the control of many different kinases. Hence activation of a phosphatase may allow dephosphorylation of many sites without having to inhibit all of the kinases responsible for their phosphorylations. It would be interesting to know if other PI3K family members use phosphatases in a similar way.

CHAPTER VII

Combination studies of mTOR and cytotoxic chemotherapy in SCLC cells

VII.1 Introduction

Combination drug treatment regimens in cancer, acquired immune deficiency syndrome (AIDS) and tuberculosis often achieve a greater therapeutic efficacy than that achieved with monotherapy. Previous work from this thesis has potentially shown that in SCLC cells, mTOR inhibitors can block a growth factor induced chemoresistance pathway. This leads to the question of whether mTOR inhibitors when added to cytotoxic chemotherapy produce additive, synergistic or inhibitory effects. It is possible that sequencing of the drugs may effect whether additive/synergistic as opposed to antagonistic effects are seen. This would appear to be pertinent here since mTOR inhibitors arrest the cell cycle (G1) (Cafferkey et al., 1994; Fingar et al., 2004) and etoposide, a semisynthetic podophyllotoxin-derived antineoplastic agent used in SCLC, is active when cells are in cycle and killing cells in G2 and S phases (Hande, 1992; Joel et al., 1994). Theoretically at least it would seem that administering the two drugs at the same time may lead to antagonistic effects. Instead, it might seem logical to treat with the mTOR inhibitor after etoposide to prevent regrowth of cells between cycles of chemotherapy.

These questions are important to ask at the in vitro level since if drug combinations lead to clinical trials be that phase II or III, determining the ideal sequence in patients is difficult at these stages because there is little flexibility allowed in these trials. The real problem here is that clinical trials are expensive and exploring sequencing of drugs is often thought by the pharmaceutical industry, whether rightly or wrongly, to not be very cost-effective.

The success of drug combination in the clinic may be aided by pre-clinical studies examining sequencing. (Chang et al., 2006; Chou & Dong, 2005; Chou, 1998).

Studies to answer these types of questions have been researched and validated by Ting-Chao Chou of the Memorial Sloan Kettering Cancer Center in the United States. His derivation of

the median effect equation from the law of mass action has been extensively used in combination studies in well over 2600 papers (Chou, 2006).

This equation describes dose-effect relationships in the simplest form:

$$f_a/f_u = (D/D_m)^m$$

Where D is the dose (concentration) of a drug, f_a is the fraction affected by D (i.e. percentage inhibition/100), and f_u is the fraction unaffected (i.e., $f_u = 1 - f_a$). D_m is the median-effect dose (IC_{50}) that inhibits the system under study by 50% and m is the coefficient signifying the shape of the dose-effect relationship, where $m=1$, >1 , and <1 to indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curves, respectively (Chou, 1976). Chou describes this equation as the general theory of dose and effect.

This equation can be extended to any drug system, for example for first order system i.e. $m=1$:

$$(f_a)_{1,2}/(f_u)_{1,2} = (f_a)_1/(f_u)_1 + (f_a)_2/(f_u)_2 = (D)_1/(D_m)_1 + (D)_2/(D_m)_2$$

In 1983 Chou and Talalay introduced the term combination index (CI) for determination of the synergism or antagonism for two drugs (Chou et al., 1983):

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 = (D)_1/(D_m)_1 + (D)_2/(D_m)_2 \{f_a/(1-f_a)\}^{1/m_2}$$

Where $CI < 1$, $= 1$, and > 1 indicate synergism, additive effect, and antagonism, respectively.

The prerequisite for synergism or antagonism determination is to know both the potency and the shape of the dose-effect curve for each drug alone and for the two drug combinations. From the individual drug dose-response curves IC_{50} s are determined and are used in fixed dose and equipotency combinations so that the contributions of effects of each drug are roughly equal as described in the methods section. This method allows for reduced numbers of data points and allows for maximal amount of useful information.

From these equations Chou developed software to allow for automated analysis of data. CalcuSyn (Chou & Hayball, 1997) generates graphics and can handle data analysis of large-scale drug combination studies.

In this chapter we studied the combination of the mTOR inhibitor RAD001 with etoposide or cisplatin in two SCLC cell lines- H510 and H69. Etoposide and cisplatin have clinical activity in SCLC and with mTOR inhibitors have no overlapping toxicities, important when administered to patients. The hypothesis is that the combination of an mTOR inhibitor and cytotoxic chemotherapy (cisplatin or etoposide) will be able to elicit more cell killing. However with the drugs acting at different points of the cell cycle, it is important to know in what sequence these drugs work best.

Previous work from this laboratory and a collaborator showed that H-69 SCLC cells were sensitive to RAD001 whilst H510 SCLC cells were more resistant and that when combined together with etoposide the H-69 cells showed at least additive effects whilst H-510 cells did not show any obvious benefit of the combination. However, these studies were rather preliminary as they: (1) did not perform formal isobologram/combination analysis, (2) only looked at etoposide and not cisplatin and (3) did not assess the question of sequencing of the drugs (Marinov, 2009).

VII.2 RESULTS

VII.2.1 Dose response curves for H69 and H510 for cisplatin, etoposide and RAD001

The cytotoxicities of cisplatin, etoposide and RAD001 in 2 small cell lung cancer cell lines- H69 and H510 are shown in figures 7.1 and 7.2. The results were obtained from dose response curves plotted using Calcosyn software. Cells (H69/H510) were seeded in 24 well plates and treated with varying concentrations as described of RAD001 or cisplatin or etoposide. After 4 days of treatments, growth inhibition was measured by cell counting. IC₅₀'s obtained (Table 7.1) from these curves were used in subsequent combination study experiments.

Cell Line	Cisplatin IC₅₀ μM (mean $\pm$ SEM)	Etoposide IC₅₀ μM (mean $\pm$ SEM)	RAD001 IC₅₀ ng/ml (mean $\pm$ SEM)
H69	193 $\pm$ 12	0.48 $\pm$ 0.04	123 $\pm$ 8
H510	141 $\pm$ 21	0.12 $\pm$ 0.02	95 $\pm$ 11

Table 7.1 In vitro growth inhibitory effect of cisplatin, etoposide and RAD001 in human SCLC cell lines.

SCLC cells were seeded at 1,500 per well in 24-well plates; at 24 hours after seeding, cells were treated with varying concentrations of RAD001, cisplatin or etoposide. After 4 days of treatments, growth inhibition was measured by cell counting. The experiments were performed in triplicate. Results were plotted on graphs by using Calcosyn software and used to determine respective inhibitory concentration IC₅₀ for combination studies. Each value is the mean $\pm$ Standard Error of the Mean (SEM) of the IC₅₀ of three or four independent experiments.

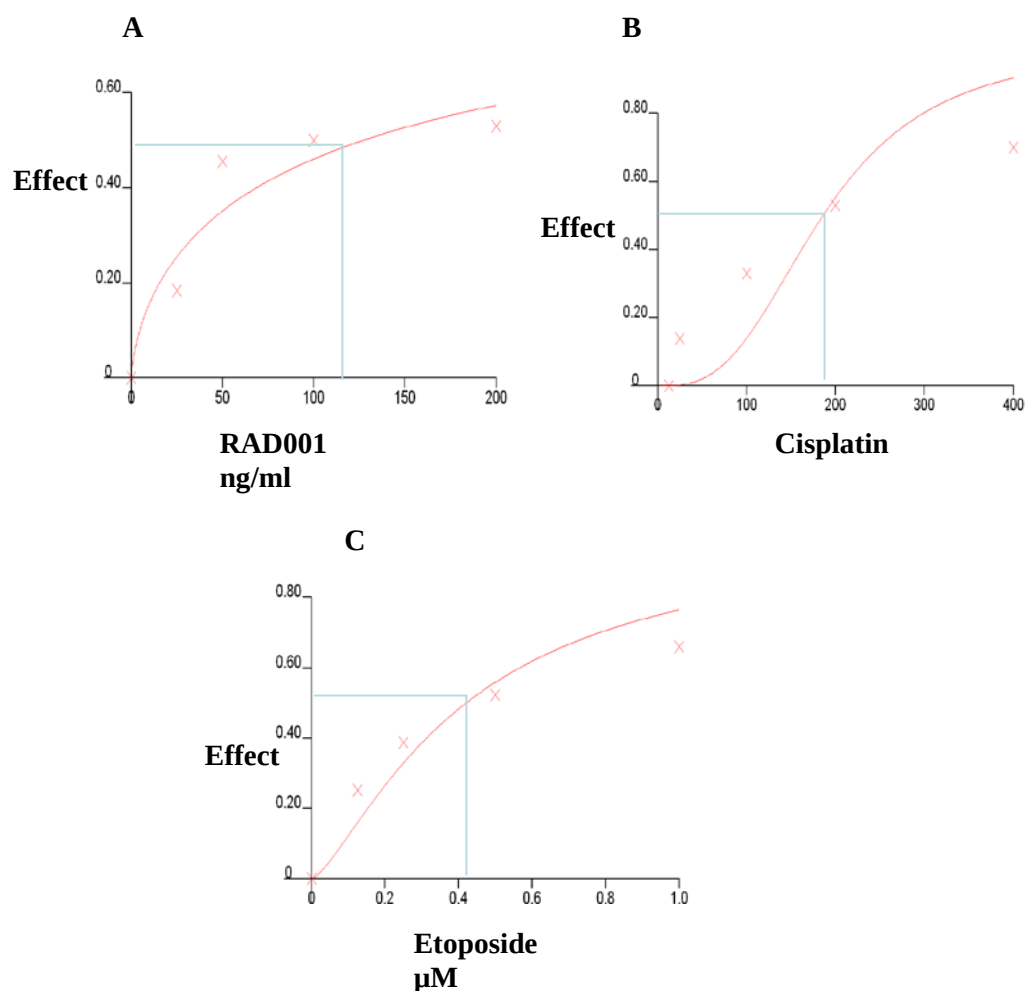


Figure 7.1 H69 SCLC cells and dose-response effects with RAD001, Cisplatin and Etoposide.

The H69 SCLC cells were seeded (1,500 per well) in 24-well plates; at 24 h after seeding, cells were treated with varying concentrations of RAD001(A) or Cisplatin (B) or etoposide (C). After 4 days of treatments, growth inhibition was measured by cell counting. Blue lines indicate IC50. The results shown are the mean of three independent experiments with each condition performed in triplicate $\pm$ the SEM.

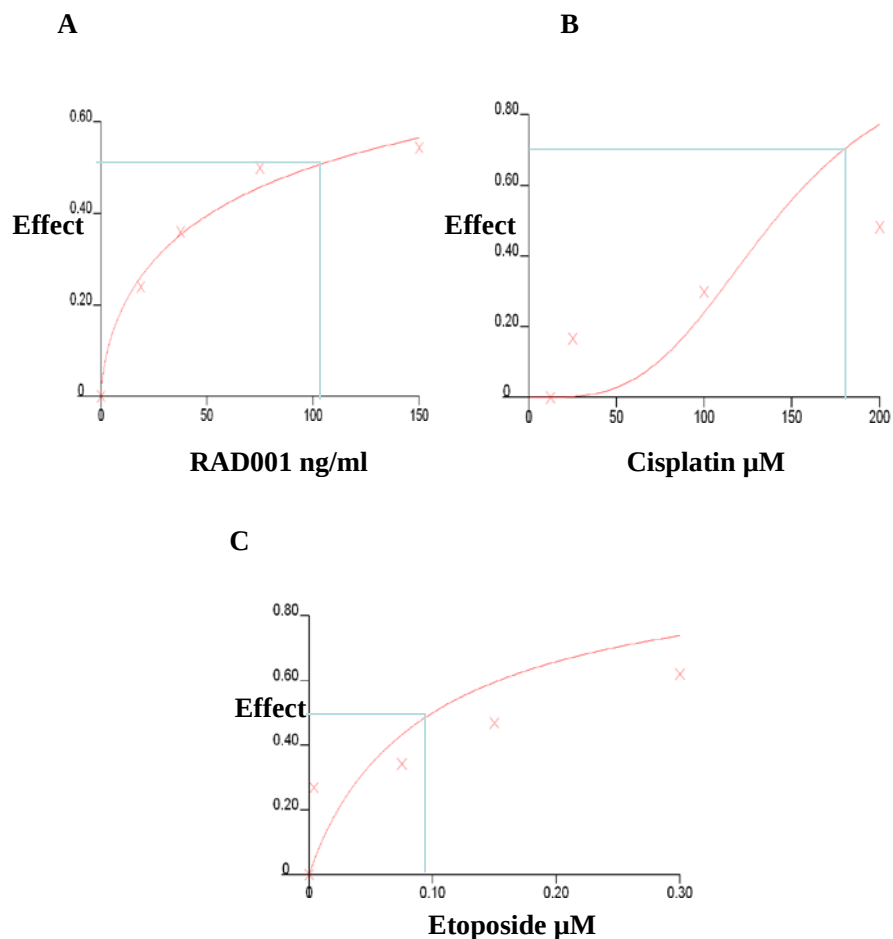


Figure 7.2 H510 SCLC cells and dose-response effects with RAD001, Cisplatin and Etoposide.

The H510 SCLC cells were seeded (1,500 per well) in 24-well plates; at 24 h after seeding, cells were treated with varying concentrations of RAD001(A) or Cisplatin (B) or etoposide (C). After 4 days of treatments, growth inhibition was measured by cell counting. Blue lines indicate IC₅₀. The results shown are the mean of three independent experiments with each condition performed in triplicate $\pm$ the SEM.

VII.2.2 Combination studies of H69 SCLC with RAD001 and Etoposide.

Next we wanted to find out the effect of combining each cytotoxic agent with RAD001 and determine the most effective schedule. To determine synergy, additivity or antagonism of combinations of these drugs the isobologram/combination index method was used which relies upon the calculation of the fixed dose combination that causes a given effect. We chose the following fixed dose combinations: IC_{12.5}, IC₂₅, IC₅₀, IC₁₀₀ and IC₂₀₀ i.e. the IC_{12.5} of etoposide in H69 cells is administered with the IC_{12.5} of RAD001 in H69 cells. The IC₅₀'s were determined from the previous experiments. Each experiment generated a set of combination index (CI) values for a particular effect. The CI was used to determine additivity, synergy, or antagonism of the combination depending on interaction index = 1, <1, or >1, respectively.

Figure 7.3 shows the combined effect of etoposide administered to H69 cells 2 days before RAD001 (A), etoposide and RAD001 given simultaneously (B) and RAD001 2 days before etoposide (C). Table 7.2 shows the CI's for each fixed dose.

Fixed Dose	CI Etoposide before RAD001	CI Etoposide and RAD001 simultaneously	CI RAD001 before Etoposide
IC _{12.5}	<1	<1	1
IC ₂₅	<1	<1	<1
IC ₅₀	1	1	<1
IC ₁₀₀	>1	>1	<1
IC ₂₀₀	>1	>1	>1

Table 7.2 CI values of combinations of etoposide and RAD001 in H69 SCLC cells. Additivity, synergy, or antagonism of the combination depends on the interaction index = 1, <1, or >1, respectively.

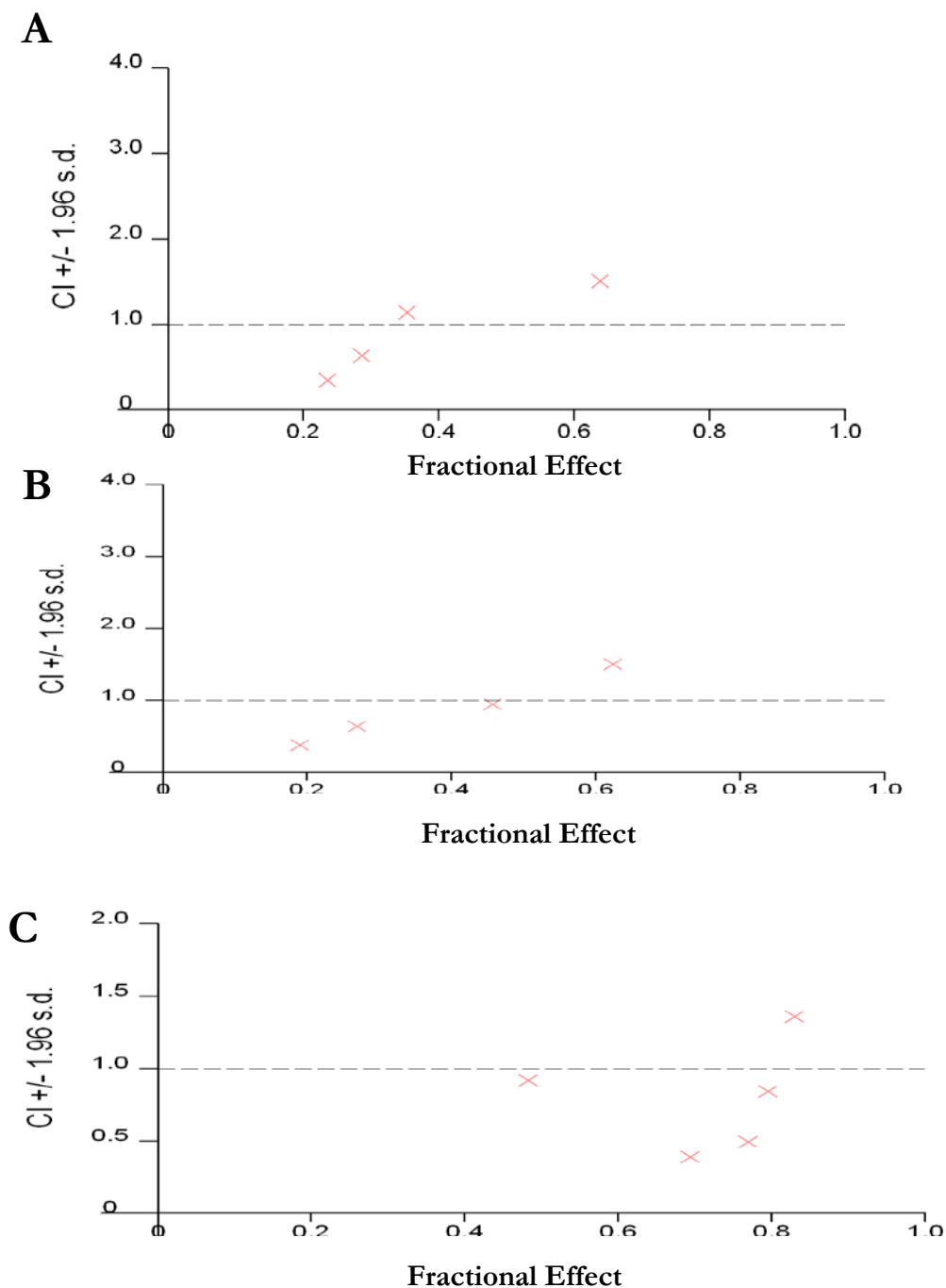


Figure 7.3 Combination studies of H69 SCLC with RAD001 and Etoposide over 4 days.

(A) Etoposide 2 days before RAD001, (B) Etoposide and RAD001 given simultaneously and (C) RAD001 2 days before Etoposide. H69 cells in serum free media were seeded in 12 well plates, five fixed drug ratios above and below the IC_{50} were explored by incubating the drug combinations with cells for 4 days and then determining the degree of cytotoxicity by cell counting with trypan blue exclusion was used to assess cell viability. The combination index (CI) was used to determine additivity, synergy, or antagonism of the combination depending on interaction index = 1, <1, or >1, respectively. The results shown are the mean of three independent experiments performed in triplicate.

From these results when etoposide is administered to H69 SCLC cells before RAD001, low fixed dose combinations are synergistic whilst higher doses are additive (IC_{50}) and higher still is antagonistic. A similar pattern is seen when the two drugs are given at the same time. However when RAD001 is administered before etoposide; low fixed dose combination ($IC_{12.5}$) is additive, higher combinations are synergistic until the IC_{200} combination where it was found to be antagonistic.

VII.2.3 Combination studies of H510 SCLC with RAD001 and Etoposide

Next we performed similar studies using etoposide and RAD001, with another SCLC cell line- H510.

Figure 7.4 shows the combined effect of etoposide administered to H510 cells 2 days before RAD001 (A), etoposide and RAD001 given simultaneously (B) and RAD001 2 days before etoposide (C). Table 7.3 shows the CI's for each fixed dose.

Fixed Dose	CI Etoposide before RAD001	CI Etoposide and RAD001 simultaneously	CI RAD001 before Etoposide
$IC_{12.5}$	<1	>1	>1
IC_{25}	1	>1	>1
IC_{50}	>1	>1	>1
IC_{100}	>1	>1	>1
IC_{200}	>1	>1	>1

Table 7.3 CI values of combinations of etoposide and RAD001 in H510 SCLC cells. Additivity, synergy, or antagonism of the combination depends on the interaction index = 1, <1, or >1, respectively.

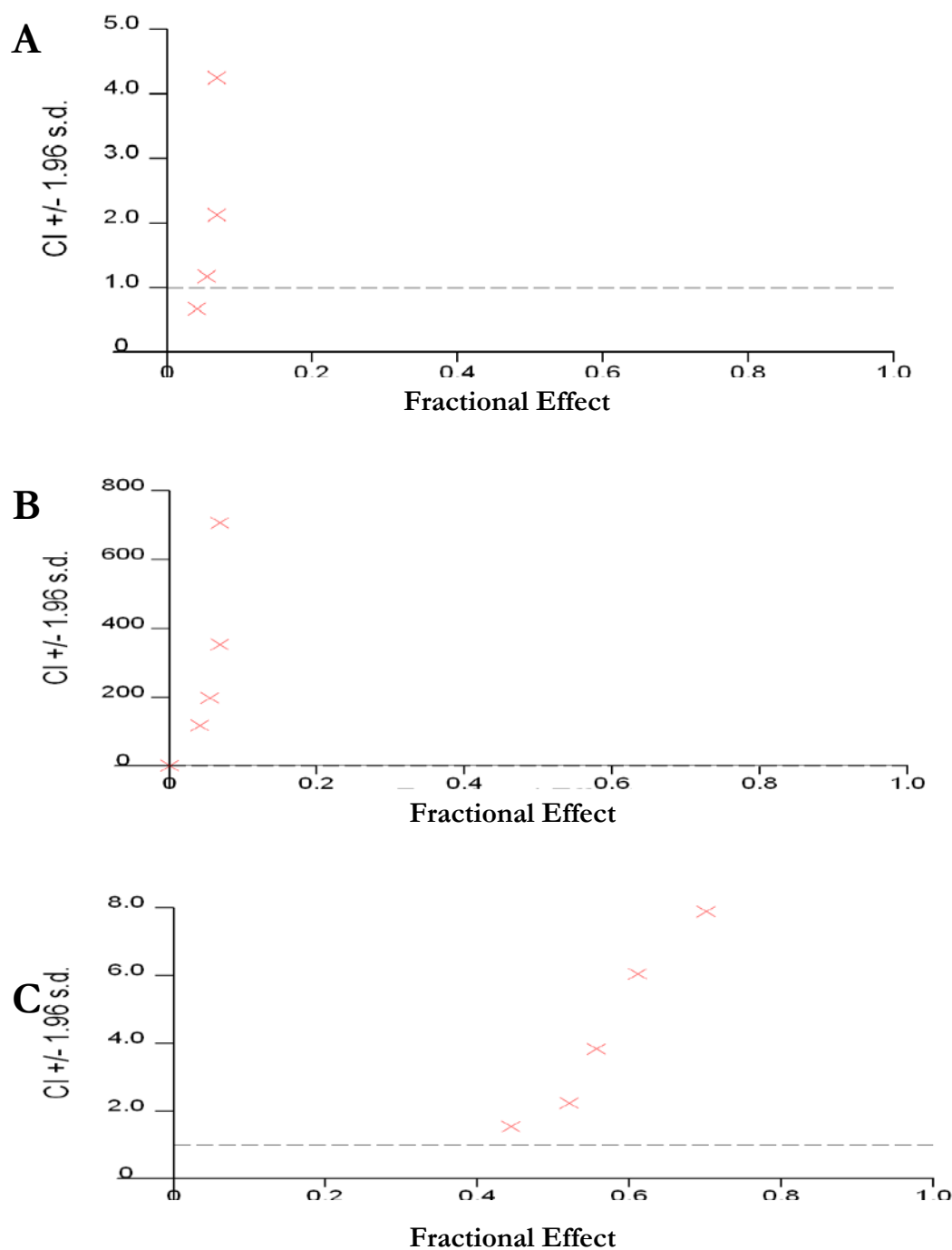


Figure 7.4 Combination studies of H510 SCLC with RAD001 and Etoposide.

(A) Etoposide 2 days before RAD001, (B) Etoposide and RAD001 given simultaneously and (C) RAD001 2 days before Etoposide. H510 cells in serum free media were seeded in 12 well plates, five fixed drug ratios above and below the IC_{50} were explored by incubating the drug combinations with cells for 4 days and then determining the degree of cytotoxicity by cell counting with trypan blue exclusion was used to assess cell viability. The combination index (CI) was used to determine additivity, synergy, or antagonism of the combination depending on interaction index = 1, <1, or >1, respectively. The results shown are the mean of three independent experiments performed in triplicate.

From these results administering etoposide 2 days before RAD001, low doses are synergistic or additive ($IC_{12.5}$ and IC_{25}) whilst higher dose concentrations when combined lead to antagonistic effects. When etoposide and RAD001 are given together and when RAD001 was given before etoposide at all doses examined antagonistic effects were only seen. Our previous paper (Marinov, 2009) showed that the H510 cells are not sensitized to etoposide killing by RAD001 is largely true except perhaps at low dose pairs when RAD001 is given before etoposide.

VII.2.4 Combination studies of H69 SCLC with RAD001 and Cisplatin.

Another drug commonly used in clinical practise to treat SCLC is cisplatin. We wanted to see the effects of combining cisplatin and RAD001. We started with the H69 SCLC cell line. Figure 7.5 shows the combined effect of cisplatin administered to H69 cells 2 days before RAD001 (A), cisplatin and RAD001 given simultaneously (B) and RAD001 2 days before cisplatin (C). Table 7.4 shows the CI's for each fixed dose.

Fixed Dose	CI Cisplatin before RAD001	CI Cisplatin and RAD001 simultaneously	CI RAD001 before Cisplatin
$IC_{12.5}$	<1	>1	<1
IC_{25}	<1	>1	<1
IC_{50}	<1	>1	<1
IC_{100}	<1	<1	<1
IC_{200}	<1	<1	<1

Table 7.4 CI values of combinations of cisplatin and RAD001 in H69 SCLC cells. Additivity, synergy, or antagonism of the combination depends on the interaction index = 1, <1, or >1, respectively.

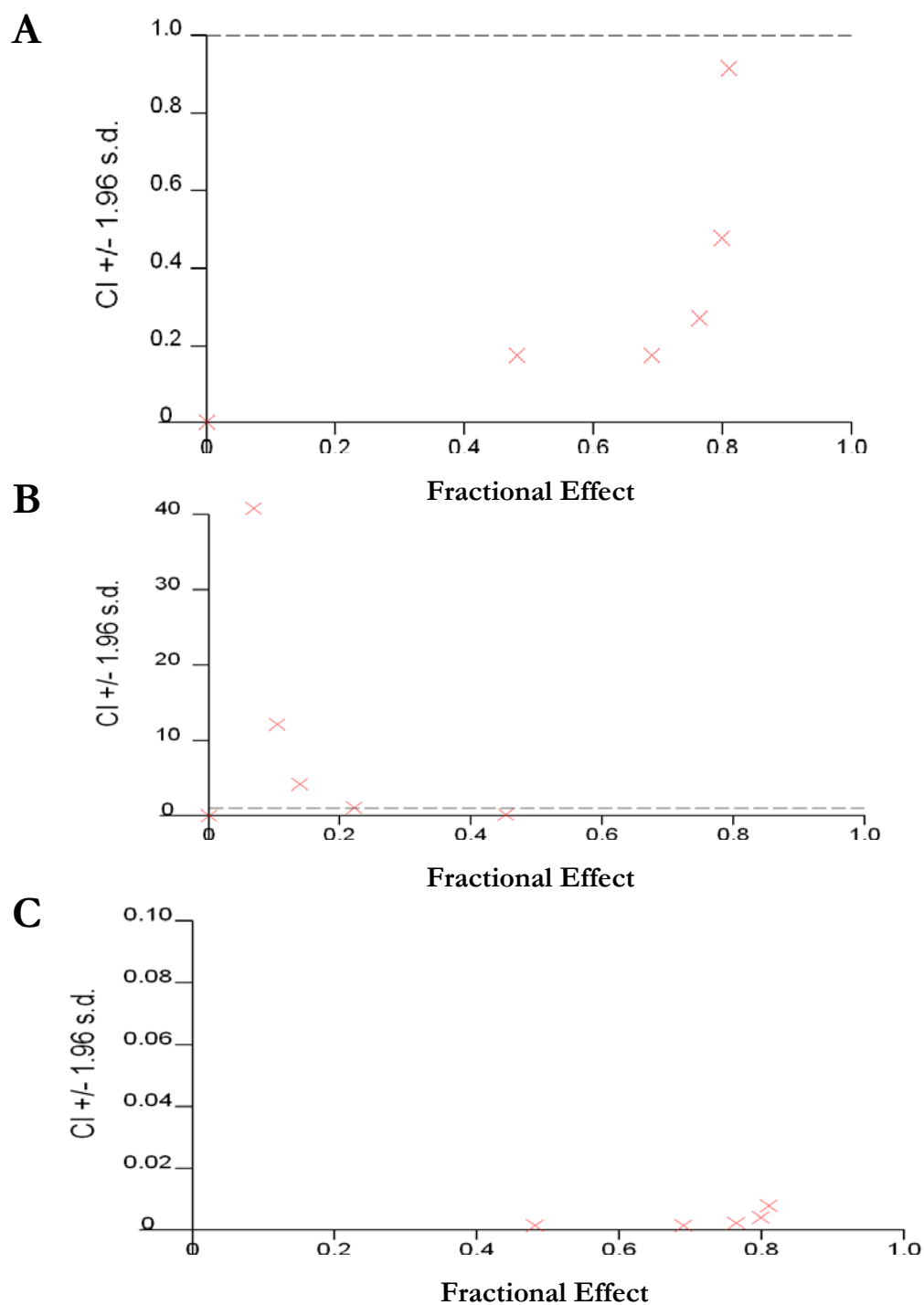


Figure 7.5 Combination studies of H69 SCLC with RAD001 and Cisplatin.

(A) Cisplatin 2 days before RAD001, (B) Cisplatin and RAD001 given simultaneously and (C) RAD001 2 days before Etoposide. H69 cells in serum free media were seeded in 12 well plates, five fixed drug ratios above and below the IC_{50} were explored by incubating the drug combinations with cells for 4 days and then determining the degree of cytotoxicity by cell counting with trypan blue exclusion was used to assess cell viability. The combination index (CI) was used to determine additivity, synergy, or antagonism of the combination depending on interaction index = 1, <1, or >1, respectively. The results shown are the mean of three independent experiments performed in triplicate.

From these results in H69 cells, giving cisplatin before RAD001 leads to synergistic effects. Low doses of cisplatin and RAD001 when given simultaneously are antagonistic whilst higher doses (IC_{100} and IC_{200}) lead to synergistic effects. RAD001 when given 2 days before cisplatin at all doses examined, synergistic effects were observed.

VII.2.5 Combination studies of H510 SCLC with RAD001 and Cisplatin.

We next examined whether similar effects were seen with cisplatin and RAD001 in the H510 cell line. Figure 7.6 shows the combined effect of cisplatin administered to H510 cells 2 days before RAD001 (A), cisplatin and RAD001 given simultaneously (B) and RAD001 2 days before cisplatin (C). Table 7.5 shows the CI's for each fixed dose.

Fixed Dose	CI Cisplatin before RAD001	CI Cisplatin and RAD001 simultaneously	CI RAD001 before Cisplatin
$IC_{12.5}$	<1	>1	>1
IC_{25}	<1	>1	>1
IC_{50}	<1	<1	1
IC_{100}	<1	<1	<1
IC_{200}	<1	<1	<1

Table 7.5 CI values of combinations of cisplatin and RAD001 in H510 SCLC cells. Additivity, synergy, or antagonism of the combination depends on the interaction index = 1, <1, or >1, respectively.

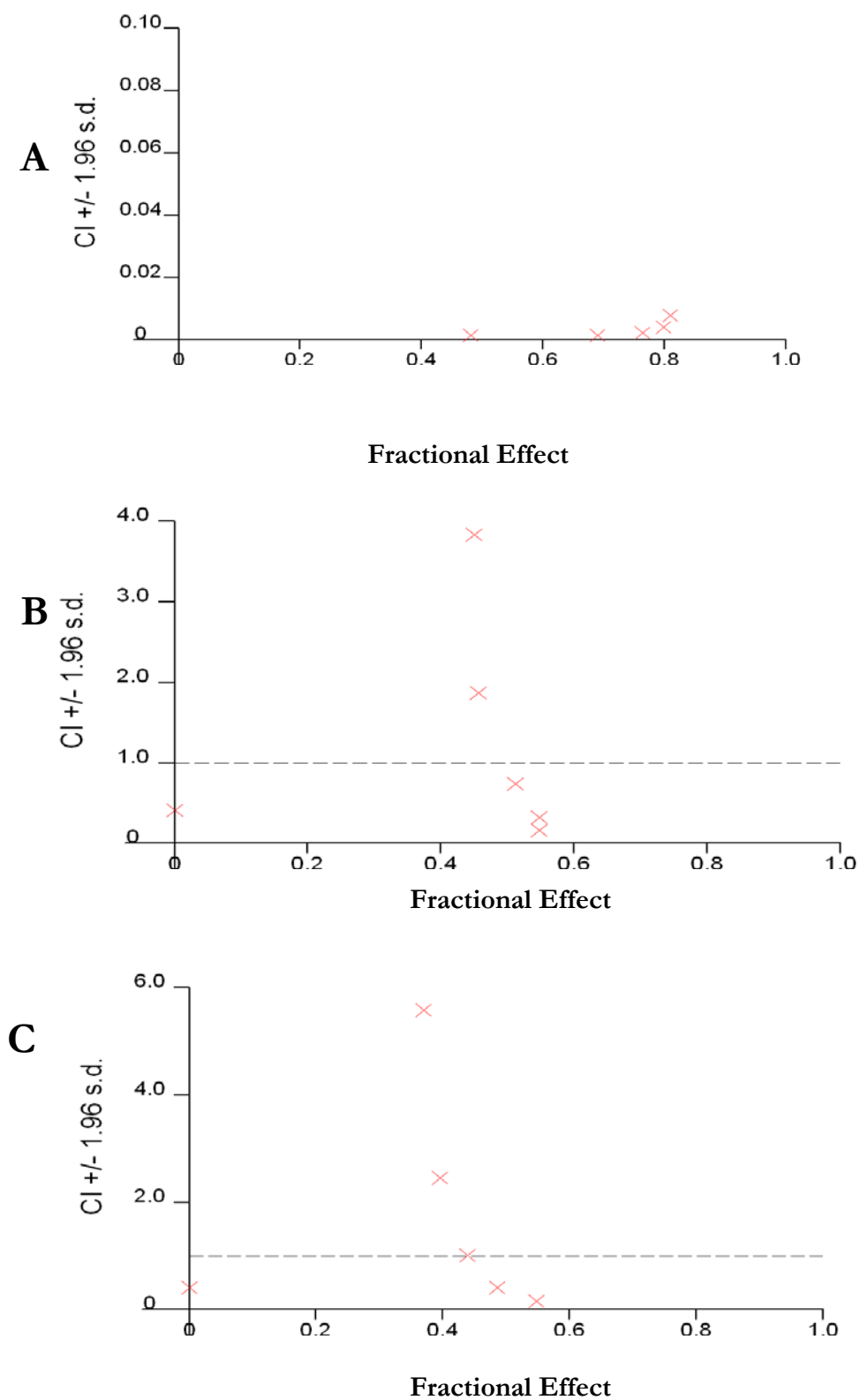


Figure 7.6 Combination studies of H510 SCLC with RAD001 and Cisplatin.

(A) Cisplatin 2 days before RAD001, (B) Cisplatin and RAD001 given simultaneously and (C) RAD001 2 days before Cisplatin. H69 cells in serum free media were seeded in 12 well plates, Cisplatin and RAD001 at 12.5%, 25%, 50%, 100% and 200% of their respective IC_{50} s were added to the wells in fixed dose combinations. Cell counting with trypan blue exclusion was used to assess cell viability. The results shown are the mean of three independent experiments performed in triplicate.

In H510 cells drug combination studies of cisplatin and RAD001, cisplatin given before RAD001, all doses examined led to synergistic effects. When the drugs were given simultaneously low dose drug combinations are antagonistic whilst higher doses (IC_{100} and IC_{200}) are synergistic. Similar results were observed when RAD001 was given before cisplatin except when the drugs were combined at the IC_{50} where additive effects were seen.

VII.3 Discussion

We studied the effects of combinations of etoposide or cisplatin and the mTOR inhibitor RAD001 in two SCLC cell lines- H69 and H510. To do this we used the tried and tested fixed dose isobologram/combination index method of Chou (Chou, 1998). We also examined the scheduling of two drug combinations.

To summarize when etoposide and RAD001 are combined in H69 SCLC mostly synergistic effects are seen regardless of the schedule in contrast with H510 SCLC cells where mostly antagonistic effects were observed.

When cisplatin was exchanged for etoposide, in H69 cells again synergistic effects were observed. In the H510 cell line a different picture was seen when cisplatin was given before RAD001 only synergism was seen. Synergism was only seen at higher doses when cisplatin was given simultaneously with RAD001 and RAD001 given before cisplatin.

It seems from these results that in the H69 cell line scheduling of the drugs does not seem to matter with regard to response. H510 cells are not very responsive to the etoposide /RAD001 combination and more sensitive to the cisplatin/RAD001 combination. How can these observations be explained? Previous work confirms the sensitivity of H69 compared to H510 with respect to etoposide/RAD001, at least when the drugs are given at the same time (Marinov, 2009). Another study using the mTOR inhibitor CCI-779, SCLC were sensitized to cisplatin induced apoptosis (Wu et al., 2005). RAD001 seems to induce apoptosis, as evidenced by induction of caspase-3, Poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase-3- activated DNase (IACD) cleavage in H69 cells but not in H510 cells. mTOR inhibitors induce autophagy (Blommaert EF, 1995; Kamada Y, 2000) and in this afore mentioned study, an autophagy inhibitor L 3-methyladenine could block the cytotoxic effects of RAD001 alone and the combination of etoposide and RAD001 (Marinov, 2009).

Our results are somewhat surprising with respect to scheduling of the drugs in the cell lines. When H69 cells were used regardless of cytotoxic agent used and scheduling, most of the effects seen were additive or synergistic, whilst in H510 cells synergistic effects were seen with cisplatin and RAD001 regardless of scheduling and antagonistic effects seen with etoposide and RAD001. The scheduling does not seem to matter but more so the drug, namely etoposide or cisplatin or the cell line i.e. H69 or H510.

We have mentioned in the introduction that relatively high doses of rapamycin can block FGF-2 induced chemoresistance to etoposide (Pardo et al., 2001), in our studies here when RAD001 was administered 2 days before etoposide in H510 SCLC cells only antagonistic effects were observed, somewhat counter intuitive to the previous data. No growth factors were present in the media so maybe in vivo growth factor/FGF-2 activation of chemoresistance mechanism may give a different result.

So how do these results help us with further studies? These results suggest that different types of small cell lung cancer respond in different ways to combination treatment at least at the in vitro level. Further studies are needed to understand the molecular mechanisms for these differences which as mentioned above are being elucidated.

Ideally combination chemotherapy for lung cancer should take advantage of synergistic interactions, as these would enhance therapeutic efficacy and lower the risk of chemoresistance. If the drugs in combination are antagonistic, the efficacies of such regimens might be compromised and the chances of resistance and spread increase, as less effective drugs may be allowing weakly resistant clones to survive. It is difficult to predict in vivo drug interactions in humans on the basis of findings in vitro studies, although the findings from studies with animal models may be more predictive (Chou, 1998).

Further work leading to animal studies would include using other SCLC cell lines, combinations of etoposide, cisplatin and RAD001 together and changing the number of days between the administrations of the drugs.

CHAPTER VIII

Final discussion and future directions

In the course of this thesis, I have attempted to highlight the role of mTOR in the biology of lung cancer. We have focused on certain aspects: The expression of mTOR in early stage NSCLC and its link to prognosis and the expression of mTOR in SCLC; the role of mTOR and mTOR inhibitors/rapalogues in chemoresistance to the cytotoxic drug etoposide in SCLC and its potential role in NSCLC, and finally the effect of combinations and scheduling of mTOR inhibitors and cytotoxic chemotherapy in SCLC. Our findings have potentially exciting implications.

1. Overexpression of mTOR in early NSCLC cases.

Our study, looking at 134 cases of surgically treated NSCLC, showed that overexpression of mTOR was linked to prognosis. Clearly this study needs repeating in at least another cohort; however if these results are reproduced-studies are clearly needed to look at the use of mTOR inhibitors and or cytotoxic chemotherapy as adjuvant therapy in these early cases that overexpress mTOR. It may be that overexpression of mTOR leads to increased sensitivity to mTOR inhibitors. Stage 1A NSCLC patients after surgery only have a 70% chance to be alive after 5 years (Tsuchiya et al., 2007) so any way of increasing this percentage would be welcome. If these results are repeated and consistent in other lung cancer cohorts, it would seem reasonable to think about phase III clinical trials looking at mTOR inhibitors and/or chemotherapy as adjuvant treatment in early NSCLC patients that overexpress mTOR.

The supplementary study looking at how phospho-signals deteriorate with time from when cells, formalin fixed cells and tissue are removed from media/blood supply led us to use total mTOR expression in our formal study on the 134 early NSCLC cases. Nearly all the papers published looking at phospho-proteins in immunohistochemistry do not cite at what

time point the samples were fixed in formalin. This study again needs to be repeated in other tumour types and phospho-proteins. The potential implication of this particular aspect of this work is large since immunohistochemistry of phospho-protein studies in cancer are common and many conclusions drawn from these studies. In the future, immunohistochemistry studies using tissue looking at phospho-proteins must state at what time point specimens were fixed in formalin.

2. The role of mTOR and mTOR inhibitors in chemoresistance to etoposide in SCLC

We confirmed that high doses of the clinically relevant mTOR inhibitor RAD001 could block a FGF-2 induced chemoresistance pathway in SCLC cells. This is a potentially novel therapeutic management strategy. Chemoresistance is the ultimate cause of therapeutic failure in SCLC, in particular to etoposide and cisplatin, which together are the standard first line treatments in SCLC. Any way to subvert chemoresistance is greatly welcome for SCLC patients, in whom no major advance in treatment has been seen for 30 years.

There could be many targets to block in this chemoresistance pathway from FGF-2 to MEK/ERK to the multiprotein complex of PKC ϵ , B-Raf and S6K2. MEK antagonists are in development however mTOR inhibitors have been around for a while including the new 'TORKinhibs' which target the ATP binding site on mTOR and have predictable and tolerable toxicities in patients. Using these drugs seems a rational approach to block a chemoresistance pathway.

Specifically targeting the FGF-2 signaling pathway, although seductive, may not be a promising strategy for the treatment of SCLC. Indeed, a whole panel of growth factors, both neuro- and polypeptide will challenge SCLC *in vivo*. The MEK/ERK and the S6K1 signaling pathways are common to many growth factors, and so it may be more attractive to

target these common molecules, such as mTOR, rather than the individual growth factors receptors.

There is some data to suggest that increased protein expression of S6K2 in SCLC and NSCLC patient biopsies appear to correlate with the development of chemoresistance (Pardo et al., 2006). We did not look at S6K2 protein expression in our early NSCLC patient biopsies, which would have been a good idea; however in the 2 NSCLC cell lines examined, growth factor induced stimulation of MEK/ERK signaling (upstream of S6K2), could not be blocked by high doses of RAD001.

First of all it is not clear if a chemoresistance pathway/complex similar to the one described in H510 SCLC cell line exists in the 2 NSCLC cell lines examined. However FGF-2 in the squamous cell NSCLC cell line HCC95 could block etoposide induced cell killing and PDB in the adenocarcinoma cell NSCLC line HCC78 could also block etoposide killing over 4 days. In the HCC78 cell line relatively high doses of RAD001 could block this observed chemoresistance. The mechanism for this needs further clarification and could be the basis of future work. PDB activates by 2nd messengers such as classical and non-classical PKCs and they in turn have many substrates so the mechanisms of chemoresistance in this particular case could be complex.

The mechanism of how high doses of mTOR inhibitors/rapalogues block growth factor induced MEK/ERK signaling was investigated. Clarification is needed on the role of mTOR and FKBP12 since RNAi knockdown experiments can never reduce protein levels to zero. This therefore makes conclusions difficult to interpret. An alternative would be to utilize the new 'TORKinhibs' which target the ATP binding site on mTOR and hence de-activate mTOR and do not require FKBP12 to function to determine if signaling through mTOR is essential in reducing growth factor activation of ERK1/2.

To determine whether the RAD001 inhibitory effect on MEK/ERK signaling was specifically due to RAD001 inhibition of mTORC1 signaling, a mTOR mutant could be used which has reduced binding affinity for RAD001/rapamycin-FKBP12 complex (Ser²⁰³⁵→I1e)(Brunn et al., 1997). This mutant is then RAD001/rapamycin resistant, but still would maintain mTORC1 signaling.

The role of PP2A was briefly investigated; our preliminary results (not shown) confirmed recent work suggesting that mTORC1 signaling could regulate growth factor activation of p44/42 MAPKs through PP2A (Harwood et al., 2008).

3. Combination studies in SCLC.

We examined at the cell line level the effect of combinations of cytotoxic chemotherapy and RAD001 in 2 SCLC cell lines and looked at what schedule was optimal. These questions are important to answer since in clinical trials it is very difficult to investigate these questions since large numbers of patients are needed and little flexibility is allowed.

Another aspect of the study was to investigate the ideal scheduling of combinations of drugs which work at different points in the cell cycle. mTOR inhibitors are cell cycle inhibitors whilst at least etoposide is active when cells are in cycle and killing cells in G2 and S phases. Surprisingly scheduling did not seem to determine response with the H69 SCLC cell line- synergistic effects were seen with etoposide and cisplatin, whilst in H510 SCLC cells, etoposide and RAD001 regardless of scheduling led to antagonistic effects and only high doses of cisplatin and RAD001 give synergistic effects.

These studies need to be repeated and in other SCLC cell lines. Studies in animal models need to be performed as these are often quoted as being more representative of 'real' patients than in vitro work.

Final Comments

This work has allowed us to investigate the role of mTOR in lung cancer. Much work is still required to understand in greater detail mTOR's involvement in the biology of lung cancer.

This work suffers from the limitations imposed by all in vitro work: only in vivo experiments in animal models could confirm the in vitro work. Therefore, this work should only be viewed as the starting point on which further work will be based.

REFERENCES

- Akashi, T., Ito, E., Eishi, Y., Koike, M., Nakamura, K. & Burgeson, R.E. (2001). Reduced expression of laminin alpha 3 and alpha 5 chains in non-small cell lung cancers. *Jpn J Cancer Res*, **92**, 293-301.
- Alberola, V., Camps, C., Provencio, M., Isla, D., Rosell, R., Vadell, C., Bover, I., Ruiz-Casado, A., Azagra, P., Jimenez, U., Gonzalez-Larriba, J.L., Diz, P., Cardenal, F., Artal, A., Carrato, A., Morales, S., Sanchez, J.J., de las Penas, R., Felip, E. & Lopez-Vivanco, G. (2003). Cisplatin plus gemcitabine versus a cisplatin-based triplet versus nonplatinum sequential doublets in advanced non-small-cell lung cancer: a Spanish Lung Cancer Group phase III randomized trial. *J Clin Oncol*, **21**, 3207-13.
- Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. & Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo J*, **15**, 6541-51.
- Alessi, D.R., Saito, Y., Campbell, D.G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C.J. & Cowley, S. (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *Embo J*, **13**, 1610-9.
- Amos, C.I., Wu, X., Broderick, P., Gorlov, I.P., Gu, J., Eisen, T., Dong, Q., Zhang, Q., Gu, X., Vijayakrishnan, J., Sullivan, K., Matakidou, A., Wang, Y., Mills, G., Doheny, K., Tsai, Y.Y., Chen, W.V., Shete, S., Spitz, M.R. & Houlston, R.S. (2008). Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet*, **40**, 616-22.
- Anagnostou, V.K., Bepler, G., Syrigos, K.N., Tanoue, L., Gettinger, S., Homer, R.J., Boffa, D., Detterbeck, F. & Rimm, D.L. (2009). High expression of mammalian target of rapamycin is associated with better outcome for patients with early stage lung adenocarcinoma. *Clin Cancer Res*, **15**, 4157-64.
- Andrade, M.A. & Bork, P. (1995). HEAT repeats in the Huntington's disease protein. *Nat Genet*, **11**, 115-6.
- Apsel, B., Blair, J.A., Gonzalez, B., Nazif, T.M., Feldman, M.E., Aizenstein, B., Hoffman, R., Williams, R.L., Shokat, K.M. & Knight, Z.A. (2008). Targeted polypharmacology: discovery of dual inhibitors of tyrosine and phosphoinositide kinases. *Nat Chem Biol*, **4**, 691-9.
- Arcaro, A., Khanzada, U.K., Vanhaesebroeck, B., Tetley, T.D., Waterfield, M.D. & Seckl, M.J. (2002). Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. *Embo J*, **21**, 5097-108.
- Arriagada, R., Bergman, B., Dunant, A., Le Chevalier, T., Pignon, J.P. & Vansteenkiste, J. (2004). Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med*, **350**, 351-60.

Arsham, A.M., Howell, J.J. & Simon, M.C. (2003). A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem*, **278**, 29655-60.

Atkins, M.B., Hidalgo, M., Stadler, W.M., Logan, T.F., Dutcher, J.P., Hudes, G.R., Park, Y., Liou, S.H., Marshall, B., Boni, J.P., Dukart, G. & Sherman, M.L. (2004). Randomized phase II study of multiple dose levels of CCI-779, a novel mammalian target of rapamycin kinase inhibitor, in patients with advanced refractory renal cell carcinoma. *J Clin Oncol*, **22**, 909-18.

Auperin, A., Arriagada, R., Pignon, J.P., Le Pechoux, C., Gregor, A., Stephens, R.J., Kristjansen, P.E., Johnson, B.E., Ueoka, H., Wagner, H. & Aisner, J. (1999). Prophylactic cranial irradiation for patients with small-cell lung cancer in complete remission. Prophylactic Cranial Irradiation Overview Collaborative Group. *N Engl J Med*, **341**, 476-84.

Avila, G., Lee, E.H., Perez, C.F., Allen, P.D. & Dirksen, R.T. (2003). FKBP12 binding to RyR1 modulates excitation-contraction coupling in mouse skeletal myotubes. *J Biol Chem*, **278**, 22600-8.

Baker, A.F., Dragovich, T., Ihle, N.T., Williams, R., Fenoglio-Preiser, C. & Powis, G. (2005). Stability of phosphoprotein as a biological marker of tumor signaling. *Clin Cancer Res*, **11**, 4338-40.

Barata, J.T., Cardoso, A.A., Nadler, L.M. & Boussiotis, V.A. (2001). Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). *Blood*, **98**, 1524-31.

Bellacosa, A., de Feo, D., Godwin, A.K., Bell, D.W., Cheng, J.Q., Altomare, D.A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Benedetti Panici, P., Mancuso, S., Neri, G. & Testa, J.R. (1995). Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer*, **64**, 280-5.

Bellacosa, A., Testa, J.R., Staal, S.P. & Tsichlis, P.N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, **254**, 274-7.

Berger, W., Elbling, L., Hauptmann, E. & Micksche, M. (1997). Expression of the multidrug resistance-associated protein (MRP) and chemoresistance of human non-small-cell lung cancer cells. *Int J Cancer*, **73**, 84-93.

Berkel, H.J., Turbat-Herrera, E.A., Shi, R. & de Benedetti, A. (2001). Expression of the translation initiation factor eIF4E in the polyp-cancer sequence in the colon. *Cancer Epidemiol Biomarkers Prev*, **10**, 663-6.

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H.A. & Thomas, G. (2005). The mTOR inhibitor RAD001 sensitizes

tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. *Cell*, **120**, 747-59.

Bjornsti, M.A. & Houghton, P.J. (2004). The TOR pathway: a target for cancer therapy. *Nat Rev Cancer*, **4**, 335-48.

Bland, J.M. & Altman, D.G. (1998). Survival probabilities (the Kaplan-Meier method). *Bmj*, **317**, 1572.

Blommaert EF, L.J., Blommaert PJ, van Woerkom GM, Meijer AJ. (1995). Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J Biol Chem*, **270**, 2320-6

Blumberg, P.M. (1988). Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. *Cancer Res*, **48**, 1-8.

Boffa, D.J., Luan, F., Thomas, D., Yang, H., Sharma, V.K., Lagman, M. & Suthanthiran, M. (2004). Rapamycin inhibits the growth and metastatic progression of non-small cell lung cancer. *Clin Cancer Res*, **10**, 293-300.

Bosotti, R., Isacchi, A. & Sonnhhammer, E.L. (2000). FAT: a novel domain in PIK-related kinases. *Trends Biochem Sci*, **25**, 225-7.

Bottomley, M.J., Salim, K. & Panayotou, G. (1998). Phospholipid-binding protein domains. *Biochim Biophys Acta*, **1436**, 165-83.

Bremnes, R.M., Sundstrom, S., Aasebo, U., Kaasa, S., Hatlevoll, R. & Aamdal, S. (2003). The value of prognostic factors in small cell lung cancer: results from a randomised multicenter study with minimum 5 year follow-up. *Lung Cancer*, **39**, 303-13.

Brennan, J., O'Connor, T., Makuch, R.W., Simmons, A.M., Russell, E., Linnoila, R.I., Phelps, R.M., Gazdar, A.F., Ihde, D.C. & Johnson, B.E. (1991). myc family DNA amplification in 107 tumors and tumor cell lines from patients with small cell lung cancer treated with different combination chemotherapy regimens. *Cancer Res*, **51**, 1708-12.

Brill, A., Torchinsky, A., Carp, H. & Toder, V. (1999). The role of apoptosis in normal and abnormal embryonic development. *J Assist Reprod Genet*, **16**, 512-9.

Brivanlou, A.H. & Darnell, J.E., Jr. (2002). Signal transduction and the control of gene expression. *Science*, **295**, 813-8.

Brognard, J., Clark, A.S., Ni, Y. & Dennis, P.A. (2001). Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. *Cancer Res*, **61**, 3986-97.

- Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. & Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, **369**, 756-8.
- Brown, E.J., Beal, P.A., Keith, C.T., Chen, J., Shin, T.B. & Schreiber, S.L. (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature*, **377**, 441-6.
- Brugarolas, J. & Kaelin, W.G., Jr. (2004). Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. *Cancer Cell*, **6**, 7-10.
- Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr. & Abraham, R.T. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science*, **277**, 99-101.
- Buck, E., Eyzaguirre, A., Brown, E., Petti, F., McCormack, S., Haley, J.D., Iwata, K.K., Gibson, N.W. & Griffin, G. (2006). Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. *Mol Cancer Ther*, **5**, 2676-84.
- Bull, H.A., Brickell, P.M. & Dowd, P.M. (1994). Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. *FEBS Lett*, **351**, 41-4.
- Burbee, D.G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A.F., Lerman, M.I., Zbarovsky, E., White, M. & Minna, J.D. (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst*, **93**, 691-9.
- Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H. & Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A*, **95**, 1432-7.
- Cafferkey, R., McLaughlin, M.M., Young, P.R., Johnson, R.K. & Livi, G.P. (1994). Yeast TOR (DRR) proteins: amino-acid sequence alignment and identification of structural motifs. *Gene*, **141**, 133-6.
- Cagini, L., Monacelli, M., Giustozzi, G., Moggi, L., Bellezza, G., Sidoni, A., Bucciarelli, E., Darwish, S., Ludovini, V., Pistola, L., Gregorc, V. & Tonato, M. (2000). Biological prognostic factors for early stage completely resected non-small cell lung cancer. *J Surg Oncol*, **74**, 53-60.
- Campbell, P.M. & Der, C.J. (2004). Oncogenic Ras and its role in tumor cell invasion and metastasis. *Semin Cancer Biol*, **14**, 105-14.
- Campling, B.G. & El-Deiry, W.S. (2003). Clinical implication of p53 mutation in lung cancer. *Mol Biotechnol*, **24**, 141-56.

Chan, S., Scheulen, M.E., Johnston, S., Mross, K., Cardoso, F., Ditttrich, C., Eiermann, W., Hess, D., Morant, R., Semiglazov, V., Borner, M., Salzberg, M., Ostapenko, V., Illiger, H.J., Behringer, D., Bardy-Bouxin, N., Boni, J., Kong, S., Cincotta, M. & Moore, L. (2005). Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. *J Clin Oncol*, **23**, 5314-22.

Chang, C.C., Liang, Y.C., Klutz, A., Hsu, C.I., Lin, C.F., Mold, D.E., Chou, T.C., Lee, Y.C. & Huang, R.C. (2006). Reversal of multidrug resistance by two nordihydroguaiaretic acid derivatives, M4N and maltose-M3N, and their use in combination with doxorubicin or paclitaxel. *Cancer Chemother Pharmacol*, **58**, 640-53.

Chen, J., Zheng, X.F., Brown, E.J. & Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc Natl Acad Sci U S A*, **92**, 4947-51.

Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi, T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N. & Testa, J.R. (1992). AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A*, **89**, 9267-71.

Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K. & Testa, J.R. (1996). Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci U S A*, **93**, 3636-41.

Chiang, G.G. & Abraham, R.T. (2005). Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. *J Biol Chem*, **280**, 25485-90.

Chiang, G.G. & Abraham, R.T. (2007). Targeting the mTOR signaling network in cancer. *Trends Mol Med*, **13**, 433-42.

Chmara, M., Wozniak, A., Ochman, K., Kobierska, G., Dziadziuszko, R., Sosinska-Mielcarek, K., Jassem, E., Skokowski, J., Jassem, J. & Limon, J. (2004). Loss of heterozygosity at chromosomes 3p and 17p in primary non-small cell lung cancer. *Anticancer Res*, **24**, 4259-63.

Choi, J., Chen, J., Schreiber, S.L. & Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science*, **273**, 239-42.

Choi, J.H., Bertram, P.G., Drenan, R., Carvalho, J., Zhou, H.H. & Zheng, X.F. (2002). The FKBP12-rapamycin-associated protein (FRAP) is a CLIP-170 kinase. *EMBO Rep*, **3**, 988-94.

- Chou, J., Chou, T. & Talalay, P. (1983). Computer simulation of drug effects: quantitation of synergism, summation and antagonism of multiple drugs. *Pharmacologist*, **25**, 175.
- Chou, T. & Dong, H. (2005). Design, experimentation and computerised automated data analysis of synergistic drug combinations against xenograft tumours and T-900607. In *AACR*, Vol. 46. pp. 1167. Proc Am Assoc Cancer Res: USA.
- Chou, T. & Hayball, M. (1997). CalcuSyn for windows: Multiple-Drug Dose-Effect Analyzer and Manual. Biosoft, Cambridge, UK: Cambridge, UK.
- Chou, T.C. (1976). Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. *J Theor Biol*, **59**, 253-76.
- Chou, T.C. (1998). Drug combinations: from laboratory to practice. *J Lab Clin Med*, **132**, 6-8.
- Chou, T.C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*, **58**, 621-81.
- Chua, Y.J., Steer, C. & Yip, D. (2004). Recent advances in management of small-cell lung cancer. *Cancer Treat Rev*, **30**, 521-43.
- Chun, K.H., Kosmider, J.W., 2nd, Sun, S., Pezzuto, J.M., Lotan, R., Hong, W.K. & Lee, H.Y. (2003). Effects of deguelin on the phosphatidylinositol 3-kinase/Akt pathway and apoptosis in premalignant human bronchial epithelial cells. *J Natl Cancer Inst*, **95**, 291-302.
- Chung, J., Kuo, C.J., Crabtree, G.R. & Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell*, **69**, 1227-36.
- Coffer, P.J. & Woodgett, J.R. (1991). Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem*, **201**, 475-81.
- Couch, F.J., Wang, X.Y., Wu, G.J., Qian, J., Jenkins, R.B. & James, C.D. (1999). Localization of PS6K to chromosomal region 17q23 and determination of its amplification in breast cancer. *Cancer Res*, **59**, 1408-11.
- Crew, J.P., Fuggle, S., Bicknell, R., Cranston, D.W., de Benedetti, A. & Harris, A.L. (2000). Eukaryotic initiation factor-4E in superficial and muscle invasive bladder cancer and its correlation with vascular endothelial growth factor expression and tumour progression. *Br J Cancer*, **82**, 161-6.
- Crino, P.B., Nathanson, K.L. & Henske, E.P. (2006). The tuberous sclerosis complex. *N Engl J Med*, **355**, 1345-56.

- Dancey, J.E. (2002). Clinical development of mammalian target of rapamycin inhibitors. *Hematol Oncol Clin North Am*, **16**, 1101-14.
- Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231-41.
- David, O., Jett, J., LeBeau, H., Dy, G., Hughes, J., Friedman, M. & Brody, A.R. (2004). Phospho-Akt overexpression in non-small cell lung cancer confers significant stage-independent survival disadvantage. *Clin Cancer Res*, **10**, 6865-71.
- Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B.A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G.J., Bigner, D.D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J.W., Leung, S.Y., Yuen, S.T., Weber, B.L., Seigler, H.F., Darrow, T.L., Paterson, H., Marais, R., Marshall, C.J., Wooster, R., Stratton, M.R. & Futreal, P.A. (2002). Mutations of the BRAF gene in human cancer. *Nature*, **417**, 949-54.
- de Castro Junior, G., Puglisi, F., de Azambuja, E., El Saghir, N.S. & Awada, A. (2006). Angiogenesis and cancer: A cross-talk between basic science and clinical trials (the "do ut des" paradigm). *Crit Rev Oncol Hematol*, **59**, 40-50.
- Dehing-Oberije, C., De Ruyscher, D., van der Weide, H., Hochstenbag, M., Bootsma, G., Geraedts, W., Pitz, C., Simons, J., Teule, J., Rahmy, A., Thimister, P., Steck, H. & Lambin, P. (2008). Tumor volume combined with number of positive lymph node stations is a more important prognostic factor than TNM stage for survival of non-small-cell lung cancer patients treated with (chemo)radiotherapy. *Int J Radiat Oncol Biol Phys*, **70**, 1039-44.
- Demmy, T.L. & Curtis, J.J. (1999). Minimally invasive lobectomy directed toward frail and high-risk patients: a case-control study. *Ann Thorac Surg*, **68**, 194-200.
- Dilling, M.B., Dias, P., Shapiro, D.N., Germain, G.S., Johnson, R.K. & Houghton, P.J. (1994). Rapamycin selectively inhibits the growth of childhood rhabdomyosarcoma cells through inhibition of signaling via the type I insulin-like growth factor receptor. *Cancer Res*, **54**, 903-7.
- Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E. & Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science*, **314**, 467-71.
- Douillard, J.Y., Rosell, R., De Lena, M., Carpagnano, F., Ramlau, R., Gonzales-Larriba, J.L., Grodzki, T., Pereira, J.R., Le Groumellec, A., Lorusso, V., Clary, C.,

- Torres, A.J., Dahabreh, J., Souquet, P.J., Astudillo, J., Fournel, P., Artal-Cortes, A., Jassem, J., Koubkova, L., His, P., Riggi, M. & Hurteloup, P. (2006). Adjuvant vinorelbine plus cisplatin versus observation in patients with completely resected stage IB-IIIa non-small-cell lung cancer (Adjuvant Navelbine International Trialist Association [ANITA]): a randomised controlled trial. *Lancet Oncol*, **7**, 719-27.
- Douros, J. & Suffness, M. (1981). New antitumor substances of natural origin. *Cancer Treat Rev*, **8**, 63-87.
- Dumont, F.J., Staruch, M.J., Koprak, S.L., Melino, M.R. & Sigal, N.H. (1990). Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J Immunol*, **144**, 251-8.
- Easton, J.B. & Houghton, P.J. (2006). mTOR and cancer therapy. *Oncogene*, **25**, 6436-46.
- Eng, C.P., Sehgal, S.N. & Vezina, C. (1984). Activity of rapamycin (AY-22,989) against transplanted tumors. *J Antibiot (Tokyo)*, **37**, 1231-7.
- Ettinger, D.S., Berkey, B.A., Abrams, R.A., Fontanesi, J., Machtay, M., Duncan, P.J., Curran, W.J., Jr., Movsas, B. & Byhardt, R.W. (2005). Study of paclitaxel, etoposide, and cisplatin chemotherapy combined with twice-daily thoracic radiotherapy for patients with limited-stage small-cell lung cancer: a Radiation Therapy Oncology Group 9609 phase II study. *J Clin Oncol*, **23**, 4991-8.
- Favre, B., Turowski, P. & Hemmings, B.A. (1997). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. *J Biol Chem*, **272**, 13856-63.
- Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D. & Shokat, K.M. (2009). Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. *PLoS Biol*, **7**, e38.
- Fennell, D.A. (2005). Caspase regulation in non-small cell lung cancer and its potential for therapeutic exploitation. *Clin Cancer Res*, **11**, 2097-105.
- Fingar, D.C., Richardson, C.J., Tee, A.R., Cheatham, L., Tsou, C. & Blenis, J. (2004). mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol Cell Biol*, **24**, 200-16.
- Flanagan, W.M. & Crabtree, G.R. (1993). Rapamycin inhibits p34cdc2 expression and arrests T lymphocyte proliferation at the G1/S transition. *Ann N Y Acad Sci*, **696**, 31-7.
- Forgacs, E., Biesterveld, E.J., Sekido, Y., Fong, K., Muneer, S., Wistuba, II, Milchgrub, S., Brezinschek, R., Virmani, A., Gazdar, A.F. & Minna, J.D. (1998). Mutation analysis of the PTEN/MMAC1 gene in lung cancer. *Oncogene*, **17**, 1557-65.

Fouladi, M., Laningham, F., Wu, J., O'Shaughnessy, M.A., Molina, K., Broniscer, A., Spunt, S.L., Lockett, I., Stewart, C.F., Houghton, P.J., Gilbertson, R.J. & Furman, W.L. (2007). Phase I study of everolimus in pediatric patients with refractory solid tumors. *J Clin Oncol*, **25**, 4806-12.

Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A. & Sabatini, D.M. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol*, **16**, 1865-70.

Fried, D.B., Morris, D.E., Poole, C., Rosenman, J.G., Halle, J.S., Detterbeck, F.C., Hensing, T.A. & Socinski, M.A. (2004). Systematic review evaluating the timing of thoracic radiation therapy in combined modality therapy for limited-stage small-cell lung cancer. *J Clin Oncol*, **22**, 4837-45.

Frodin, M. & Gammeltoft, S. (1999). Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol*, **151**, 65-77.

Fruman, D.A., Meyers, R.E. & Cantley, L.C. (1998). Phosphoinositide kinases. *Annu Rev Biochem*, **67**, 481-507.

Fuchs, B.C. & Bode, B.P. (2005). Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? *Semin Cancer Biol*, **15**, 254-66.

Gao, H.G., Chen, J.K., Stewart, J., Song, B., Rayappa, C., Whong, W.Z. & Ong, T. (1997). Distribution of p53 and K-ras mutations in human lung cancer tissues. *Carcinogenesis*, **18**, 473-8.

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R.S., Ru, B. & Pan, D. (2002). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol*, **4**, 699-704.

Garnett, M.J. & Marais, R. (2004). Guilty as charged: B-RAF is a human oncogene. *Cancer Cell*, **6**, 313-9.

Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. & Hay, N. (1998). 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev*, **12**, 502-13.

Gore, M.E. & Escudier, B. (2006). Emerging efficacy endpoints for targeted therapies in advanced renal cell carcinoma. *Oncology (Williston Park)*, **20**, 19-24.

Granville, C.A., Warfel, N., Tsurutani, J., Hollander, M.C., Robertson, M., Fox, S.D., Veenstra, T.D., Issaq, H.J., Linnoila, R.I. & Dennis, P.A. (2007). Identification of a highly effective rapamycin schedule that markedly reduces the size, multiplicity, and phenotypic progression of tobacco carcinogen-induced murine lung tumors. *Clin Cancer Res*, **13**, 2281-9.

- Group, N.-s.C.L.C.C. (1995). Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. *Bmj*, **311**, 899-909.
- Group, P.M.-a.T. (1998). Postoperative radiotherapy in non-small-cell lung cancer: systematic review and meta-analysis of individual patient data from nine randomised controlled trials. PORT Meta-analysis Trialists Group. *Lancet*, **352**, 257-63.
- Guba, M., von Breitenbuch, P., Steinbauer, M., Koehl, G., Flegel, S., Hornung, M., Bruns, C.J., Zuelke, C., Farkas, S., Anthuber, M., Jauch, K.W. & Geissler, E.K. (2002). Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med*, **8**, 128-35.
- Guertin, D.A. & Sabatini, D.M. (2007). Defining the role of mTOR in cancer. *Cancer Cell*, **12**, 9-22.
- Gupta, A.K., Soto, D.E., Feldman, M.D., Goldsmith, J.D., Mick, R., Hahn, S.M., Machtay, M., Muschel, R.J. & McKenna, W.G. (2004). Signaling pathways in NSCLC as a predictor of outcome and response to therapy. *Lung*, **182**, 151-62.
- Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*, **100**, 57-70.
- Hande, K.R. (1992). Etoposide pharmacology. *Semin Oncol*, **19**, 3-9.
- Hanna, N., Shepherd, F.A., Fossella, F.V., Pereira, J.R., De Marinis, F., von Pawel, J., Gatzemeier, U., Tsao, T.C., Pless, M., Muller, T., Lim, H.L., Desch, C., Szondy, K., Gervais, R., Shaharyar, Manegold, C., Paul, S., Paoletti, P., Einhorn, L. & Bunn, P.A., Jr. (2004). Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J Clin Oncol*, **22**, 1589-97.
- Hansen, H. (2008). *Textbook of Lung Cancer*. Informa Healthcare: London.
- Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. & Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell*, **110**, 177-89.
- Hart, I.R. & Saini, A. (1992). Biology of tumour metastasis. *Lancet*, **339**, 1453-7.
- Harwood, F.C., Shu, L. & Houghton, P.J. (2008). mTORC1 signaling can regulate growth factor activation of p44/42 mitogen-activated protein kinases through protein phosphatase 2A. *J Biol Chem*, **283**, 2575-85.
- Hashemolhosseini, S., Nagamine, Y., Morley, S.J., Desrivieres, S., Mercep, L. & Ferrari, S. (1998). Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem*, **273**, 14424-9.
- Haydon, M.S., Googe, J.D., Sorrells, D.S., Ghali, G.E. & Li, B.D. (2000). Progression of eIF4e gene amplification and overexpression in benign and malignant tumors of the head and neck. *Cancer*, **88**, 2803-10.

- Heasley, L.E. (2001). Autocrine and paracrine signaling through neuropeptide receptors in human cancer. *Oncogene*, **20**, 1563-9.
- Hecht, S.S. (1999). Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst*, **91**, 1194-210.
- Heitman, J., Movva, N.R. & Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253**, 905-9.
- Helliwell, S.B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. & Hall, M.N. (1994). TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol Biol Cell*, **5**, 105-18.
- Hennessy, B.T., Smith, D.L., Ram, P.T., Lu, Y. & Mills, G.B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov*, **4**, 988-1004.
- Hensel, C.H., Hsieh, C.L., Gazdar, A.F., Johnson, B.E., Sakaguchi, A.Y., Naylor, S.L., Lee, W.H. & Lee, E.Y. (1990). Altered structure and expression of the human retinoblastoma susceptibility gene in small cell lung cancer. *Cancer Res*, **50**, 3067-72.
- Herberger, B., Puhalla, H., Lehnert, M., Wrba, F., Novak, S., Brandstetter, A., Gruenberger, B., Gruenberger, T., Pirker, R. & Filipits, M. (2007). Activated mammalian target of rapamycin is an adverse prognostic factor in patients with biliary tract adenocarcinoma. *Clin Cancer Res*, **13**, 4795-9.
- Herbst, R.S., Heymach, J.V. & Lippman, S.M. (2008). Lung cancer. *N Engl J Med*, **359**, 1367-80.
- Heriche, J.K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E.M. & Goldberg, Y. (1997). Regulation of protein phosphatase 2A by direct interaction with casein kinase 2alpha. *Science*, **276**, 952-5.
- Hirsch, F.R. & Witta, S. (2005). Biomarkers for prediction of sensitivity to EGFR inhibitors in non-small cell lung cancer. *Curr Opin Oncol*, **17**, 118-22.
- Hodkinson, P.S., Mackinnon, A. & Sethi, T. (2008). Targeting growth factors in lung cancer. *Chest*, **133**, 1209-16.
- Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K. & Wong, A.J. (1996). A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*, **379**, 560-4.
- Holgado-Madruga, M., Moscatello, D.K., Emlet, D.R., Dieterich, R. & Wong, A.J. (1997). Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proc Natl Acad Sci U S A*, **94**, 12419-24.

- Honkanen, R.E. & Golden, T. (2002). Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr Med Chem*, **9**, 2055-75.
- Hosoi, H., Dilling, M.B., Liu, L.N., Danks, M.K., Shikata, T., Sekulic, A., Abraham, R.T., Lawrence, J.C., Jr. & Houghton, P.J. (1998). Studies on the mechanism of resistance to rapamycin in human cancer cells. *Mol Pharmacol*, **54**, 815-24.
- Houchens, D.P., Ovejera, A.A., Riblet, S.M. & Slagel, D.E. (1983). Human brain tumor xenografts in nude mice as a chemotherapy model. *Eur J Cancer Clin Oncol*, **19**, 799-805.
- Hu, P., Margolis, B., Skolnik, E.Y., Lammers, R., Ullrich, A. & Schlessinger, J. (1992). Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol Cell Biol*, **12**, 981-90.
- Huang, S., Liu, L.N., Hosoi, H., Dilling, M.B., Shikata, T. & Houghton, P.J. (2001). p53/p21(CIP1) cooperate in enforcing rapamycin-induced G(1) arrest and determine the cellular response to rapamycin. *Cancer Res*, **61**, 3373-81.
- Huang, S., Shu, L., Dilling, M.B., Easton, J., Harwood, F.C., Ichijo, H. & Houghton, P.J. (2003). Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21(Cip1). *Mol Cell*, **11**, 1491-501.
- Huber, M., Helgason, C.D., Damen, J.E., Scheid, M., Duronio, V., Liu, L., Ware, M.D., Humphries, R.K. & Krystal, G. (1999). The role of SHIP in growth factor induced signalling. *Prog Biophys Mol Biol*, **71**, 423-34.
- Hudes, G., Carducci, M., Tomczak, P., Dutcher, J., Figlin, R., Kapoor, A., Staroslawska, E., Sosman, J., McDermott, D., Bodrogi, I., Kovacevic, Z., Lesovoy, V., Schmidt-Wolf, I.G., Barbarash, O., Gokmen, E., O'Toole, T., Lustgarten, S., Moore, L. & Motzer, R.J. (2007). Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. *N Engl J Med*, **356**, 2271-81.
- Huffman, T.A., Mothe-Satney, I. & Lawrence, J.C., Jr. (2002). Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin. *Proc Natl Acad Sci U S A*, **99**, 1047-52.
- Hung, J.J., Wang, C.Y., Huang, M.H., Huang, B.S., Hsu, W.H. & Wu, Y.C. (2007). Prognostic factors in resected stage I non-small cell lung cancer with a diameter of 3 cm or less: visceral pleural invasion did not influence overall and disease-free survival. *J Thorac Cardiovasc Surg*, **134**, 638-43.
- Hunter, T. (1987). A thousand and one protein kinases. *Cell*, **50**, 823-9.
- Hunter, T. (1989). Protein-tyrosine phosphatases: the other side of the coin. *Cell*, **58**, 1013-6.

Huser, M., Lockett, J., Chiloeches, A., Mercer, K., Iwobi, M., Giblett, S., Sun, X.M., Brown, J., Marais, R. & Pritchard, C. (2001). MEK kinase activity is not necessary for Raf-1 function. *Embo J*, **20**, 1940-51.

Imoto, H., Osaki, T., Taga, S., Ohgami, A., Ichiyoshi, Y. & Yasumoto, K. (1998). Vascular endothelial growth factor expression in non-small-cell lung cancer: prognostic significance in squamous cell carcinoma. *J Thorac Cardiovasc Surg*, **115**, 1007-14.

Inoki, K., Li, Y., Zhu, T., Wu, J. & Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*, **4**, 648-57.

Inoki, K., Zhu, T. & Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell*, **115**, 577-90.

J. C. Yao, A.P., D. Z. Chang, R. A. Wolff, C. Jacobs, J. E. Mares, S. Gupta, F. Meric-Bernstam, A. Rashid. (2007). Phase II study of RAD001 (everolimus) and depot octreotide (sandostatin LAR) in advanced low grade neuroendocrine carcinoma (LGNET). In *2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement)*, 2007: 4503.

Jacinto, E. & Hall, M.N. (2003). Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol*, **4**, 117-26.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A. & Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*, **6**, 1122-8.

Jacobs, D., Glossip, D., Xing, H., Muslin, A.J. & Kornfeld, K. (1999). Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev*, **13**, 163-75.

Jaiswal, R.K., Moodie, S.A., Wolfman, A. & Landreth, G.E. (1994). The mitogen-activated protein kinase cascade is activated by B-Raf in response to nerve growth factor through interaction with p21ras. *Mol Cell Biol*, **14**, 6944-53.

James, S.R., Downes, C.P., Gigg, R., Grove, S.J., Holmes, A.B. & Alessi, D.R. (1996). Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem J*, **315 (Pt 3)**, 709-13.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C. & Thun, M.J. (2006). Cancer statistics, 2006. *CA Cancer J Clin*, **56**, 106-30.

Jiang, B.H., Jiang, G., Zheng, J.Z., Lu, Z., Hunter, T. & Vogt, P.K. (2001). Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ*, **12**, 363-9.

Joel, S.P., Shah, R. & Slevin, M.L. (1994). Etoposide dosage and pharmacodynamics. *Cancer Chemother Pharmacol*, **34 Suppl**, S69-75.

Johnson, B.E., Bridges, J.D., Sobczek, M., Gray, J., Linnoila, R.I., Gazdar, A.F., Hankins, L., Steinberg, S.M., Edison, M., Frame, J.N., Pass, H., Nesbitt, J., Holden, D., Mulshine, J.L., Glatstein, E. & Ihde, D.C. (1996). Patients with limited-stage small-cell lung cancer treated with concurrent twice-daily chest radiotherapy and etoposide/cisplatin followed by cyclophosphamide, doxorubicin, and vincristine. *J Clin Oncol*, **14**, 806-13.

Kaira, K., Oriuchi, N., Imai, H., Shimizu, K., Yanagitani, N., Sunaga, N., Hisada, T., Tanaka, S., Ishizuka, T., Kanai, Y., Endou, H., Nakajima, T. & Mori, M. (2008). Prognostic significance of L-type amino acid transporter 1 expression in resectable stage I-III nonsmall cell lung cancer. *Br J Cancer*, **98**, 742-8.

Kamada Y, F.T., Shintani T, Nagano K, Ohsumi M, Ohsumi Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J cell Biol*, **150**, 1507-13.

Kawauchi, K., Lazarus, A.H., Sanghera, J.S., Man, G.L., Pelech, S.L. & Delovitch, T.L. (1996). Regulation of BCR- and PKC/Ca(2+)-mediated activation of the Raf1/MEK/MAPK pathway by protein-tyrosine kinase and -tyrosine phosphatase activities. *Mol Immunol*, **33**, 287-96.

Kerr, J.F. (2002). History of the events leading to the formulation of the apoptosis concept. *Toxicology*, **181-182**, 471-4.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*, **110**, 163-75.

Kiriakogiani-Psaropoulou, P., Malamou-Mitsi, V., Martinopoulou, U., Legaki, S., Tamvakis, N., Vrettou, E., Fountzilias, G., Skarlos, D., Kosmidis, P. & Pavlidis, N. (1994). The value of neuroendocrine markers in non-small cell lung cancer: a comparative immunohistopathologic study. *Lung Cancer*, **11**, 353-64.

Kok, K., Osinga, J., Carritt, B., Davis, M.B., van der Hout, A.H., van der Veen, A.Y., Landsvater, R.M., de Leij, L.F., Berendsen, H.H., Postmus, P.E. & et al. (1987). Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature*, **330**, 578-81.

Kolch, W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J*, **351 Pt 2**, 289-305.

Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M.J., Sauter, G. & Kallioniemi, O.P. (1998). Tissue

microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med*, **4**, 844-7.

Krug, L.M., Crapanzano, J.P., Azzoli, C.G., Miller, V.A., Rizvi, N., Gomez, J., Kris, M.G., Pizzo, B., Tyson, L., Dunne, M. & Heelan, R.T. (2005). Imatinib mesylate lacks activity in small cell lung carcinoma expressing c-kit protein: a phase II clinical trial. *Cancer*, **103**, 2128-31.

Krystal, G.W., Sulanke, G. & Litz, J. (2002). Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. *Mol Cancer Ther*, **1**, 913-22.

Kurup, A. & Hanna, N.H. (2004). Treatment of small cell lung cancer. *Crit Rev Oncol Hematol*, **52**, 117-26.

Laack, E., Dickgreber, N., Muller, T., Knuth, A., Benk, J., Lorenz, C., Gieseler, F., Durk, H., Engel-Riedel, W., Dalhoff, K., Kortsik, C., Graeven, U., Burk, M., Dierlamm, T., Welte, T., Burkholder, I., Edler, L. & Hossfeld, D.K. (2004). Randomized phase III study of gemcitabine and vinorelbine versus gemcitabine, vinorelbine, and cisplatin in the treatment of advanced non-small-cell lung cancer: from the German and Swiss Lung Cancer Study Group. *J Clin Oncol*, **22**, 2348-56.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-5.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C. & Semenza, G.L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol*, **21**, 3995-4004.

Lee-Fruman, K.K., Kuo, C.J., Lippincott, J., Terada, N. & Blenis, J. (1999). Characterization of S6K2, a novel kinase homologous to S6K1. *Oncogene*, **18**, 5108-14.

Li, B.D., Gruner, J.S., Abreo, F., Johnson, L.W., Yu, H., Nawas, S., McDonald, J.C. & DeBenedetti, A. (2002). Prospective study of eukaryotic initiation factor 4E protein elevation and breast cancer outcome. *Ann Surg*, **235**, 732-8; discussion 738-9.

Lin, X., Bohle, A.S., Dohrmann, P., Leuschner, I., Schulz, A., Kremer, B. & Fandrich, F. (2001). Overexpression of phosphatidylinositol 3-kinase in human lung cancer. *Langenbecks Arch Surg*, **386**, 293-301.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. & Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*, **10**, 457-68.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. (2005a). Rheb binds and regulates the mTOR kinase. *Curr Biol*, **15**, 702-13.

Long, X., Ortiz-Vega, S., Lin, Y. & Avruch, J. (2005b). Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J Biol Chem*, **280**, 23433-6.

Lukanova, A., Toniolo, P., Akhmedkhanov, A., Hunt, K., Rinaldi, S., Zeleniuch-Jacquotte, A., Haley, N.J., Riboli, E., Stattin, P., Lundin, E. & Kaaks, R. (2001). A cross-sectional study of IGF-I determinants in women. *Eur J Cancer Prev*, **10**, 443-52.

Macchiarini, P., Fontanini, G., Hardin, M.J., Chuanchieh, H., Bigini, D., Vignati, S., Pingitore, R. & Angeletti, C.A. (1993). Blood vessel invasion by tumor cells predicts recurrence in completely resected T1 N0 M0 non-small-cell lung cancer. *J Thorac Cardiovasc Surg*, **106**, 80-9.

MacKinnon, A.C., Tufail-Hanif, U., Lucas, C.D., Jodrell, D., Haslett, C. & Sethi, T. (2005). Expression of V1A and GRP receptors leads to cellular transformation and increased sensitivity to substance-P analogue-induced growth inhibition. *Br J Cancer*, **92**, 522-31.

Mader, S., Lee, H., Pause, A. & Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol Cell Biol*, **15**, 4990-7.

Machama, T. & Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*, **273**, 13375-8.

Manda, R., Kohno, T., Niki, T., Yamada, T., Takenoshita, S., Kuwano, H. & Yokota, J. (2000). Differential expression of the LAMB3 and LAMC2 genes between small cell and non-small cell lung carcinomas. *Biochem Biophys Res Commun*, **275**, 440-5.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J. & Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell*, **10**, 151-62.

Marinov, M., Ziogas, A., Pardo, O., Tan, L., Dhillon, T., Mauri, F., Lane, H., Lemoine, N., Zangemeister-Wittke, U., Seckl, M., and Arcaro, A. (2009). AKT/mTOR Pathway activation and BCL-2 family proteins modulate the sensitivity of human small cell lung cancer cells to RAD001. *Clin Cancer Res*, 1277-1287.

Marshall, C.J. (1996). Cell signalling. Raf gets it together. *Nature*, **383**, 127-8.

Martini, R. (1994). Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. *J Neurocytol*, **23**, 1-28.

Mascaux, C., Iannino, N., Martin, B., Paesmans, M., Berghmans, T., Dusart, M., Haller, A., Lothaire, P., Meert, A.P., Noel, S., Lafitte, J.J. & Sculier, J.P. (2005). The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer*, **92**, 131-9.

Massion, P.P., Taflan, P.M., Shyr, Y., Rahman, S.M., Yildiz, P., Shakhour, B., Edgerton, M.E., Ninan, M., Andersen, J.J. & Gonzalez, A.L. (2004). Early involvement of the phosphatidylinositol 3-kinase/Akt pathway in lung cancer progression. *Am J Respir Crit Care Med*, **170**, 1088-94.

Mayerhofer, M., Valent, P., Sperr, W.R., Griffin, J.D. & Sillaber, C. (2002). BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1alpha, through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. *Blood*, **100**, 3767-75.

Mellor, H. & Parker, P.J. (1998). The extended protein kinase C superfamily. *Biochem J*, **332 (Pt 2)**, 281-92.

Minna, J.D., Kurie, J.M. & Jacks, T. (2003). A big step in the study of small cell lung cancer. *Cancer Cell*, **4**, 163-6.

Molhoek, K.R., Brautigan, D.L. & Slingluff, C.L., Jr. (2005). Synergistic inhibition of human melanoma proliferation by combination treatment with B-Raf inhibitor BAY43-9006 and mTOR inhibitor Rapamycin. *J Transl Med*, **3**, 39.

Molina, J.R. & Adjei, A.A. (2006). The Ras/Raf/MAPK pathway. *J Thorac Oncol*, **1**, 7-9.

Moriya, Y., Niki, T., Yamada, T., Matsuno, Y., Kondo, H. & Hirohashi, S. (2001). Increased expression of laminin-5 and its prognostic significance in lung adenocarcinomas of small size. An immunohistochemical analysis of 102 cases. *Cancer*, **91**, 1129-41.

Mukohara, T., Kudoh, S., Yamauchi, S., Kimura, T., Yoshimura, N., Kanazawa, H., Hirata, K., Wanibuchi, H., Fukushima, S., Inoue, K. & Yoshikawa, J. (2003). Expression of epidermal growth factor receptor (EGFR) and downstream-activated peptides in surgically excised non-small-cell lung cancer (NSCLC). *Lung Cancer*, **41**, 123-30.

Murayama, T., Inokuchi, M., Takagi, Y., Yamada, H., Kojima, K., Kumagai, J., Kawano, T. & Sugihara, K. (2009). Relation between outcomes and localisation of p-mTOR expression in gastric cancer. *Br J Cancer*.

Musashi, M., Ota, S. & Shiroshita, N. (2000). The role of protein kinase C isoforms in cell proliferation and apoptosis. *Int J Hematol*, **72**, 12-9.

Naeem, M., Dahiya, M., Clark, J.I., Creech, S.D. & Alkan, S. (2002). Analysis of c-kit protein expression in small-cell lung carcinoma and its implication for prognosis. *Hum Pathol*, **33**, 1182-7.

Nave, B.T., Ouwers, M., Withers, D.J., Alessi, D.R. & Shepherd, P.R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*, **344 Pt 2**, 427-31.

Naylor, S.L., Johnson, B.E., Minna, J.D. & Sakaguchi, A.Y. (1987). Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. *Nature*, **329**, 451-4.

Nesbit, C.E., Tersak, J.M. & Prochownik, E.V. (1999). MYC oncogenes and human neoplastic disease. *Oncogene*, **18**, 3004-16.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., Zwartkruis, F.J. & Thomas, G. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci U S A*, **102**, 14238-43.

Noguchi, M., Hirohashi, S., Hara, F., Kojima, A., Shimosato, Y., Shinkai, T. & Tsuchiya, R. (1990). Heterogenous amplification of myc family oncogenes in small cell lung carcinoma. *Cancer*, **66**, 2053-8.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J. & Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem*, **278**, 15461-4.

Nurcombe, V., Smart, C.E., Chipperfield, H., Cool, S.M., Boilly, B. & Hondermarck, H. (2000). The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates. *J Biol Chem*, **275**, 30009-18.

O'Brien, M.E., Ciuleanu, T.E., Tsekov, H., Shparyk, Y., Cucevia, B., Juhasz, G., Thatcher, N., Ross, G.A., Dane, G.C. & Crofts, T. (2006). Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer. *J Clin Oncol*, **24**, 5441-7.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L., Baselga, J. & Rosen, N. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res*, **66**, 1500-8.

Okamoto, H., Watanabe, K., Kunikane, H., Yokoyama, A., Kudoh, S., Asakawa, T., Shibata, T., Kunitoh, H., Tamura, T. & Saijo, N. (2007). Randomised phase III trial of carboplatin plus etoposide vs split doses of cisplatin plus etoposide in elderly or

poor-risk patients with extensive disease small-cell lung cancer: JCOG 9702. *Br J Cancer*, **97**, 162-9.

Olaussen, K.A., Dunant, A., Fouret, P., Brambilla, E., Andre, F., Haddad, V., Taranchon, E., Filipits, M., Pirker, R., Popper, H.H., Stahel, R., Sabatier, L., Pignon, J.P., Tursz, T., Le Chevalier, T. & Soria, J.C. (2006). DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*, **355**, 983-91.

Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J. & Lax, I. (2001). Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. *Proc Natl Acad Sci U S A*, **98**, 6074-9.

Ory, S., Zhou, M., Conrads, T.P., Veenstra, T.D. & Morrison, D.K. (2003). Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol*, **13**, 1356-64.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N. & et al. (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell*, **65**, 91-104.

Otterson, G.A., Kratzke, R.A., Coxon, A., Kim, Y.W. & Kaye, F.J. (1994). Absence of p16INK4 protein is restricted to the subset of lung cancer lines that retains wildtype RB. *Oncogene*, **9**, 3375-8.

Paez, J.G., Janne, P.A., Lee, J.C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F.J., Lindeman, N., Boggon, T.J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M.J., Sellers, W.R., Johnson, B.E. & Meyerson, M. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*, **304**, 1497-500.

Pandya, K., Levy, D. & Hifalgo, M. (2005). A randomized, phase II ECOG trial of two dose levels of temsirolimus (CCI-779) in patients with extensive stage small cell lung cancer in remission after induction chemotherapy. A preliminary report. In *ASCO Annual Meetings Proceedings*.

Pardo, O.E., Arcaro, A., Salerno, G., Raguz, S., Downward, J. & Seckl, M.J. (2002). Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. *J Biol Chem*, **277**, 12040-6.

Pardo, O.E., Arcaro, A., Salerno, G., Tetley, T.D., Valovka, T., Gout, I. & Seckl, M.J. (2001). Novel cross talk between MEK and S6K2 in FGF-2 induced proliferation of SCLC cells. *Oncogene*, **20**, 7658-67.

Pardo, O.E., Lesay, A., Arcaro, A., Lopes, R., Ng, B.L., Warne, P.H., McNeish, I.A., Tetley, T.D., Lemoine, N.R., Mehmet, H., Seckl, M.J. & Downward, J. (2003).

Fibroblast growth factor 2-mediated translational control of IAPs blocks mitochondrial release of Smac/DIABLO and apoptosis in small cell lung cancer cells. *Mol Cell Biol*, **23**, 7600-10.

Pardo, O.E., Wellbrock, C., Khanzada, U.K., Aubert, M., Arozarena, I., Davidson, S., Bowen, F., Parker, P.J., Filonenko, V.V., Gout, I.T., Sebire, N., Marais, R., Downward, J. & Seckl, M.J. (2006). FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. *Embo J*, **25**, 3078-88.

Park, W.S., Moon, Y.W., Yang, Y.M., Kim, Y.S., Kim, Y.D., Fuller, B.G., Vortmeyer, A.O., Fogt, F., Lubensky, I.A. & Zhuang, Z. (1998). Mutations of the STK11 gene in sporadic gastric carcinoma. *Int J Oncol*, **13**, 601-4.

Parkin, D.M., Bray, F., Ferlay, J. & Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

Parkin, D.M., Pisani, P., Lopez, A.D. & Masuyer, E. (1994). At least one in seven cases of cancer is caused by smoking. Global estimates for 1985. *Int J Cancer*, **59**, 494-504.

Parsons, D.W., Wang, T.L., Samuels, Y., Bardelli, A., Cummins, J.M., DeLong, L., Silliman, N., Ptak, J., Szabo, S., Willson, J.K., Markowitz, S., Kinzler, K.W., Vogelstein, B., Lengauer, C. & Velculescu, V.E. (2005). Colorectal cancer: mutations in a signalling pathway. *Nature*, **436**, 792.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C., Jr. & Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature*, **371**, 762-7.

Pawson, T. & Nash, P. (2000). Protein-protein interactions define specificity in signal transduction. *Genes Dev*, **14**, 1027-47.

Pearce, L.R., Huang, X., Boudeau, J., Pawlowski, R., Wullschleger, S., Deak, M., Ibrahim, A.F., Gourlay, R., Magnuson, M.A. & Alessi, D.R. (2007). Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochem J*, **405**, 513-22.

Pedersen, M.W., Meltorn, M., Damstrup, L. & Poulsen, H.S. (2001). The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. *Ann Oncol*, **12**, 745-60.

Pedersen, N., Mortensen, S., Sorensen, S.B., Pedersen, M.W., Rieneck, K., Bovin, L.F. & Poulsen, H.S. (2003). Transcriptional gene expression profiling of small cell lung cancer cells. *Cancer Res*, **63**, 1943-53.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C. & Thomas, G. (2004). S6K1(-/-)/S6K2(-/-) mice exhibit

perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol*, **24**, 3112-24.

Pesesse, X., Deleu, S., De Smedt, F., Drayer, L. & Erneux, C. (1997). Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem Biophys Res Commun*, **239**, 697-700.

Petersen, I., Langreck, H., Wolf, G., Schwendel, A., Psille, R., Vogt, P., Reichel, M.B., Ried, T. & Dietel, M. (1997). Small-cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p. *Br J Cancer*, **75**, 79-86.

Peterson, R.T., Desai, B.N., Hardwick, J.S. & Schreiber, S.L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. *Proc Natl Acad Sci U S A*, **96**, 4438-42.

Pignon, J.P., Arriagada, R., Ihde, D.C., Johnson, D.H., Perry, M.C., Souhami, R.L., Brodin, O., Joss, R.A., Kies, M.S., Lebeau, B. & et al. (1992). A meta-analysis of thoracic radiotherapy for small-cell lung cancer. *N Engl J Med*, **327**, 1618-24.

Pignon, J.P., Tribodet, H., Scagliotti, G.V., Douillard, J.Y., Shepherd, F.A., Stephens, R.J., Dunant, A., Torri, V., Rosell, R., Seymour, L., Spiro, S.G., Rolland, E., Fossati, R., Aubert, D., Ding, K., Waller, D. & Le Chevalier, T. (2008). Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. *J Clin Oncol*, **26**, 3552-9.

Pirker, R., Pereira, J.R., Szczesna, A., von Pawel, J., Krzakowski, M., Ramlau, R., Vynnychenko, I., Park, K., Yu, C.T., Ganul, V., Roh, J.K., Bajetta, E., O'Byrne, K., de Marinis, F., Eberhardt, W., Goddemeier, T., Emig, M. & Gatzemeier, U. (2009). Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial. *Lancet*, **373**, 1525-31.

Pisick, E., Jagadeesh, S. & Salgia, R. (2004). Receptor tyrosine kinases and inhibitors in lung cancer. *ScientificWorldJournal*, **4**, 589-604.

Potti, A., Mukherjee, S., Petersen, R., Dressman, H.K., Bild, A., Koontz, J., Kratzke, R., Watson, M.A., Kelley, M., Ginsburg, G.S., West, M., Harpole, D.H., Jr. & Nevins, J.R. (2006). A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. *N Engl J Med*, **355**, 570-80.

Prochownik, E.V. (2008). c-Myc: linking transformation and genomic instability. *Curr Mol Med*, **8**, 446-58.

Pujol, J.L., Daures, J.P., Riviere, A., Quoix, E., Westeel, V., Quantin, X., Breton, J.L., Lemarie, E., Poudenx, M., Milleron, B., Moro, D., Debieuvre, D. & Le Chevalier, T. (2001). Etoposide plus cisplatin with or without the combination of 4'-

epidoxorubicin plus cyclophosphamide in treatment of extensive small-cell lung cancer: a French Federation of Cancer Institutes multicenter phase III randomized study. *J Natl Cancer Inst*, **93**, 300-8.

Rahmoune, H., Chen, H.L., Gallagher, J.T., Rudland, P.S. & Fernig, D.G. (1998). Interaction of heparan sulfate from mammary cells with acidic fibroblast growth factor (FGF) and basic FGF. Regulation of the activity of basic FGF by high and low affinity binding sites in heparan sulfate. *J Biol Chem*, **273**, 7303-10.

Rao, S., Cunningham, D., de Gramont, A., Scheithauer, W., Smakal, M., Humblet, Y., Kourteva, G., Iveson, T., Andre, T., Dostalova, J., Illes, A., Belly, R., Perez-Ruixo, J.J., Park, Y.C. & Palmer, P.A. (2004). Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *J Clin Oncol*, **22**, 3950-7.

Reeve, J.G., Payne, J.A. & Bleehen, N.M. (1990). Production of immunoreactive insulin-like growth factor-I (IGF-I) and IGF-I binding proteins by human lung tumours. *Br J Cancer*, **61**, 727-31.

Richardson, G.E. & Johnson, B.E. (1993). The biology of lung cancer. *Semin Oncol*, **20**, 105-27.

Rinehart, J., Adjei, A.A., Lorusso, P.M., Waterhouse, D., Hecht, J.R., Natale, R.B., Hamid, O., Varterasian, M., Asbury, P., Kaldjian, E.P., Gulyas, S., Mitchell, D.Y., Herrera, R., Sebolt-Leopold, J.S. & Meyer, M.B. (2004). Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol*, **22**, 4456-62.

Robinson, M.J. & Cobb, M.H. (1997). Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol*, **9**, 180-6.

Rohr, U.P., Rehfeld, N., Pflugfelder, L., Geddert, H., Muller, W., Steidl, U., Fenk, R., Graf, T., Schott, M., Thiele, K.P., Gabbert, H.E., Germing, U., Kronenwett, R. & Haas, R. (2004). Expression of the tyrosine kinase c-kit is an independent prognostic factor in patients with small cell lung cancer. *Int J Cancer*, **111**, 259-63.

Rosell, R., Moran, T., Queralt, C., Porta, R., Cardenal, F., Camps, C., Majem, M., Lopez-Vivanco, G., Isla, D., Provencio, M., Insa, A., Massuti, B., Gonzalez-Larriba, J.L., Paz-Ares, L., Bover, I., Garcia-Campelo, R., Moreno, M.A., Catot, S., Rolfo, C., Reguart, N., Palmero, R., Sanchez, J.M., Bastus, R., Mayo, C., Bertran-Alamillo, J., Molina, M.A., Sanchez, J.J. & Taron, M. (2009). Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med*, **361**, 958-67.

Rosenwald, I.B., Chen, J.J., Wang, S., Savas, L., London, I.M. & Pullman, J. (1999). Upregulation of protein synthesis initiation factor eIF-4E is an early event during colon carcinogenesis. *Oncogene*, **18**, 2507-17.

Rosenwald, I.B., Kaspar, R., Rousseau, D., Gehrke, L., Leboulch, P., Chen, J.J., Schmidt, E.V., Sonenberg, N. & London, I.M. (1995). Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. *J Biol Chem*, **270**, 21176-80.

Rosner, M., Hanneder, M., Siegel, N., Valli, A., Fuchs, C. & Hengstschlager, M. (2008). The mTOR pathway and its role in human genetic diseases. *Mutat Res*, **659**, 284-92.

Roux, P.P. & Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev*, **68**, 320-44.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. & Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature*, **363**, 83-5.

Ruotsalainen, T., Joensuu, H., Mattson, K. & Salven, P. (2002). High pretreatment serum concentration of basic fibroblast growth factor is a predictor of poor prognosis in small cell lung cancer. *Cancer Epidemiol Biomarkers Prev*, **11**, 1492-5.

Rusch, V., Baselga, J., Cordon-Cardo, C., Orazem, J., Zaman, M., Hoda, S., McIntosh, J., Kurie, J. & Dmitrovsky, E. (1993). Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res*, **53**, 2379-85.

Rygaard, K., Nakamura, T. & Spang-Thomsen, M. (1993). Expression of the proto-oncogenes c-met and c-kit and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts. *Br J Cancer*, **67**, 37-46.

Sandler, A., Gray, R., Brahmer, J., Dowlati, J., Schiller, Perry, M. & Johnson, D. (2005). Randomized phase II/III Trial of paclitaxel (P) plus carboplatin (C) with or without bevacizumab (NSC # 704865) in patients with advanced non-squamous non-small cell lung cancer (NSCLC): An Eastern Cooperative Oncology Group (ECOG) Trial - E4599. In *ASCO. Journal of Clinical Oncology*, 2005 ASCO Annual Meeting Proceedings. Vol 23, No. 16S, Part I of II (June 1 Supplement), 2005: 4: USA.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*, **14**, 1296-302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. & Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*, **22**, 159-68.

- Sarbassov, D.D., Guertin, D.A., Ali, S.M. & Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*, **307**, 1098-101.
- Sattler, M. & Salgia, R. (2003). Molecular and cellular biology of small cell lung cancer. *Semin Oncol*, **30**, 57-71.
- Scagliotti, G.V., Parikh, P., von Pawel, J., Biesma, B., Vansteenkiste, J., Manegold, C., Serwatowski, P., Gatzemeier, U., Digumarti, R., Zukin, M., Lee, J.S., Mellemegaard, A., Park, K., Patil, S., Rolski, J., Goksel, T., de Marinis, F., Simms, L., Sugarman, K.P. & Gandara, D. (2008). Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naïve patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol*, **26**, 3543-51.
- Schalm, S.S. & Blenis, J. (2002). Identification of a conserved motif required for mTOR signaling. *Curr Biol*, **12**, 632-9.
- Schalm, S.S., Fingar, D.C., Sabatini, D.M. & Blenis, J. (2003). TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol*, **13**, 797-806.
- Schmelzle, T. & Hall, M.N. (2000). TOR, a central controller of cell growth. *Cell*, **103**, 253-62.
- Scott, P.H., Brunn, G.J., Kohn, A.D., Roth, R.A. & Lawrence, J.C., Jr. (1998). Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc Natl Acad Sci U S A*, **95**, 7772-7.
- Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., Leopold, W.R. & Saltiel, A.R. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med*, **5**, 810-6.
- Secombe, J., Pierce, S.B. & Eisenman, R.N. (2004). Myc: a weapon of mass destruction. *Cell*, **117**, 153-6.
- Sekulic, A., Hudson, C.C., Homme, J.L., Yin, P., Otterness, D.M., Karnitz, L.M. & Abraham, R.T. (2000). A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res*, **60**, 3504-13.
- Selvaggi, G., Novello, S., Torri, V., Leonardo, E., De Giuli, P., Borasio, P., Mossetti, C., Ardisson, F., Lausi, P. & Scagliotti, G.V. (2004). Epidermal growth factor receptor overexpression correlates with a poor prognosis in completely resected non-small-cell lung cancer. *Ann Oncol*, **15**, 28-32.
- Sethi, T., Rintoul, R.C., Moore, S.M., MacKinnon, A.C., Salter, D., Choo, C., Chilvers, E.R., Dransfield, I., Donnelly, S.C., Strieter, R. & Haslett, C. (1999).

Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. *Nat Med*, **5**, 662-8.

Sethi, T. & Rozengurt, E. (1991). Multiple neuropeptides stimulate clonal growth of small cell lung cancer: effects of bradykinin, vasopressin, cholecystokinin, galanin, and neurotensin. *Cancer Res*, **51**, 3621-3.

Seufferlein, T. & Rozengurt, E. (1996a). Galanin, neurotensin, and phorbol esters rapidly stimulate activation of mitogen-activated protein kinase in small cell lung cancer cells. *Cancer Res*, **56**, 5758-64.

Seufferlein, T. & Rozengurt, E. (1996b). Rapamycin inhibits constitutive p70s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells. *Cancer Res*, **56**, 3895-7.

Shantz, L.M. & Pegg, A.E. (1994). Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res*, **54**, 2313-6.

Shaw, R.J., Kosmatka, M., Bardeesy, N., Hurley, R.L., Witters, L.A., DePinho, R.A. & Cantley, L.C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A*, **101**, 3329-35.

Shepherd, F.A., Dancey, J., Ramlau, R., Mattson, K., Gralla, R., O'Rourke, M., Levitan, N., Gressot, L., Vincent, M., Burkes, R., Coughlin, S., Kim, Y. & Berille, J. (2000). Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. *J Clin Oncol*, **18**, 2095-103.

Shepherd, F.A., Rodrigues Pereira, J., Ciuleanu, T., Tan, E.H., Hirsh, V., Thongprasert, S., Campos, D., Maoleekoonpiroj, S., Smylie, M., Martins, R., van Kooten, M., Dediu, M., Findlay, B., Tu, D., Johnston, D., Bezjak, A., Clark, G., Santabarbara, P. & Seymour, L. (2005). Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med*, **353**, 123-32.

Shi, S.R., Key, M.E. & Kalra, K.L. (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem*, **39**, 741-8.

Shi, Y., Yan, H., Frost, P., Gera, J. & Lichtenstein, A. (2005). Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. *Mol Cancer Ther*, **4**, 1533-40.

Shields, T.W. (1983). Prognostic significance of parenchymal lymphatic vessel and blood vessel invasion in carcinoma of the lung. *Surg Gynecol Obstet*, **157**, 185-90.

Shivapurkar, N., Reddy, J., Chaudhary, P.M. & Gazdar, A.F. (2003). Apoptosis and lung cancer: a review. *J Cell Biochem*, **88**, 885-98.

Siemiatycki, J., Richardson, L., Straif, K., Latreille, B., Lakhani, R., Campbell, S., Rousseau, M.C. & Boffetta, P. (2004). Listing occupational carcinogens. *Environ Health Perspect*, **112**, 1447-59.

Simon, G.R., Sharma, S., Cantor, A., Smith, P. & Bepler, G. (2005). ERCC1 expression is a predictor of survival in resected patients with non-small cell lung cancer. *Chest*, **127**, 978-83.

Simon, G.R. & Wagner, H. (2003). Small cell lung cancer. *Chest*, **123**, 259S-271S.
Slotman, B., Faivre-Finn, C., Kramer, G., Rankin, E., Snee, M., Hatton, M., Postmus, P., Collette, L., Musat, E. & Senan, S. (2007). Prophylactic cranial irradiation in extensive small-cell lung cancer. *N Engl J Med*, **357**, 664-72.

Smith, E.M., Finn, S.G., Tee, A.R., Browne, G.J. & Proud, C.G. (2005). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J Biol Chem*, **280**, 18717-27.

Sorrells, D.L., Meschonat, C., Black, D. & Li, B.D. (1999). Pattern of amplification and overexpression of the eukaryotic initiation factor 4E gene in solid tumor. *J Surg Res*, **85**, 37-42.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H. & Tavtigian, S.V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet*, **15**, 356-62.

Strimpakos, A.S., Karapanagiotou, E.M., Saif, M.W. & Syrigos, K.N. (2008). The role of mTOR in the management of solid tumors: An overview. *Cancer Treat Rev*.
Strumberg, D. (2005). Preclinical and clinical development of the oral multikinase inhibitor sorafenib in cancer treatment. *Drugs Today (Barv)*, **41**, 773-84.

Sun, S.Y., Rosenberg, L.M., Wang, X., Zhou, Z., Yue, P., Fu, H. & Khuri, F.R. (2005). Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res*, **65**, 7052-8.

Sundstrom, S., Bremnes, R.M., Kaasa, S., Aasebo, U., Hatlevoll, R., Dahle, R., Boye, N., Wang, M., Vigander, T., Vilsvik, J., Skovlund, E., Hannisdal, E. & Aamdal, S. (2002). Cisplatin and etoposide regimen is superior to cyclophosphamide, epirubicin, and vincristine regimen in small-cell lung cancer: results from a randomized phase III trial with 5 years' follow-up. *J Clin Oncol*, **20**, 4665-72.

Takai, Y., Sasaki, T. & Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev*, **81**, 153-208.

Tee, A.R., Anjum, R. & Blenis, J. (2003a). Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin. *J Biol Chem*, **278**, 37288-96.

Tee, A.R. & Blenis, J. (2005). mTOR, translational control and human disease. *Semin Cell Dev Biol*, **16**, 29-37.

Tee, A.R., Fingar, D.C., Manning, B.D., Kwiatkowski, D.J., Cantley, L.C. & Blenis, J. (2002). Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc Natl Acad Sci U S A*, **99**, 13571-6.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C. & Blenis, J. (2003b). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol*, **13**, 1259-68.

Testa, J.R. & Bellacosa, A. (2001). AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci U S A*, **98**, 10983-5.

Therneau T, G.P., Fleming T. (1990). Martingale-based residuals for survival models. *Biometrika*, **77**, 147-160.

Travis, W., TV, C., B, C., Sobin, L., Shimosato, Y. & Brambilla, E. (1999). *Histological Typing of Lung and Pleural Tumours (WHO - World Health Organization - International Histological Classification of Tumours)*. Vol. . Springer: Berlin.

Tsao, M.S., Sakurada, A., Cutz, J.C., Zhu, C.Q., Kamel-Reid, S., Squire, J., Lorimer, I., Zhang, T., Liu, N., Daneshmand, M., Marrano, P., da Cunha Santos, G., Lagarde, A., Richardson, F., Seymour, L., Whitehead, M., Ding, K., Pater, J. & Shepherd, F.A. (2005). Erlotinib in lung cancer - molecular and clinical predictors of outcome. *N Engl J Med*, **353**, 133-44.

Tsuchiya, T., Akamine, S., Muraoka, M., Kamohara, R., Tsuji, K., Urabe, S., Honda, S. & Yamasaki, N. (2007). Stage IA non-small cell lung cancer: vessel invasion is a poor prognostic factor and a new target of adjuvant chemotherapy. *Lung Cancer*, **56**, 341-8.

Tsurutani, J., Steinberg, S.M., Ballas, M., Robertson, M., LoPiccolo, J., Soda, H., Kohno, S., Egilsson, V. & Dennis, P.A. (2007). Prognostic significance of clinical factors and Akt activation in patients with bronchioloalveolar carcinoma. *Lung Cancer*, **55**, 115-21.

Valovka, T., Verdier, F., Cramer, R., Zhyvoloup, A., Fenton, T., Rebholz, H., Wang, M.L., Gzhegotsky, M., Lutsyk, A., Matsuka, G., Filonenko, V., Wang, L., Proud, C.G., Parker, P.J. & Gout, I.T. (2003). Protein kinase C phosphorylates ribosomal

protein S6 kinase betaII and regulates its subcellular localization. *Mol Cell Biol*, **23**, 852-63.

Van Cutsem, E., van de Velde, H., Karasek, P., Oettle, H., Vervenne, W.L., Szawlowski, A., Schoffski, P., Post, S., Verslype, C., Neumann, H., Safran, H., Humblet, Y., Perez Ruixo, J., Ma, Y. & Von Hoff, D. (2004). Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol*, **22**, 1430-8.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J. & Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol*, **9**, 316-23.

Vandermoere, F., El Yazidi-Belkoura, I., Adriaenssens, E., Lemoine, J. & Hondermarck, H. (2005). The antiapoptotic effect of fibroblast growth factor-2 is mediated through nuclear factor-kappaB activation induced via interaction between Akt and IkappaB kinase-beta in breast cancer cells. *Oncogene*, **24**, 5482-91.

Veikkola, T., Karkkainen, M., Claesson-Welsh, L. & Alitalo, K. (2000). Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res*, **60**, 203-12.

Vezina, C., Kudelski, A. & Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot (Tokyo)*, **28**, 721-6.

Wakelee, H., Dubey, S. & Gandara, D. (2007). Optimal adjuvant therapy for non-small cell lung cancer--how to handle stage I disease. *Oncologist*, **12**, 331-7.

Wan, X., Harkavy, B., Shen, N., Grohar, P. & Helman, L.J. (2007). Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene*, **26**, 1932-40.

Wang, S., Lloyd, R.V., Hutzler, M.J., Rosenwald, I.B., Safran, M.S., Patwardhan, N.A. & Khan, A. (2001). Expression of eukaryotic translation initiation factors 4E and 2alpha correlates with the progression of thyroid carcinoma. *Thyroid*, **11**, 1101-7.

Waters, C.M., MacKinnon, A.C., Cummings, J., Tufail-Hanif, U., Jodrell, D., Haslett, C. & Sethi, T. (2003). Increased gastrin-releasing peptide (GRP) receptor expression in tumour cells confers sensitivity to [Arg6,D-Trp7,9,NmePhe8]-substance P (6-11)-induced growth inhibition. *Br J Cancer*, **88**, 1808-16.

Way, K.J., Chou, E. & King, G.L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci*, **21**, 181-7.

Weber, S., Zuckerman, J.E., Bostwick, D.G., Bensch, K.G., Sikic, B.I. & Raffin, T.A. (1985). Gastrin releasing peptide is a selective mitogen for small cell lung carcinoma in vitro. *J Clin Invest*, **75**, 306-9.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes--the Achilles heel of cancer. *Science*, **297**, 63-4.

Weinstein, I.B. & Joe, A.K. (2006). Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol*, **3**, 448-57.

Wert, M.M. & Palfrey, H.C. (2000). Divergence in the anti-apoptotic signalling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: rescue by bFGF involves protein kinase C delta. *Biochem J*, **352 Pt 1**, 175-82.

WHO. (2007). *International Statistical Classification of Diseases and Related Health Problems (ICD-10)*. WHO: Geneva

Winton, T., Livingston, R., Johnson, D., Rigas, J., Johnston, M., Butts, C., Cormier, Y., Goss, G., Inculet, R., Vallieres, E., Fry, W., Bethune, D., Ayoub, J., Ding, K., Seymour, L., Graham, B., Tsao, M.S., Gandara, D., Kesler, K., Demmy, T. & Shepherd, F. (2005). Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *N Engl J Med*, **352**, 2589-97.

Wistuba, II, Gazdar, A.F. & Minna, J.D. (2001). Molecular genetics of small cell lung carcinoma. *Semin Oncol*, **28**, 3-13.

Wistuba, II, Lam, S., Behrens, C., Virmani, A.K., Fong, K.M., LeRiche, J., Samet, J.M., Srivastava, S., Minna, J.D. & Gazdar, A.F. (1997). Molecular damage in the bronchial epithelium of current and former smokers. *J Natl Cancer Inst*, **89**, 1366-73.

Wittinghofer, A. & Pai, E.F. (1991). The structure of Ras protein: a model for a universal molecular switch. *Trends Biochem Sci*, **16**, 382-7.

Witzig, T.E., Geyer, S.M., Ghobrial, I., Inwards, D.J., Fonseca, R., Kurtin, P., Ansell, S.M., Luyun, R., Flynn, P.J., Morton, R.F., Dakhil, S.R., Gross, H. & Kaufmann, S.H. (2005). Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. *J Clin Oncol*, **23**, 5347-56.

Wong, A.S., Kim, S.O., Leung, P.C., Auersperg, N. & Pelech, S.L. (2001). Profiling of protein kinases in the neoplastic transformation of human ovarian surface epithelium. *Gynecol Oncol*, **82**, 305-11.

Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. & Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol*, **13**, 2004-8.

Wu, C., Wangpaichitr, M., Feun, L., Kuo, M.T., Robles, C., Lampidis, T. & Savaraj, N. (2005). Overcoming cisplatin resistance by mTOR inhibitor in lung cancer. *Mol Cancer*, **4**, 25.

Wullschlegel, S., Loewith, R. & Hall, M.N. (2006). TOR signaling in growth and metabolism. *Cell*, **124**, 471-84.

Wyllie, A.H., Kerr, J.F. & Currie, A.R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol*, **68**, 251-306.

Wymann, M.P., Zvelebil, M. & Laffargue, M. (2003). Phosphoinositide 3-kinase signalling--which way to target? *Trends Pharmacol Sci*, **24**, 366-76.

Xing, Y., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B. & Shi, Y. (2006). Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell*, **127**, 341-53.

Xu, Y., Xing, Y., Chen, Y., Chao, Y., Lin, Z., Fan, E., Yu, J.W., Strack, S., Jeffrey, P.D. & Shi, Y. (2006). Structure of the protein phosphatase 2A holoenzyme. *Cell*, **127**, 1239-51.

Yokogami, K., Wakisaka, S., Avruch, J. & Reeves, S.A. (2000). Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. *Curr Biol*, **10**, 47-50.

Yokomizo, A., Tindall, D.J., Drabkin, H., Gemmill, R., Franklin, W., Yang, P., Sugio, K., Smith, D.I. & Liu, W. (1998). PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. *Oncogene*, **17**, 475-9.

Yoon, S. & Seger, R. (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors*, **24**, 21-44.

Yu, D., Zhang, X., Liu, J., Yuan, P., Tan, W., Guo, Y., Sun, T., Zhao, D., Yang, M., Liu, J., Xu, B. & Lin, D. (2008). Characterization of functional excision repair cross-complementation group 1 variants and their association with lung cancer risk and prognosis. *Clin Cancer Res*, **14**, 2878-86.

Yu, H., Spitz, M.R., Mistry, J., Gu, J., Hong, W.K. & Wu, X. (1999). Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. *J Natl Cancer Inst*, **91**, 151-6.

Yu, Y. & Merlino, G. (2002). Constitutive c-Met signaling through a nonautocrine mechanism promotes metastasis in a transgenic transplantation model. *Cancer Res*, **62**, 2951-6.

Yuan, T.L. & Cantley, L.C. (2008). PI3K pathway alterations in cancer: variations on a theme. *Oncogene*, **27**, 5497-510.

Zabarovsky, E.R., Lerman, M.I. & Minna, J.D. (2002). Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. *Oncogene*, **21**, 6915-35.

- Zamble, D.B. & Lippard, S.J. (1995). Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem Sci*, **20**, 435-9.
- Zarbo, R.J. & Hammond, M.E. (2003). Conference summary, Strategic Science symposium. Her-2/neu testing of breast cancer patients in clinical practice. *Arch Pathol Lab Med*, **127**, 549-53.
- Zheng, Z., Chen, T., Li, X., Haura, E., Sharma, A. & Bepler, G. (2007). DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. *N Engl J Med*, **356**, 800-8.
- Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W. & Semenza, G.L. (2000). Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res*, **60**, 1541-5.
- Zhou, B., Wang, Z.X., Zhao, Y., Brautigan, D.L. & Zhang, Z.Y. (2002). The specificity of extracellular signal-regulated kinase 2 dephosphorylation by protein phosphatases. *J Biol Chem*, **277**, 31818-25.
- Zhou, X., Tan, M., Stone Hawthorne, V., Klos, K.S., Lan, K.H., Yang, Y., Yang, W., Smith, T.L., Shi, D. & Yu, D. (2004). Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clin Cancer Res*, **10**, 6779-88.
- Zou, Y., Komuro, I., Yamazaki, T., Aikawa, R., Kudoh, S., Shiojima, I., Hiroi, Y., Mizuno, T. & Yazaki, Y. (1996). Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. *J Biol Chem*, **271**, 33592-7.
- Zuber, J., Tchernitsa, O.I., Hinzmann, B., Schmitz, A.C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A. & Schafer, R. (2000). A genome-wide survey of RAS transformation targets. *Nat Genet*, **24**, 144-52.