EGFR and related therapeutic targets in Malignant Pleural Mesothelioma

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Abstract

Introduction

Malignant pleural mesothelioma (MPM) is a rare but aggressive disease and the current first line treatment is associated with a survival rate of 40%. There is currently no second line therapy. This study aimed to explore the expression of proteins in the arachidonic acid pathway, VEGFR-2 pathway, EGFR/HER2 pathway, c-MET pathway and the PI3K/AKT/MTOR pathway in MPM archival tissue samples and cell lines. We also investigated the cytotoxic effect of inhibitors for each individual pathway as single agents and in some instances as combinations.

Materials and Methods

Immunohistochemical analysis was performed in 93 archival MPM tissue samples to determine the expression of the HER2, 5-LOX, 12-LOX, VEGFR-2 and c-MET proteins. Mesothelioma cell lines NCI-H2452, NCI-H2052, MSTO-211H and a non-small cell lung cancer cell line A549 were used for western blot analysis, MTS assay and *in vitro* scratch assay. Western blot analysis was used to evaluate 5-LOX, 12-LOX, VEGFR-2, EGFR, p-ERK, ERK, c-MET, PTEN and p70S6K protein expression in the cell lines. The antiproliferative effect of Baicalein (12-LOX), Zileuton (5-LOX), MK886 (FLAP inhibitor), Celecoxib (COX-2), Cediranib (VEGFR-2), MGCD265 (c-MET/VEGFR inhibitor), Afatinib (EGFR/HER2), Gefitinib (EGFR), Selumetinib (MEK), Tivantinib (c-MET), Crizotinib (c-MET/ALK), SU11274 (c-MET), Onartuzumab (MET monoclonal antibody), NVPBEZ235 (PI3K/AKT/MTOR), VS5584 (PI3K/AKT/MTOR), Ku0063794 (MTOR1/MTOR2), XL388 (MTOR1/MTOR2) was assessed as single agents and in combinations and analysed using the MTS proliferation assay.

Results

Positive 5-LOX and 12-LOX protein expression was seen in 73% (56/77) and 83% (69/83) of archival MPM tissue samples respectively. NCI-H2452, NCI-H2052, MSTO-211H and A549 cells also expressed 5-LOX and 12-LOX proteins. Baicalein was effective in all cell lines. Combination of celecoxib (3 µM) and baicalein (10 µM) was synergistic in the MSTO-211H cell line. Positive VEGFR-2 protein expression was seen in 93.8% (75/80) of archival tissue samples. Cediranib demonstrated cytotoxic effect at doses higher than the clinical relevant dose. MGCD265 also reduced cell proliferation in all cell lines. Positive HER2 expression was seen in 86.2% (69/80) of archival tissue samples. All cell lines expressed EGFR, p-ERK and ERK protein. Gefitinib and Afatinib demonstrated cytotoxic effects at doses significantly higher than their therapeutically relevant doses. Positive c-MET expression was seen in 82%

(58/71) of archival tissue samples. NCI-H2452, NCI-H2052, MSTO-211H and A549 cells also expressed c-MET protein. Crizotinib inhibited cell growth by 50% in MSTO-211H cells within its clinically relevant dose. SU11274 also reduced cell growth by 50%. Tivantinib reduced cell growth by 50% in all cell lines at doses significantly lower than its clinically achievable dose of 4 μM. A549 and MSTO-211H cells positively expressed the p70S6K protein and loss of PTEN was also observed in the MSTO-211H cells. PI3K/AKT/MTOR inhibitors, NVPBEZ235 and VS-5584 significantly reduced cell growth by 50% at low nanomolar IC50 values. VS-5584 was combined in turn with Tivantinib and Afatinib. The combination of VS-5584 with Tivantinib demonstrated enhanced growth inhibition in all cell lines compared to either inhibitors alone. Combination of Tivantinib and Afatinib also enhanced the inhibition of cell growth in all cell lines compared to either inhibitors alone. The addition of cisplatin to the tyrosine kinase combinations produced a synergistic effect.

Conclusions

Our findings suggest that multiple signalling pathways are active in a significant proportion of MPM samples. Co-targeting the c-MET and PI3K/MTOR pathway might be a potential therapeutic strategy for mesothelioma patients. Further work is required to explore the combination of an EGFR inhibitor and a PI3K/MTOR inhibitor when the EGFR inhibitor is fixed at a therapeutically relevant dose. In addition, understanding the molecular mechanism of Tivantinib, Afatinib and VS-5584, through the use of comparative proteomic platforms, could potentially identify predictive biomarkers of response to these anti-cancer agents in mesothelioma patients.

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Author's declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS code of practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Presentations and Publications

Oguh L, Ranatunge D, Agarwal V, Lind MJ, Cawkwell L. *Lipoxygenase (LOX) expression and inhibition in Malignant Pleural Mesothelioma*. Oral presentation – Hull York Medical School Research Network Conference (June 2014, York, UK).

Oguh L, Cawkwell L, Lind MJ. *Potential therapeutic strategies in Malignant Pleural Mesothelioma*. Oral presentation – Daisy seminar series (August 2014, Hull, UK)

Oguh L, Ranatunge D, Agarwal V, Campbell A, Lind MJ, Cawkwell L. *Immunohistochemical study of Lipoxygenase (LOX) expression in MPM and the effect of LOX inhibitors on Mesothelioma cell lines using MTS assay*. Poster presentation – International Mesothelioma Interest Group (IMIG) (October 2014, Cape Town, South Africa).

Oguh L, Lind MJ, Cawkwell L. *The In vitro effect of novel receptor tyrosine kinase inhibitors on mesothelioma cells.* Poster presentation – International Mesothelioma Interest Group (IMIG) (October 2014, Cape Town, South Africa)

Oguh L, Ranatunge D, Agarwal V, Campbell A, Lind MJ, Cawkwell L. *Effect of c-Met and MTOR inhibitors in malignant pleural mesothelioma (MPM) cells*. Poster presentation – EACR-AACR-SIC Special Conference (June 2015, Florence, Italy)

Ranatunge D, **Oguh L**, Lind MJ, Upadhyay S, El-Mahdawi N. *A Retrospective study of a single centre clinical experience in maintenance Pemetrexed in Advanced non-squamous non-small cell lung cancer*. Poster presentation – European Society for Medical Oncology) (September 2016, Vienna, Austria)

Oguh L, Agarwal V, Ranatunge D, Laufer S, Campbell A, Lind MJ, Cawkwell L. *The investigation of lipoxygenases as therapeutic targets in malignant pleural mesothelioma.* – **under review**

Oguh L, Ranatunge D, Campbell A, Lind MJ, Cawkwell L. *Novel therapeutic strategies in Malignant Pleural Mesothelioma*. Poster presentation – Hull York Medical School Research Network Conference (May 2015, Hull, UK).

Abbreviations

AKT	Protein kinase B
ATP	Adenosine triphosphate
ATTC	American Type Culture Collection
BAX	Bcl-2-associated X protein
BRAF	V –raf murine sarcoma viral oncogene homolog B1
Bcl-xL	B-cell lymphoma-extra large
BID	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
c-MET	Mesenchymal Epithelial Transition factor
CALGB	Cancer and Leukamia Group B
CO2	Carbon dioxide
COX	Cyclooxygenase
CR	Complete response
DAB	3,3-Diamino Benzidine Tetrahydrochloride
DEP	Differentially Expressed Protein
DH2O	Distilled Water
DMF	Dimethylformanide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECACC	European Collection of Cell Cultures
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicine Agency
EP	Prostaglandin E Receptor
EORTC	European Organisation for Research and Treatment of Cancer
FDA	Food and Drug Administration
FLAP	5LOX activating protein
FGF	Fibroblast Growth Factor
GAP	GTPase-Activating Protein
GTP	Guanosine triphosphate
Gy	Gray
H2O2	Hydrogen Peroxide
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitor
HER	Human Epidermal Growth factor Receptor
HETE	Hydroxyeicosateraenoic
HGF	Hepatocyte Growth Factor
HNSCC	Head and Neck Squamous cell cancer
HRP	Horseradish Peroxidase

HUVEC	Human Umbilical Vein Endothelial Cells
IC50	Half maximal inhibitory concentration
IGF-IR	Insulin-like Growth Factor 1 Receptor
IHC	Immunohistochemistry
IMIG	International Mesothelioma Interest Group
ISEL	Ingenuity Pathway Analysis
IRSI	Insulin Receptor Substrate 1
kDa	Kilo-daltons
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
Mins	Minutes
ml	Millilitre
MM	Matrix Metalloproteinases
mM	Millimolar
MPF	Megakaryocyte Potentiating factor
MPM	Malignant Pleural Mesothelioma
MTOR	Mammalian Target of Rapamycin
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(-3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFkβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PDGFR	Platelet Derived Growth Factor Receptor
PFS	Progressive Free Survival
PG	Prostaglandins
PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphoinositide-3-kinase catalytic alpha polypeptide
PIP	Phosphatidylinositol phosphate
PKA	Protein Kinase A
PLGF	Placental Growth Factor
PR	Partial Response
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RR	Response rate

RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Stable response
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
si-RNA	Small interfering ribonucleic acid
SV40	Simian Virus 40
TBS	Tris Buffered Saline
TGF-α	Transforming Growth Factor alpha
TKI	Tyrosine Kinase Inihibitors
TNF-R1	Tumour necrosis factor alpha receptors
TNFα	Tumour necrosis factor alpha
TRAIL	TNF related apoptosis-inducing ligand
TSC	Tuberous Sclerosis Complex
µg	Microgram
µl	Microlitre
µm	Micrometre/micron
µM	Micromolar
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

CHAPTER ONE

MALIGNANT PLEURAL MESOTHELIOMA

Chapter 1 Introduction to Malignant Pleural Mesothelioma

1.1 Epidemiology

Malignant Mesothelioma (MM) is a rare, aggressive tumour of the protective lining (mesothelium) of the pleural, peritoneal (including the tunica vaginalis) and pericardial cavities (Donington et al., 1995; Crispi et al., 2010; Carbone et al., 2011). Malignant Pleural Mesothelioma (MPM) is the most common type of MM accounting for 80% of mesothelioma cases, followed by peritoneal malignancy characterised by a shorter survival time of 6 months (van Meerbeeck et al., 2011) Over several decades, MPM has become a major public and occupational health problem with a survival period of 9-12 months (Galateau-Salle, 2006) and higher mortality rate in men (Carbone et al., 2011).

Several attempts to estimate the global incidence of the disease has been futile due to unreported cases in developing countries known to use asbestos extensively. This could be as a result of inadequate resources for the diagnosis of mesothelioma or/and lack of awareness of its carcinogenic effects (Park et al., 2011). The global incidence of mesothelioma still remains vague and the continuous increase in its incidence is attributed to the extensive use of asbestos in the past century (Yang et al., 2009). Despite the ban on asbestos use in developed countries since the end of the 1980s, the incidence of MPM is still on the rise in various parts of the world.

In a recently analysed 10-year record of global mesothelioma deaths obtained from the World Health Organisation (WHO) database, 83 countries reported a total of 92253 deaths (71975 males and 20248 females) most of which occurred in high-income countries (Figure 1.1). The United Kingdom and Australia had significantly high age-adjusted mortality rate of 17.8 and 16.5 cases per million respectively (Delgermaa et al., 2011). This correlates with the high consumption rate of asbestos by these countries between the late 1960s and early 1970s (Peto et al., 1999). In 2014, the Health and Safety Executive (HSE) reported the number of mesothelioma deaths over a 45-year period attributed to asbestos exposure in Great Britain (Figure 1.2). This report showed an exponential increase from 153 deaths in 1968 to 2538 deaths in 2013 and over 80% were among men with prior occupational asbestos exposure (HSE, 2014). Several studies have also predicted a peak in mortality rates at 1950-2450 annual deaths between 2011 and 2015 with over 60000 deaths from 2002 to 2050. An estimate of about 91000 deaths from mesothelioma are predicted to be recorded for the period 1968-2050 in the UK (Hodgson et al., 2005; Tan et al., 2010). Nevertheless, certain cities in England are noticeably more affected than others. Most of these affected cities are those with the history of asbestos use within the shipping industry. Hull is an example of a city in the

Yorkshire and Humber region of England whose economy was built on trades within the shipping industry. The extensive use of asbestos in its dockyard could be attributed to the increase in the number of mesothelioma cases within the region over the past decade (Figure 1.3). Although the incidence of MPM is predicted to have reached its peak in some countries, this hypothesis is unlikely to be global. In the United States of America there has been a decline in the incidence of MPM for over two decades as reported by the Surveillance, Epidemiology and End Results program (SEER). Incidences in France, Netherlands and Australia are predicted to peak in the years 2025-40, 2017 and 2010 respectively (Weill et al., 2004). These predictions are yet to be established and may not be attainable considering country-specific variations relating to asbestos consumption, legislation and genetic factors (Bianchi & Bianchi, 2007; van Meerbeeck et al., 2011; Carbone et al., 2012). Asbestos use is still on the increase in Asia and the incidence of MPM is expected to increase over the next decade in such areas (Robinson and Lake 2005; Le et al., 2011). A recent study reported an increase in the number of MPM cases in Western Australia associated with home renovations and maintenance in both men and women (Olsen et al., 2011); similar cases might become noticeable in other developed countries in the attempt to eradicate asbestos.

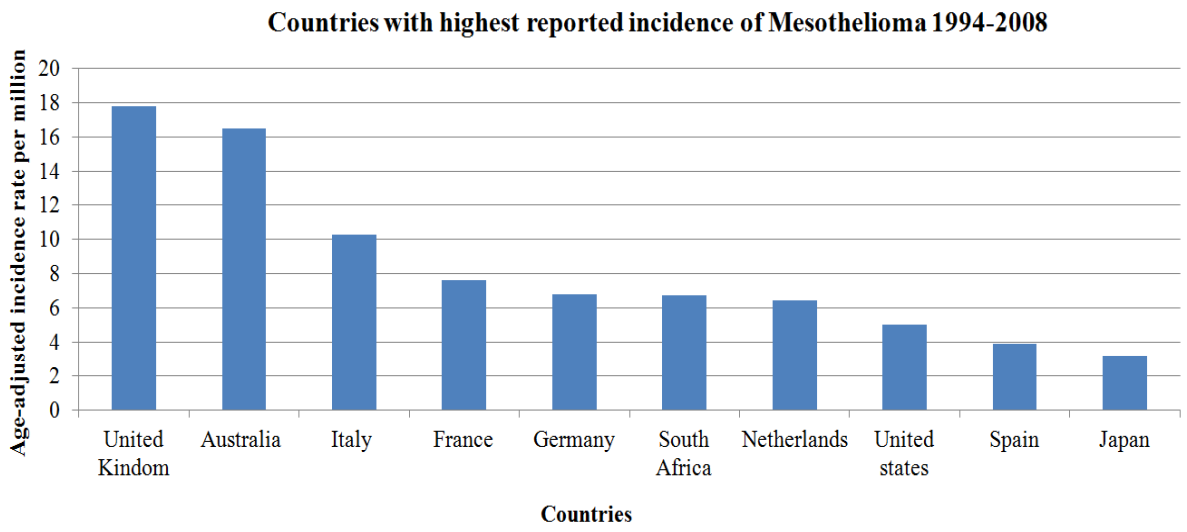


Figure 1.1 Incidence of mesothelioma in some countries. This table shows the estimated age-adjusted mortality rate per million, with the United Kingdom having the highest incidence. (Adapted from Bianchi & Bianchi 2007)

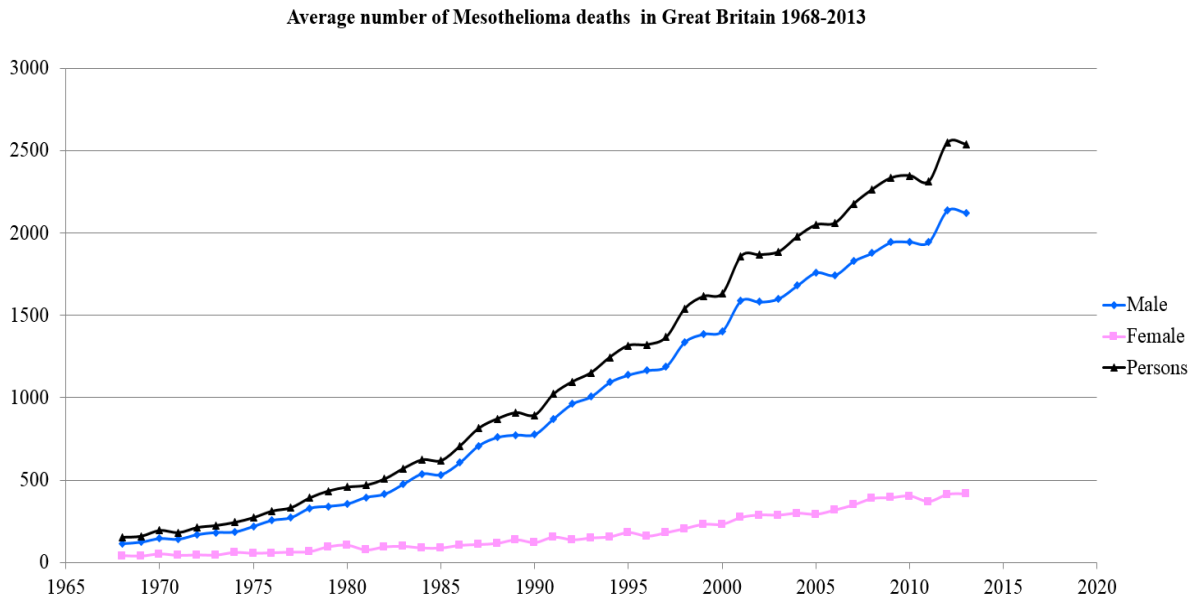


Figure 1.2 A line graph showing number of deaths from mesothelioma in Great Britain. This shows the rapid increase in the incidence of mesothelioma among men from 1968-2013 and very low incidence in women. (Data reproduced with permission from Health and Safety Executive, *Mesothelioma Mortality in Great Britain 1968-2013*. HSE, 2014)

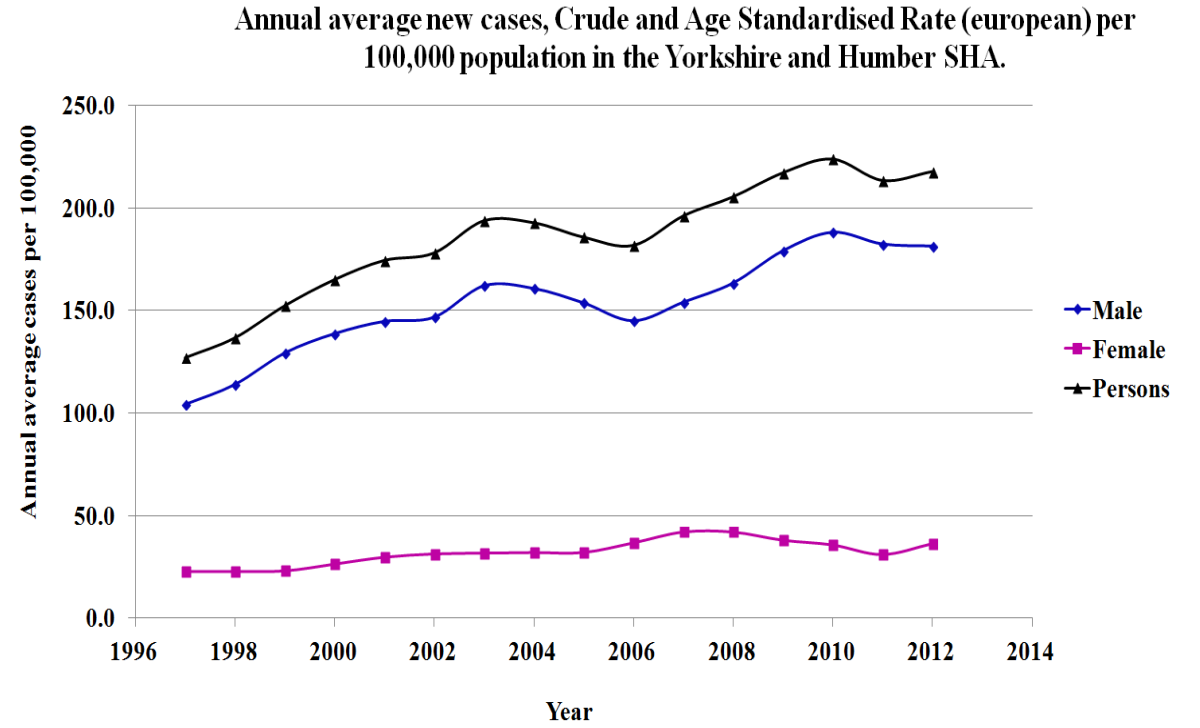


Figure 1.3 Mesothelioma incidence within the Yorkshire and Humber health authority. This graph shows a continuous increase in the number of mesothelioma cases since 1997 up to 2012. As represented globally, there is higher incidence in males than females (Data produced with permission from the Yorkshire and Humber Strategic Health Authority).

1.1.1 Aetiology

The established aetiological factor associated with mesothelioma is exposure to asbestos fibres in both occupational and non-occupational settings which accounts for about 80% of mesothelioma cases (Spirtas et al., 1994). Its association with the disease was first described by Wagner and colleagues in the 1960s (Wagner et al., 1960), with a mean latency period of 32 years (Pass et al., 2004). The Simian virus (SV40), a DNA monkey virus has also been implicated as a co-carcinogen in the development of MPM (Carbone et al., 1997; Bocchetta et al., 2000). Familial cases have been reported which supports the role of genetic predisposition in the development of the malignancy following exposure to a non-asbestos mineral- Eronite in some villages in Turkey (Bianchi et al., 2004; Dogan et al., 2006; Carbone et al., 2007).

1.1.1.1 Asbestos

Asbestos is a group of naturally occurring hydrated silicate mineral fibres with inextinguishable properties as the name denotes. The two main groups are: the serpentines and amphibole. Serpentine asbestos refers to those made up of long, curly fibres while amphiboles are those made up of long, straight fibres that are easy to inhale. Chrysotile belongs to the serpentine group and accounts for about 95% of global asbestos production. The commercially used amphiboles are amosite, crocidolite, actinolite, tremolite, and anthophyllite. The commercial exploit of asbestos in modern times dates back to early 1800s and rapidly increased as a result of improved industrialization when asbestos was used to replace other materials due to its tensile strength and high resistance to electricity, chemicals, heat and fire (Virta, 2006; Carbone et al., 2011). Chrysotile fibres are thought to be the least carcinogenic because they are easily degraded and dissolved in the lungs whilst the amphiboles; with thinner and longer fibres are more readily trapped in the pleura particularly the crocidolites and amosites (Donington et al., 1995; Hoogsteden et al., 1997). The implication of asbestos in the development of MPM was first reported in 1960 when Wagner and his colleagues described 33 cases (22 males and 11 females) of malignant mesothelioma of the pleura in South Africans with prior occupational and environmental exposure to crocidolite asbestos (Wagner et al., 1960). This led to several studies on the carcinogenicity of the mineral fibres; their effect on the increasing incidence of MPM was later followed by a consensus by international organisations to ban the global use of asbestos.

The mechanism of asbestos-induced oncogenesis is not fully understood due to its long latency period and susceptibility of human mesothelial cells to asbestos cytotoxicity.

Exposure to asbestos fibres naturally induces apoptosis in human and rabbit mesothelial cells, a mechanism that might be mediated by reactive oxygen species (ROS) in response to DNA damage. Therefore, the development of asbestos induced neoplasm would imply that the cells evade the apoptotic process which might be an important factor in the pathogenesis of MPM (Broaddus et al., 1996). Several studies support a possible mechanism demonstrated by Yang and colleagues suggesting the inhibition of apoptosis via the nuclear factor kappa-light-chain-enhancer (NF-κB) pathway. Inhalation of mineral fibres particularly 'the amphiboles' causes accumulation of macrophages in the lung and pleura and promotes inflammatory response. Small fibres are thought to be phagocytised and eliminated from the lung but larger fibres remain and are insoluble. The large asbestos fibres are phagocytised by macrophages as a form of immune defence resulting in the release of the Tumour necrosis factor-alpha (TNF-α) (a pro-inflammatory cytokine) and mutagenic changes producing deoxyribonucleic acid (DNA) strand breaks and deletions. Depending on cell types, TNF-α induces apoptosis or increases cell survival by activating the NF-κB pathway. In human mesothelial cells, exposure to asbestos fibres induces the expression of TNF-α receptor (TNF-R1) and stimulates the release of TNF- α in a paracrine and autocrine manner. Activation of the NF-κB pathway increases survival and replication of asbestos-induced DNA damaged human mesothelial cells resulting in the development of MPM (Figure 1.4) (Yang et al. 2006; Yang et al. 2009).

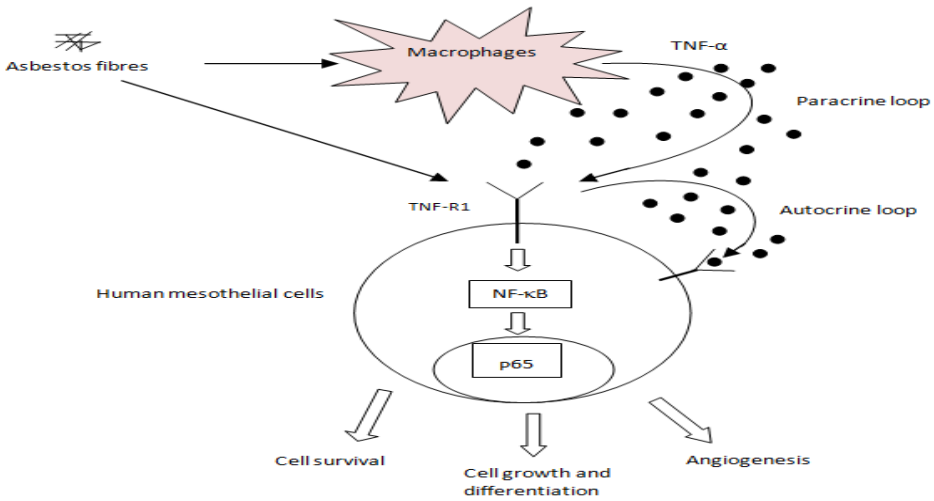


Figure 1.4 Pathogenesis of asbestos induced mesothelioma. Asbestos fibres in the lungs and pleura result in the accumulation of macrophages. Macrophages phagocytise the fibres resulting in the release of TNF-α; the fibres also stimulate human mesothelial cells to release TNF-α in an autocrine and paracrine manner and induce TNF-R1. TNF-α activates the NF-κB pathway resulting in the resistance of cells to apoptosis. (Adapted from Zucali et al. 2011)

1.1.1.2 SV40 and MPM

The Siman 40 virus (SV40) is a small, circular double-stranded DNA polyomavirus which is thought to have found its way into the human genome through the contamination of polio vaccines raised in rhesus monkeys in the 1960s (Carbone et al. 1997; Strickler et al. 1998). The molecular basis of the semi-permissive characteristics and varied susceptibility of human cells to SV40 infection remains unclear, however mesothelial cells have been reported to be uniquely prone to SV40 transformations and possibly act as co-carcinogens with asbestos (Bocchetta et al., 2000). The SV40 encodes several proteins including the small tumour antigen (tag) and the large tumour antigen (Tag) significantly associated with oncogenesis and transformation (Figure 1.5). Both proteins lie within the early region of the SV40 genome where initial transcription occurs within the host cell. Tag, a 90kDa protein mainly found in the nucleus of infected cells is a direct mutagen inducing chromosomal aberrations in order to alter stability and karyotype of the host genome.

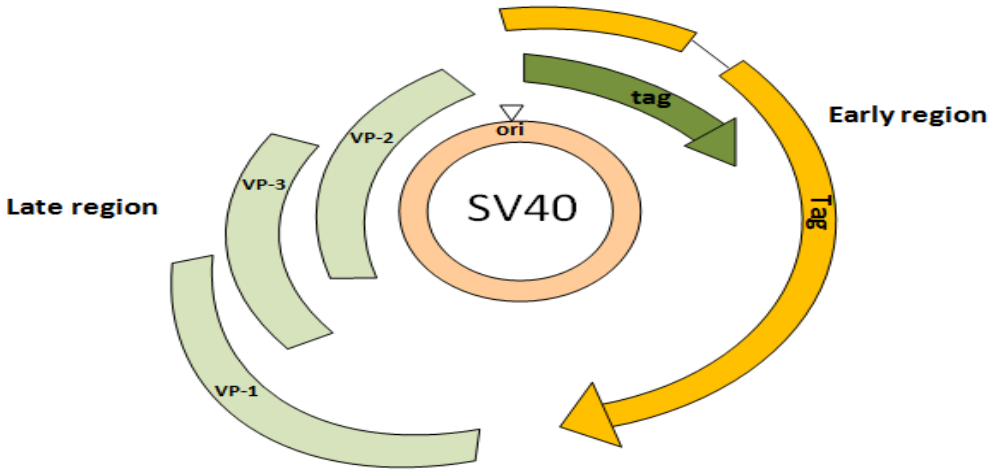


Figure 1.5 A diagrammatic representation of the genome of SV40. The SV40 genome has a circular DNA and consists of an early and a late region. The early region encodes the tumour antigens, Tag and tag responsible for DNA replication of the virus. The late region encodes the capsid proteins responsible for viral assembly: VP-1, VP-2 and VP-3. Also indicated is the ori; origin of DNA replication. (Adapted from Rizzo et al. 2001).

It binds and inactivates products of tumour suppressor genes such as p53, retinoblastoma (pRb), p107, p130/Rb2, p300, p400 allowing the replication of DNA damaged cells prior to transformation. In addition to this, it also induces the insulin-like growth factor 1 and its receptor resulting in cell proliferation and survival. The small tumour antigen tag, a 17kDa protein found in the cytoplasm of infected host cells is reported to inhibit the cellular

phosphatase 2A (PP2A) resulting in the upregulation of the transcription factor AP1 and aids Tag in the inactivation of tumour suppressor genes (Pass et al., 1998; Cacciotti et al., 2001; Rizzo et al., 2001; Powers & Carbone, 2002; Toyooka et al., 2002; Gazdar & Carbone, 2003). Carbone and colleagues were the first to report the presence of SV40 DNA sequences in human pleural mesothelioma by immunoblotting, PCR, immunohistochemical staining and immunoprecipitation. They found 60% of the mesothelioma cases contained SV40-like DNA sequences and 81% of 16 different specimens positively expressed the SV40 large T antigen. This finding was further confirmed by a multi-institutional study led by Testa et al in 1998 and other studies from different research group identifying SV40 homologous sequences in 10% - 60% of malignant mesothelioma in different geographical locations (Carbone, 1999; Ramael et al., 1999; Shivapurkar et al., 1999; De Rienzo et al., 2002; Cristaudo et al., 2005; Comar et al., 2007; Zekri et al., 2007). Microdissection experiments by Shivapurkar and colleagues further identified the specificity of SV40 to mesothelioma cells but not surrounding stroma. *In vitro* studies have demonstrated that the PI3K/Akt signalling pathway induces resistance to apoptosis in human mesothelial cells and mesothelioma cells. Progressive resistance was observed upon exposure of SV40 or Tag-positive cells to toxic agents in response to growth factors (Cacciotti et al., 2005). Another study also demonstrated that long term exposure of mesothelial cells to asbestos and SV40 could induce chemotherapy resistance, a common phenomenon in mesothelioma (Cleaver et al., 2013). On the contrary, other research groups were unable to detect SV40 DNA sequences in their population arguing against the role of SV40 in the pathogenesis of MPM and suggesting that such areas may not have been exposed to SV40 contamination from the polio vaccine despite extensive use (López-ríos et al., 2004; Manfredi et al., 2005). An epidemiology study using data from the SEER program was unable to identify a significant increase in mesothelioma rates due to exposure to the SV40 contaminated vaccines in different birth cohorts (exposed versus unexposed) (Strickler et al., 1998). The role of SV40 in MPM remains a controversy and the polyomavirus remains an important tool in the study of viral oncogenesis despite its possible pathogenic role in humans.

1.1.1.3 Other factors

Apart from SV40 virus there are several non-asbestos related factors that could cause MPM such as eronite, ionizing radiation, genetic predisposition and organic chemicals. There are a growing number of studies of the occurrence of MPM as a second primary malignancy after receiving radiotherapy for other types of malignancies. The median latency period between

the MPM development and the first cancer was 4.3 years (Cavazza et al., 1996). A study by De Bruin et al found that the risk of developing MPM was increased by almost 30-fold in Hodgkin lymphoma patients following radiation treatment when compared with the general population (De Bruin et al., 2009). This could be as a result of improved survival of patients after radiotherapy plus chemotherapy treatment. Recently, Chirieac et al (2013) reported that patients with lymphoma-associated pleural diffuse malignant mesothelioma (PDMM) have distinct histologic patterns when compared with those with asbestos-associated PDMM. Lymphoma-associated PDMM patients were also significantly younger at diagnosis with significantly improved overall survival (32.5 vs 12.7 months; p=0.018). In both malignancies, histologic subtype was an important predictor of survival as previously established (Chirieac et al., 2013)

Eronite, a naturally occurring mineral fibre found in many parts of the world has been implicated in the pathogenesis of MPM and is found to be more potent than asbestos. In an *in vitro* study by Wagner and his colleagues, 200 rats (male and female) were allocated into 5 treatment groups and injected intrapleurally with 20mg eronite, Turkish rock fibre, chrysolite, saline and non-fibrous zeolite. All rats injected with eronite developed mesothelioma while 48% of the group treated with chrysolite asbestos developed the disease (Wagner et al., 1985). Endemic malignant mesothelioma in Cappadocia led to the discovery of eronite in the lungs of its residents. Several studies have shown that eronite is a major cause of mesothelioma in areas where it is used in the construction of buildings and roads. These findings have also led to the implication of genetic predisposition due to high incidence of the disease within some families. Pedegree studies suggest the possibility of an autosomal dominant pattern of inheritance among such family members (Roushdy-Hammady et al., 2001; Dogan et al., 2006).

1.1.2 Anatomy of the lungs and pleura

The lungs are two large coned-shaped organs of the lower respiratory system which occupy most of the thoracic cavity except the mediastinum which encloses the heart (Figure 1.6). Each lung divides into distinct sections known as lobes. The main function of the lungs is the oxygenation of blood. This is carried out by maintaining a constant close relationship between inspired air and venous blood in the pulmonary capillaries. The right lung has three lobes and the left lung has two. The right lung is heavier and larger but shorter and wider than the left lung. This difference in width is due to the height of the right dome (pushed up by the

liver) in the diaphragm and the bulging of the pericardium and heart towards the left. The surface of the lungs is covered by two continuous thin protective membranes known as the Visceral and Parietal pleura.

The pleural membranes secrete a small amount of pleural fluid (5 – 10 ml) within the pleural space lubricating the surfaces of the pleura. This pleural fluid allows the visceral and parietal pleura slide on each other during respiration to avoid friction. The lung surface and the thoracic wall are also kept in contact by a cohesion force resulting from the surface tension of the pleural fluid. The parietal pleura is thicker than the visceral pleura and can be separated from the surface in surgery, this is not possible with the visceral pleura as it cannot be separated from the lungs (Snell, 2004, Moore et al., 2010). The visceral layer is lined by simple squamous epithelium known as the mesothelium. It consists of mesothelial cells which extend over the entire surface of the peritoneal, pericardial and pleural cavities. The major functions of the mesothelium include providing a protective barrier against physical damage, invading microorganisms, transport of fluids and cells, tissue repair, initiation and resolution of inflammation (Mutsaers, 2002). Mesothelial cells in response to asbestos exposure secrete several pro-, anti-inflammatory and immunomodulatory mediators such as prostaglandins, nitric oxide, reactive nitrogen and oxygen species, growth factors, cytokines and extracellular matrix molecules in an attempt to restore the normal serosal function and architecture. A study by van de Wal and colleagues in a murine model suggested that traumatized mesothelial surfaces support tumour cell adhesion and proliferation due to upregulation of adhesion molecules in response to inflammatory mediators and localized growth factor production (van der Wal et al., 1997).

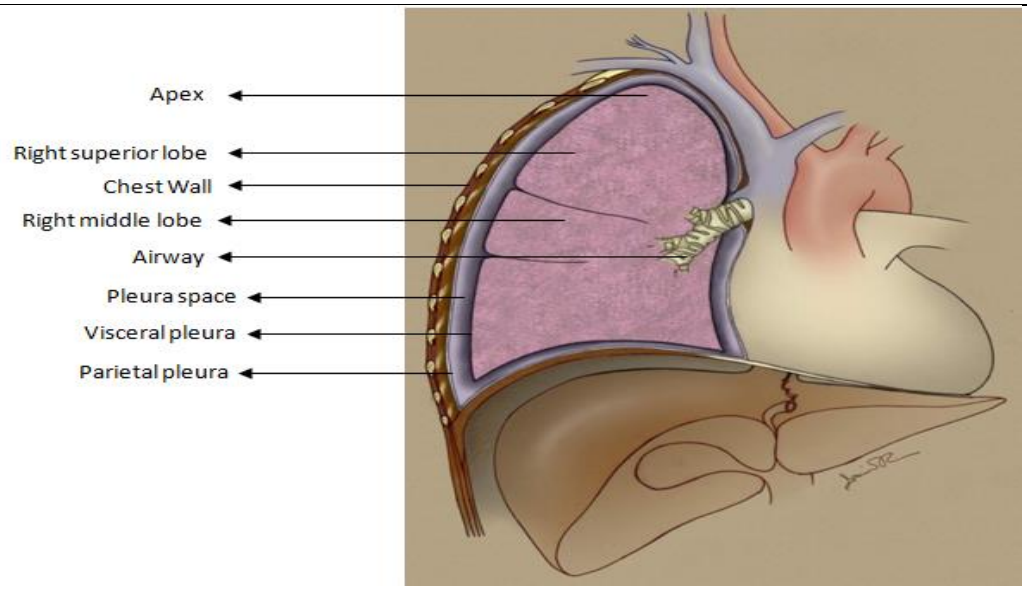


Figure 1.6 A pictoral representation of a healthy lung. This diagram shows the structure of the right lung highlighting its major features. The apex of the lung is the narrow superior end that extends to the clavicle and its base is the broader area that rests just above the diaphragm, a primary muscle of respiration that separates the thoracic cavity from the abdominal region. The parietal pleura line the thoracic cavity, the thoracic surface of the diaphragm and the lateral region of the mediastinum. The visceral pleura completely covers the surfaces of the lungs including the depths of the interlobar fissures. Both layers of pleura are separated by the pleural space formed from the reduction of the coelomic cavity during the formation of the lungs. Adapted from Havey I. Pass **http://www.slideshare.net/mbelamaric/harvey-pass?from_search=1**

1.1.3 Pathology

1.1.3.1 Histology of the normal pleura

The normal pleura of a healthy individual is a thin tissue consisting of a layer of mesothelial cells overlying a thin layer of vascularised connective tissue (Figure 1.7) (Mescher, 2010).

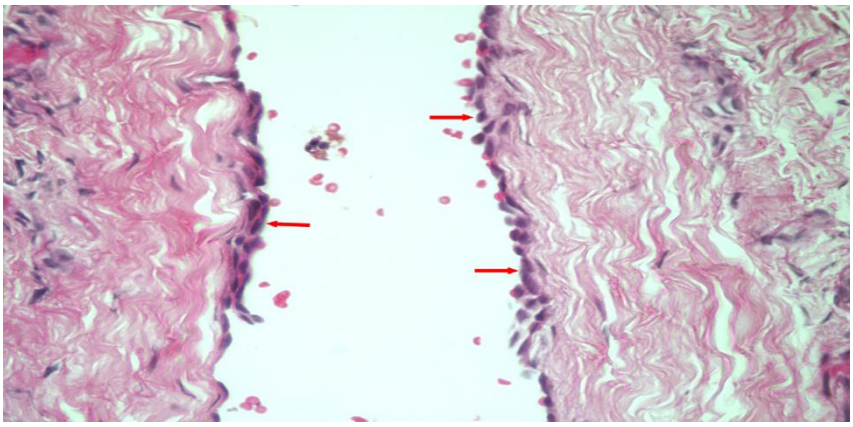


Figure 1.7 Hematoxylin and Eosin staining of the visceral pleura. This picture shows the thin layer of mesothelial cells (red arrows) overlying the connective tissue. The connective tissue is rich in both elastic fibres and collagen and also contains both blood vessels and lymphatics.

1.1.3.2 Histology of Malignant pleural mesothelioma

MPM has the ability to display either epithelial or mesenchymal differentiation hence it is classified into three major histologic subtypes depending on the predominant cell type(s) that constitutes the tumour. The proportion of each is approximately epithelial (60%), sarcomatoid (20%) and biphasic (mixed) type (20%), as reviewed by (Inai, 2008) (Figure 1.8). The epithelioid subtypes consist of cells with epithelial morphology while the sarcomatoid mesotheliomas are composed of fibroblastoid tumour cells and biphasic a combination of both (Hoogsteden et al., 1997; Ambrogi & Mineo, 2012). Several myriad patterns have also been reported among the three major subtypes (Figure 1.9). The epithelial subtype is the most common and is associated with better prognosis (~12 months) while the other two have more severe prognosis (~ <6 -12 months) (Ceresoli et al., 2001; O'Kane et al., 2005).

Mesothelioma is mainly identified by routine haematoxylin & eosin staining however; the morphology of the disease does not provide a differential diagnosis between mesothelioma and other carcinomas. Distinguishing between epithelial mesothelioma and adenocarcinoma, sarcomatoid mesothelioma and other spindle cell carcinoma has been a major diagnostic dilemma which resulted to the use of various immunohistochemical staining using positive and negative markers e.g. calretinin (Tischoff et al., 2011). An update of the guidelines for pathologic diagnosis of malignant mesothelioma by the International Mesothelioma Interest Group as a reference for pathologists was published by Husain et al. 2013. It was proposed that tumour lesions should have greater than 80% specificity or sensitivity to

immunohistochemical markers and in the interpretation of positivity, localization of the stain (cytoplasmic versus nuclear) and percentage of cells stained should be taken into consideration. These proposed guidelines were as a result of technical difficulties that can be faced with immunohistochemistry such as formalin overfixation or negative immunoreactivity due to inappropriate antigen retrieval method for alcohol-fixed tissues. Variations in staining between antibody clones and different laboratories can also occur therefore it is important for laboratories to test antibodies of choice with appropriate controls and chose antibodies with 80% sensitivity or specificity (Husain et al., 2013) .

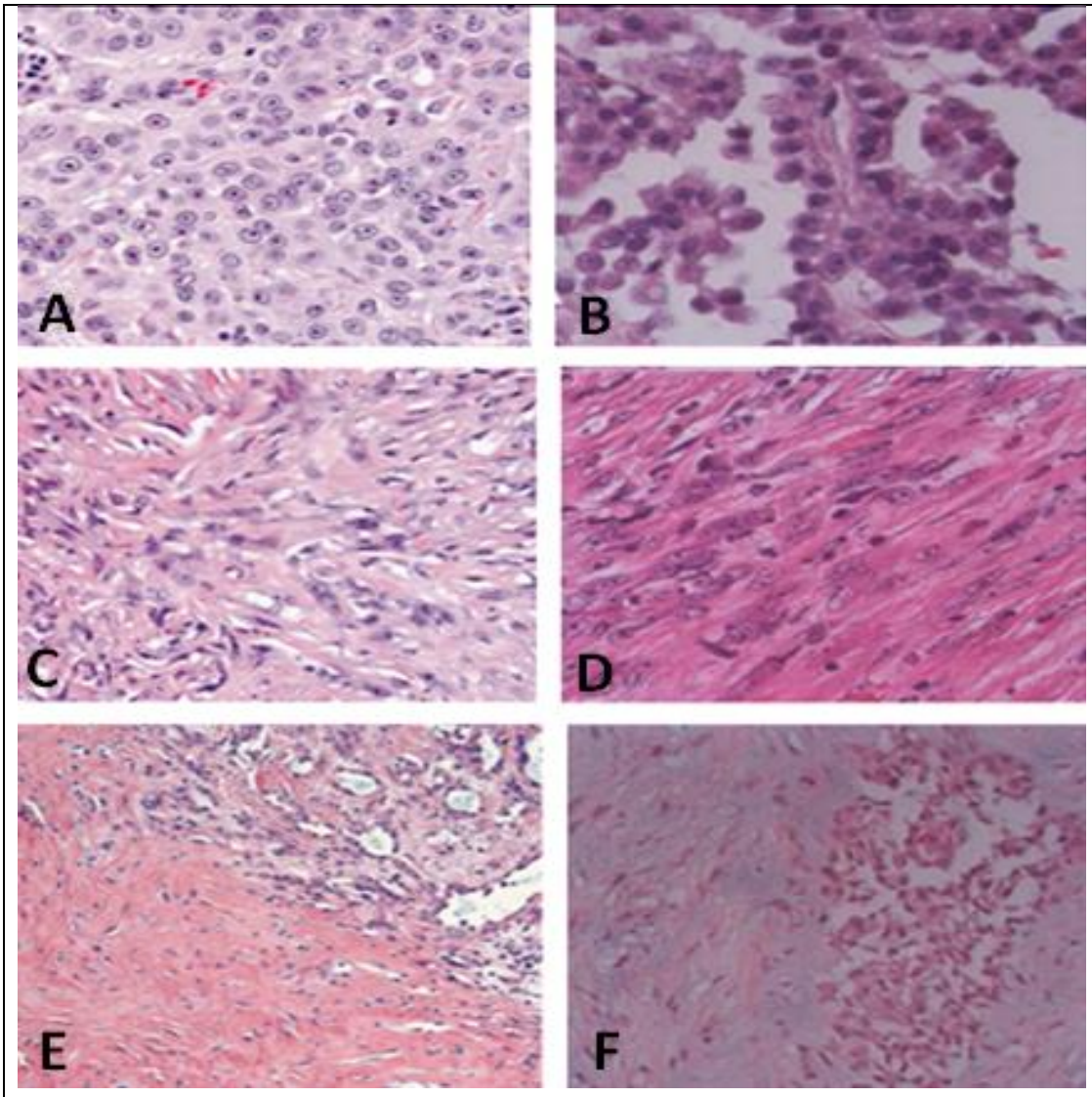


Figure 1.8 Histological illustration of the three major mesothelioma subtypes.
A & B: Haematoxylin &Eosin (H&E) staining of epithelioid mesothelioma showing tubular structures and prominent cytoplasm. Epithelial mesotheliomas are mostly formed by cuboidal cells with tubular structures and eosinophilic cytoplasm. The epithelioid cells are usually large and well differentiated with a centrally placed regular nuclei and prominent nucleoli. The histologic pattern of this subtype is similar to adenocarcinoma and reactive mesothelial hyperplasia.
C & D: H&E staining of sarcomatoid mesothelioma showing a haphazard pattern of cells. Sarcomatoid mesothelioma consists of spindle cells arranged in a haphazard manner with a strong similarity to fibrosarcoma or malignant fibrous histiocytoma.
E & F: H&E staining of biphasic mesothelioma showing both epithelial and fibrotic characteristics. Biphasic mesothelioma which accounts for 20-30% of all mesotheliomas comprises both epithelioid and sarcomatioid components, at least 10% of the tumour are represented by one of the components.

(Adapted and modified from (Pass et al., 2004; Allen, 2005).

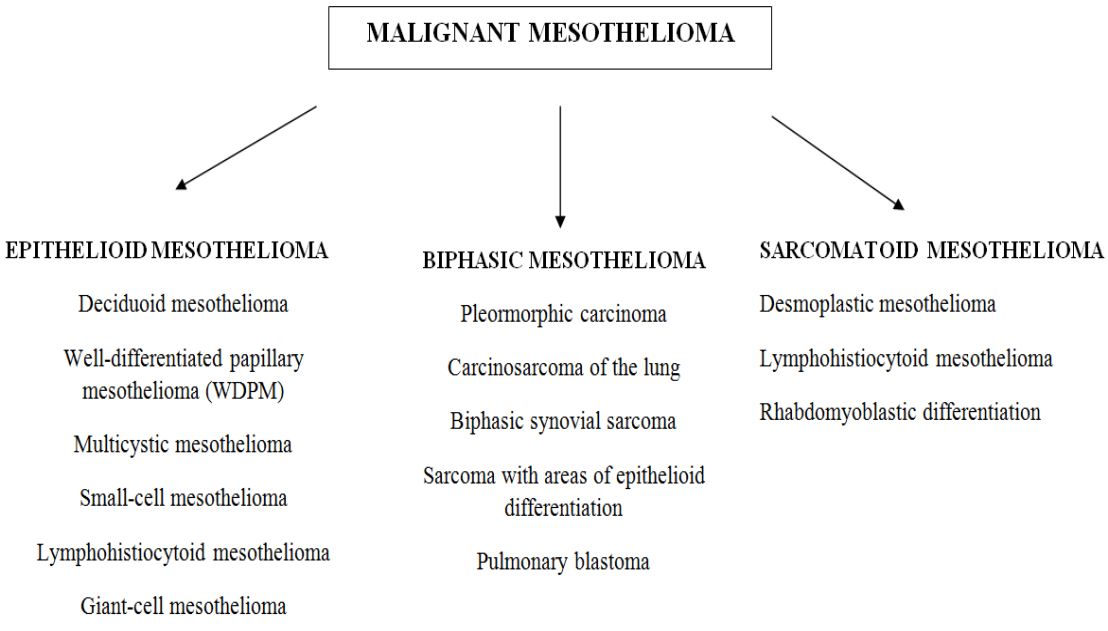


Figure 1.9 Differential diagnosis of pleural mesothelioma. This figure shows the different variants under the three major subtypes of malignant mesothelioma. (Adapted and modified from King et al., 2006).

In addition to the proposal it was recommended that at least two positive mesothelioma markers and two positive carcinoma markers should be included in the immunohistochemical panel (Husain et al., 2013). Useful markers in distinguishing mesothelioma from other neoplasms are summarized in Table 1.1.

Table 1.1 Immunohistochemical markers to distinguish mesothelioma from other carcinomas. (Adapted from Scherpereel et al. 2010; Husain et al. 2013)

Tumour type	**Positive Immunohistochemical Markers**	**Positivity**
Mesothelioma	Calretinin	80 – 100%
	Keratin CK5/6	75 – 100%
	WT-1	70 – 95%
	D2-40 (Podoplanin)	90 – 100%
Lung adenocarcinoma	CEA monoclonal	80 – 100%
	MOC-31	95 – 100%
	Ber-EP4	95 – 100%
	TTF-1	75 – 85%
	B72.3	95 – 100%
	Napsin A	75 – 85%
	BG8	90 – 100%
Squamous cell carcinoma	P63	80 – 100%
	Ber-EP4	20%
	MOC-31	2 – 10%
Breast Carcinoma	ER	~70%

1.1.3.3 Clinical staging

Malignant mesothelioma in its early stage begins as multiple nodules in the parietal pleura, as the disease progresses the nodules coalesce and fuses the two layers of the pleura. The pleural cavity is subsequently obliterated resulting in a thick coat of malignant tissue that covers, compresses and invades the lung, diaphragm and the interlobar fissures (King et al., 2006). Due to the peculiar growth pattern in MPM developing a sufficient staging system that accurately estimates precise tumour volume is very difficult hence staging systems are being constantly reviewed. The first staging system was proposed by Butchart et al in 1976; other staging systems were subsequently developed but they all failed to show accurate correlations of survival and stage. In 1995, the International Mesothelioma Interest Group developed a

universally accepted method based on the tumour node metastasis system which is now commonly used (Butchart et al., 1976; Rusch, 1996). The staging system is outlined in Table 1.2 &Table 1.3 and a pictorial representation can be found in Figure 1.10.

Table 1.2 The IMIG TNM staging system for malignant pleural mesothelioma

Primary Tumours (pT)	
T1	
T1a	Tumour limited to the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura. No involvement of the visceral pleura
T1b	Tumour limited to the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura. Scattered foci of tumour involving the visceral pleura.
T2	Tumour involving each of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: • Involvement of diaphragmatic muscle • Extension of tumour from visceral pleural or confluence of visceral pleural tumour (including the fissures) into the underlying pulmonary parenchyma
T3	Describes locally advanced but potentially resectable tumour. Tumour involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: • Involvement of the endothoracic fascia • Extension into the mediastinal fat • Solitary, completely, resectable focus of tumour extending into the soft tissues of the chest wall • Non-transmural involvement of the pericardium
T4	Describes locally advanced technically unresectable tumour. Tumour involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, visceral pleura) with at least one of the following features: • Diffuse extension or multifocal masses of tumour in the chest wall, with or without associated rib destruction • Direct transdiaphragmatic extension of tumour to the peritoneum • Direct extension of tumour to the contralateral pleura • Direct extension of tumour to mediastinal organs • Direct extension of tumour into the spine • Tumour extending through to the internal surface of the pericardium with or without a pericardial effusion, or tumour involving the myocardium

Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in the ipsilateral bronchopulmonary or hilar lymph nodes
N2	Metastases in the subcarinal or the ipsilateral mediastinal lymph nodes, including the ipsilateral internal mammary nodes
N3	Metastases in the contralateral mediastinal contralateral internal mammary, ipsilateral or contralateral supraclavicular lymph nodes
Distant metastasis (M)	
MX	Presence of distant metastases cannot be assessed
M0	No distant metastasis present
M1	Distant metastasis present

Table 1.3 Different stages of malignant pleural mesothelioma and their description

Staging	Description		
Stage I			
Ia	T_{1a}	N_0	M_0
Ib	T_{1b}	N_0	M_0
Stage II	T_2	N_0	M_0
Stage III	Any T_3	Any N_1 Any N_2	M_0
Stage IV	Any T_4	Any N_3	Any M_1

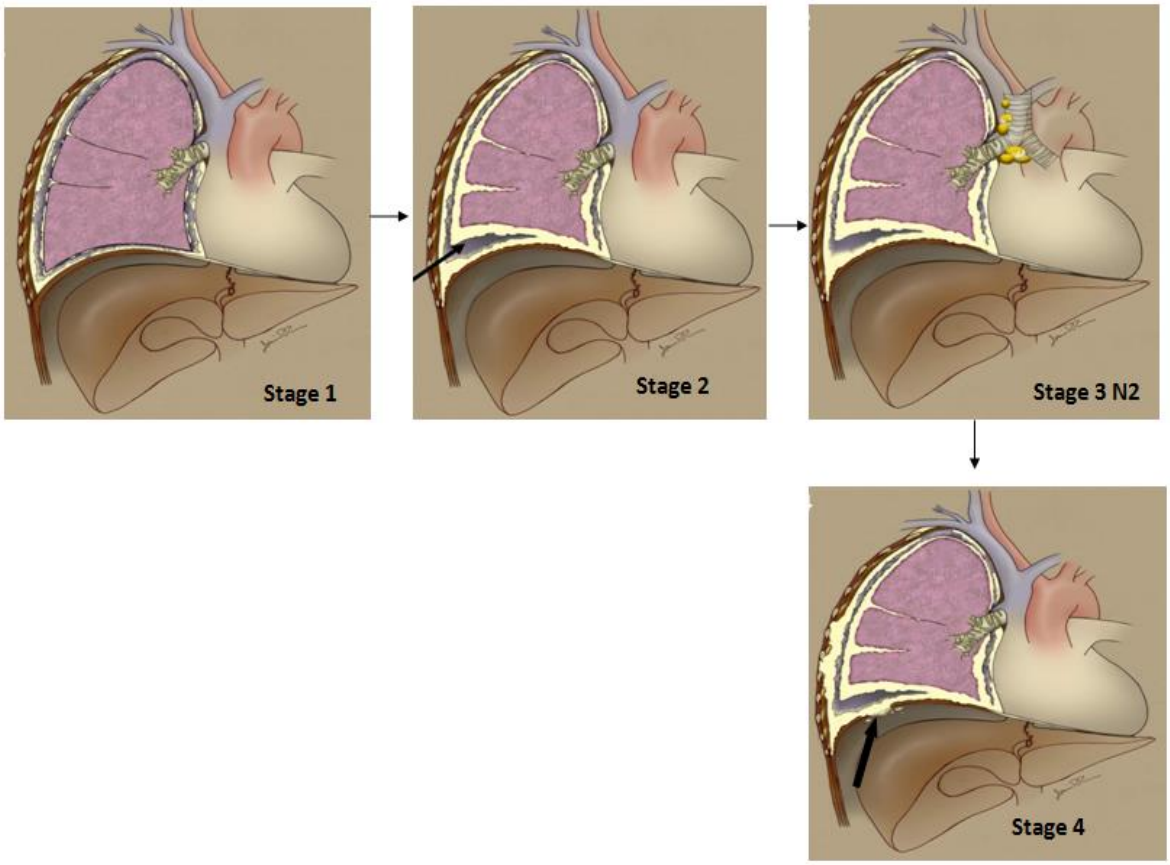


Figure 1.10 Different stages of malignant pleural mesothelioma. Each picture shows the spread of the tumour at each stage. Stage 4 shows distant metastasis to the diaphragm and pericardium. (Adapted from Havey I. Pass **http://www.slideshare.net/mbelamaric/harvey-pass?from_search=1**)

1.1.4 Biomarkers for mesothelioma

The discovery of biomarkers has been very useful in the early diagnosis, prognosis and in predicting response to therapy of several diseases. The search for biomarkers in mesothelioma is ongoing though it remains a challenge due to its rarity and variations in tumour subtypes.

1.1.4.1 Diagnostic Biomarkers

A few potential diagnostic serum markers such as soluble mesothelin-related protein (SMRP), megakaryocyte potentiating factor (MPF) and osteopontin have been identified but not without limitations.

SMRPs are breakdown products of the membrane proteins of mesothelial cells which are indicative of mesothelial cell damage. Levels of SMRPs can be measured through venous blood whereby high levels may indicate mesothelial cell damage or mesothelioma. Test for SMRP levels are not carried out as a stand-alone test but in addition with other mesothelioma

biomarkers. Concentrations of SMRP were found to be elevated in mesothelioma patients with 84% (37/44) sensitivity compared to 2% (3/160) in non-mesothelioma patients making it a potential marker in differentiating MPM from other metastatic pleura carcinomas (Robinson et al., 2003). A commercially marketed test for SMRP is available in form of a two-step immunoenzymatic assay in an enzyme-linked immunosorbent assay (ELISA) format (MESOMARK) (Beyer et al., 2007).

MPF is a soluble 31 kDa protein with cytokine activity (Kojima et al., 1995). It was also found to be upregulated in the serum of MPM patients and had been implicated as a negative prognostic factor when examined with clinical covariates but lacks specificity for the detection of non-epithelial subtypes (Onda et al., 2006; Hollevoet et al., 2012). Mesothelin and MPF show similar diagnostic data in serum and pleural effusions of MPM patient therefore can be used interchangeably (Creaney et al., 2013)

Osteopontin is a 44 kDa glycoprotein involved in cell migration, cell-matrix interactions and other diverse functions. In asbestos-induced rat models and *in vitro* cells exposed to asbestos, osteopontin was found to be upregulated (Sandhu et al., 2000). Osteopontin was also found to be a marker for asbestos exposure duration but not specific for mesothelioma (Pass et al., 2005). Expression of osteopontin in tissue samples of mesothelioma patients further revealed that low osteopontin was significantly associated with improved survival (Cappia et al., 2008). In addition, a comparison study of these three markers did not demonstrate an improvement in the accurate diagnosis of mesothelioma when combined (Creaney et al., 2013). Recently, a study by Pass et al (2012) found plasma fibulin-3 of mesothelioma patients to be significantly elevated but could not confirm it as an early diagnostic marker due to lack of plasma-based longitudinal collection (Pass et al., 2012). Serum and pleural fibulin-3 was able to discriminate between MPM and pleural metastasis of carcinoma or benign pleural effusion using ELISA in 45 patients (Agha et al., 2014). Recently, fibulin-3 was compared with soluble mesothelin and the latter provided better diagnostic accuracy but higher concentration of fibulin-3 in pleural effusion was a significant negative predictor of survival. Further investigation is warranted for a defined role of this biomarker and the identification of other diagnostic biomarkers in the understanding of the biology of malignant pleural mesothelioma (Creaney et al., 2014). Tissue biomarkers used clinically for the diagnosis of mesothelioma have been mentioned in Table 1.1

1.1.4.1.1 Potential tissue biomarkers

Several biological and genetic alterations involved in malignant pleural mesothelioma have been recently identified. These include tissue biomarkers involving different mechanism such as oxidative stress, cell-life modulation, cyclooxygenase and metalloproteinase enzymes, and growth factors (Table 1.4). An improved understanding of the biology and molecular pathways involved in mesothelioma may facilitate the clinical use of some of these biomarkers to predict prognosis. Various studies have reported the correlation of the expression of several protein biomarkers with survival. For example, cyclooxygenase-2 (COX-2) is implicated in multiple events during the tumorigenesis process, producing highly reactive products that may alter apoptosis, immunoresponse, angiogenesis and cell growth. High expression of COX-2 protein was correlated with poor survival (Edwards et al., 2002). In 77 MPM tissue samples, high COX-2 and low p21 and p27 expression was significantly associated with shorter overall survival (Mineo et al., 2010). Similarly, many growth factors have been reported to be highly expressed in malignant pleural mesothelioma and may have significant prognostic indications (Zucali et al., 2011; Davidson, 2015).

Table 1.4 Potential biomarkers identified to be associated with prognosis in MPM. **(**Adapted and modified from Ambrogi & Mineo 2012)

Function	**Correlation with poor prognosis**	
	Direct	**Inverse**
Apoptosis		TRAIL (Liu et al., 2001)
		BAX (Kokturk et al., 2005)
	Glucose transporter-1 (GLUT-1) (Fennell et al., 2004)	PTEN (Opitz et al., 2008)
Cell Cycle	p53 (Hopkins-Donaldson et al., 2006)	p27kip1 (Bongiovanni et al., 2001)
	MIB-1/Ki67 (Comin et al., 2000)	p21 (Baldi et al., 2002)
DNA repair	ERCC1 (Zimling et al., 2012)	
Growth factor	MM2 & MM9 (Edwards et al., 2003)	
	EGF (Edwards et al., 2006)	
	PLGF (Pompeo et al., 2009)	
	VEGF (Demirag et al., 2005)	
	PDGF (Filiberti et al., 2005)	
	FGF (Kumar-Singh et al., 1999)	
	HGF (Tolnay et al., 1998)	
Inflammation	COX-2 (Edwards et al., 2002)	
Lymphatic marker		D2-40 (Chu et al., 2005)
Membrane carrier	Aquaporin 1 (Kao et al., 2012)	Calretinin (Kao et al., 2011)
Oxidative stress	ERK (Buder-Hoffmann et al., 2001)	
	NF-kB (H. Yang et al., 2006)	

1.1.5 Clinical features

The report of the SEER program was one of the pioneer studies to evaluate prognostic factors in MPM. The study examined 1,475 patients with histologically confirmed mesothelioma and identified sex, age, treatment, tumour stage and geographical area as important predictive factors for patient survival (Scherpereel et al., 2010). The currently used clinical prognostic scoring system for MPM was developed by the European Organisation for Research and Treatment of Cancer (EORTC) and the Cancer and Leukemia Group B (CALGB). The CALGB group examined the prognostic significance of individual and joint pre-treatment patient characteristics using exponential regression trees and Cox survival models in 337 mesothelioma patients. Patients with best prognosis with a median survival of 13.9 months were those with PS=0 and > 49years, or PS = 0, ≥ 49 years and haemoglobin (Hb) ≥ 14.6/µL. Patients with PS=1 or PS=2, and a white blood cell count (WBC) ≥ 15.6/µL had the worse median survival time of 1.4 months (Herndon et al., 1998) (Figure 1.11).

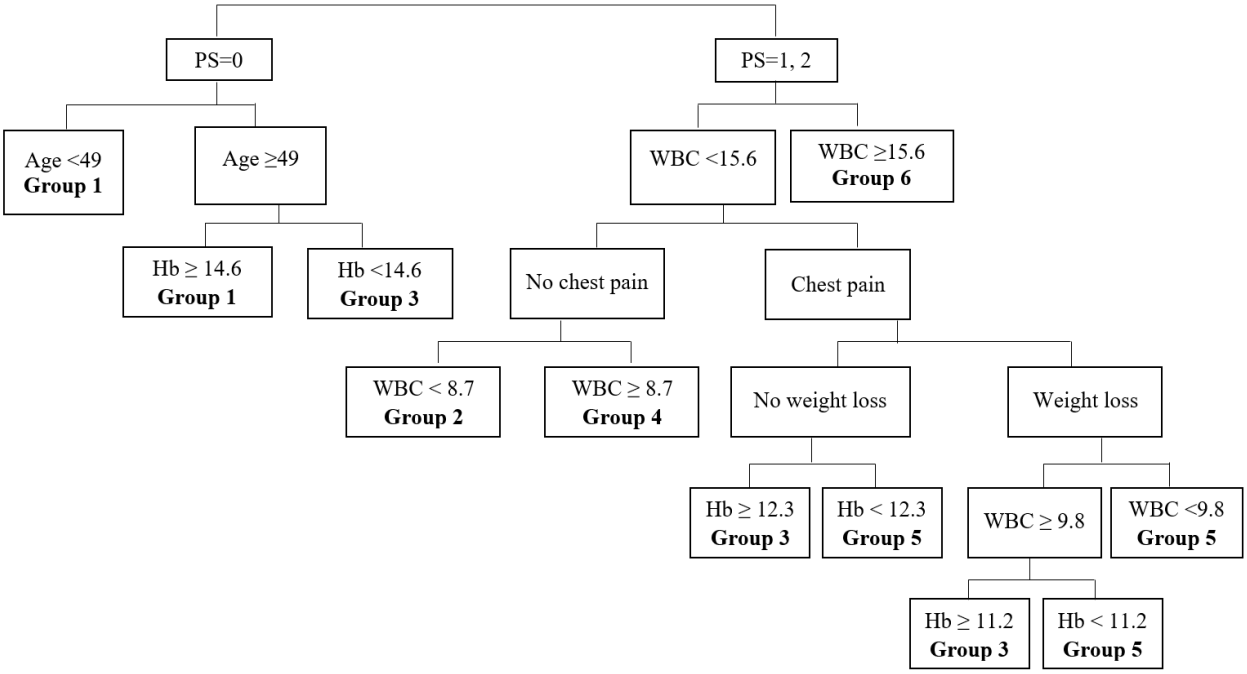


Figure 1.11 Flowchart showing the derivation of the CALGB prognostic groups for Malignant Mesothelioma. The subgroup with the best survival (13.9 months) included patients with PS=0 and age younger than 49 year, and patients with PS=0, age of 49 years or older, and Hb level ≥ 14.6 (Group 1). The worst survival time (1.4 months) was for patients with PS=1/2 and WBC ≥15.6/µL (Group 6) (Herndon et al., 1998).

The EORTC group analysed data from 204 patients with mesothelioma enrolled in five consecutive phase II clinical trials designed to assess the efficacy of different anticancer drugs for a period of nine years. Multivariate analysis revealed the association of high white blood cell count, sarcomatoid subtype, poor performance status, male gender and probability of histological diagnosis of mesothelioma with poor prognosis (Table 1.5). Patients were further divided into two groups based on these five factors; the low-risk group had a median survival time of 10.8 months and 40% 1-year survival rates. Survival time for the high risk group was 5.5 months with 12% 1-year survival rate (Curran et al., 1998). Several multicenter studies have been carried out by different authors to validate the EORTC and CALGB prognostic scoring systems (Edwards et al., 2000; Neumann et al., 2004; Fennell et al., 2005). In 2005, Van Meerbeck et al proposed a modification to the two prognostic scoring system accounting for tumour stage, non-epithelioid histology, loss of appetite and interval between symptoms and diagnosis >50days. Negative predictors of the CALGB system such as presence of chest pain, low HGB levels and thrombocytosis (>350x10^9/L) were also adapted into the new modified system (van Meerbeeck et al., 2005). A study by Tabata *et al* identified serum High-mobility group box 1 (HMGB1) as a prognostic factor in mesothelioma patients when compared to the cohort with history of asbestos exposure but no mesothelioma. *In vitro,* HMGB1 protein also was found to be upregulated in mesothelioma cell lines (H28 and H2052) (Tabata et al., 2013). Several serum or plasma biomarkers have also been identified to have prognostic values in mesothelioma but a large number of them are yet to be implemented in clinical practice. These potential biomarkers require more validation data from large cohort of MPM patient.

Table 1.5 EORTC Prognosis scoring system Malignant Mesothelioma

Factor	Group	Risk score
WBC	>8.3 x 10/L	+0.55
Performance status	1 or 2	+0.60
Histology	'probable' or 'possible'	+0.52
	sarcomatoid	+0.67
Gender	male	+0.60
Prognostic Group	Low risk	Total = <1.27
	High risk	Total = >1.27

1.1.6 Treatment

The current management of MPM involves multimodality therapy carried out with the combination of surgery with chemotherapy and/or radiotherapy depending on the stage of the tumour. Chemotherapy is however the most common form offered to patients.

1.1.6.1 Surgery

Surgery is an important tool in the diagnosis of mesothelioma through the use of video-assisted thoracoscopic surgery (VATS); an accurate diagnostic procedure that allows the safe collection of large tissue samples from multiple sites in the thoracic cavity. The two potentially curative surgical procedures in the management of MPM patients with good prognosis are pleurectomy/decortication (P/D) and extrapleural pneumonectomy (EPP). The latter involves en bloc removal of both visceral and parietal pleura, entire lung, pericardium and diaphragm with synthetic reconstruction of the diaphragm and pericardium usually followed by adjuvant chemotherapy and radiotherapy (trimodality therapy). P/D involves the resection of the parietal and visceral pleura without the removal of the lungs (Figure 1.12). Depending on the extent of the tumour, the pericardium and diaphragm may be resected and reconstructed to remove all macroscopic tumours in a similar manner to EPP. The selection of a suitable procedure remains controversial due to insufficient data on survival and quality of life of patients after surgery involving either procedure (Rice, 2012). A multi-institutional study to compare survival outcome between EPP and P/D in 663 patients reported improved survival with an overall median survival of 16 months for the 278 patients who underwent P/D compared to the EPP arm (Flores et al., 2008). It was not possible to draw firm conclusions from these findings because of selection bias as one of the institutions involved carried out P/D on patients with biologically more favourable tumours (Flores, 2009). Results of the Mesothelioma and Radical Surgery (MARS) trial in which 50 patient (26 no EPP and 24 EPP) were randomly assigned to assess the survival difference in patient undergoing EPP or no EPP in the context of trimodal therapy also revealed a low median survival time of 14.4 months as well as a lower median quality of life score in the EPP arm (Treasure et al., 2011). This trial had a very small sample size hence it was limited; however the authors concluded that EPP may not be beneficial to MPM patients within trimodal therapy. There are ongoing studies on the benefits of a less radical form of surgery – Extended pleurectomy decortication (EPD) in mesothelioma patients (MARS2 trial NCT02040272).

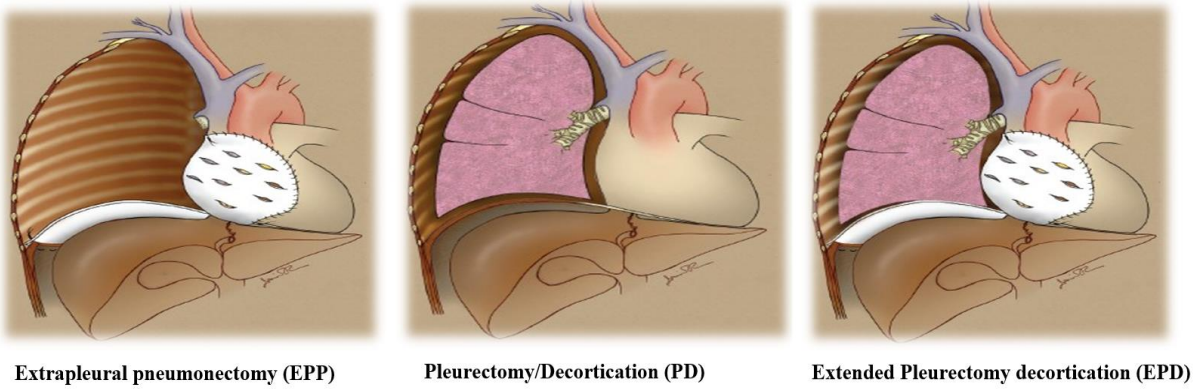


Figure 1.12 Surgical options in the management of mesothelioma. EPD is the most recent technique being investigated in the ongoing MARS2 trial (NCT02040272). (Rice, 2012)

1.1.6.2 Radiotherapy

Radiotherapy can aid the management of patients with MPM either by radical radiotherapy, prophylactic radiotherapy or palliative radiotherapy. The use of radical radiotherapy poses some challenges due to the diffuse nature of mesothelioma because it exposes other critical organs to large tumoricidal treatment volumes (Dhalluin & Scherpereel, 2011). Studies have shown that its use as single treatment has no benefit on survival and results in high toxicity (Alberts et al., 1988). However as part of a multimodal treatment, it was found to reduce local relapse post-surgery alone and in combination with chemotherapy. A phase II study conducted by Rusch and colleagues showed prolonged survival in patients with stage I and II tumours but an increased risk of early distant relapse in stage III patients (Rusch et al., 2001). A common complication after invasive diagnostic procedures is tumour cell seeding along the chest wall resulting in metastases occurring in 0% - 48% of MPM patients. Prophylactic radiotherapy was proposed to prevent these complications but there are controversies on its effectiveness based on conflicting randomised trial results which could be attributed to the use of different radiotherapy techniques and systemic therapies (Dhalluin & Scherpereel, 2011; van Thiel et al., 2011). This is the subject of ongoing clinical trial (PIT; NCT01604005).

1.1.6.3 Chemotherapy

1.1.6.3.1 First line chemotherapy

Prior to 2003, clinical trials for chemotherapy in mesothelioma yielded several discordant conclusions due to limited number of patients and non-randomised phase II trials. A

systematic review and meta-analysis of 83 eligible clinical trials between 1983 and June 2001 was published in 2002 by Berghmans et al. The qualitative evaluation was performed according to the scale of the European Lung Cancer Working Party (ELCWP). The 83 clinical trials represented 88 treatment arms; 80 of the trials were single phase II trials and 3 were randomised phase II studies. The trials were grouped into 4 categories based on the treatment regimen; cisplatin based trials without doxorubicin (n=20), doxorubicin based trials without cisplatin (n=8), combination of cisplatin and doxorubicin (n=6), regimens with other chemotherapy drugs such as vinorelbine, carboplatin, epirubicin, ifosfamide, methotrexate, immunotherapy drugs without cisplatin or doxorubicin (n=54). The result of this study revealed that the combination of cisplatin and doxorubicin was the best regimen in mesothelioma patients with an overall response of 28% (95% CI [21.3-35.7] and the most active single agent was cisplatin. It was also observed that combination therapy was more effective than single agents (22.6% *versus* 11.6% respectively; $p<0.001$) and cisplatin based treatments yielded better response rates that the carboplatin regimens (24% *vs* 11.6%; $p=0.004$) (Berghmans et al., 2002). Such analyses are of limited value because of their retrospective nature and lack of randomised controls. A major step in the chemotherapy treatment for MPM patients was the results of the two large randomised phase III trials reported by Vogelzang et al in 2003 and van Meerbeeck and colleagues in 2005.

In the first randomised phase III trial, 456 chemotherapy-naive patients not eligible for curative surgery were recruited; 226 were assigned to a combination regimen of pemetrexed (an antifolate drug) and cisplatin, 222 received cisplatin alone and 8 had no therapy. Based on previous evidences of the toxicities associated with pemetrexed, both treatment arms were supplemented with folic acid and vitamin B_{12} as evidenced by (Niyikiza et al., 2002). A better response rate was observed in the combination group compared to the cisplatin alone arm (41.3% *versus* 16.7%; $p<0.0001$), median overall survival was significantly longer (12.1 *versus* 9.3 months; $p=0.02$) and there was an improved progression free survival (5.7 months *versus* 3.9 months; $p=0.001$). The supplementation with folic acid and vitamin B_{12} also reduced toxicity significantly with no adverse effect to survival time (Vogelzang et al., 2003).

In 2005, another randomised phase III trial in 250 chemotherapy-naive patients was conducted; 126 patients received a combination of cisplatin and raltitrexed (another antifolate) and 124 received cisplatin alone. The combined chemotherapy regimen also significantly increased median overall survival when compared to cisplatin alone (11.4 months versus 8.8 months; $p=0.048$) (van Meerbeeck et al., 2005). The combination of

cisplatin and pemetrexed or raltitrexed has since been standard chemotherapeutic option in management of MPM.

1.1.6.3.2 Second line chemotherapy

Several second-line trials (SLTs) have been or are still being conducted for patients who progressed during or after the first line chemotherapy. Unfortunately this includes nearly all MPM patients. The use of second-line chemotherapy has increased in clinical practice because quite a number of patients are still fit to undergo another treatment at the time of disease progression (Ceresoli et al., 2010). The role of second-line therapies is yet to be defined in the management of mesothelioma due to limited number of patients and the absence of randomised trials. Chemotherapy agents as well as targeted agents are commonly used in second-line chemotherapy trials. In 2005, Manegold et al reported a retrospective analysis of patients treated in the phase III pemetrexed/cisplatin who had received post study chemotherapy (PSC). There was an imbalance in the percentage of patients who received PSC; 37.2% were previously treated with pemetrexed and cisplatin while 47.3% had received cisplatin alone. There was a significantly prolonged survival time in the PSC subgroup than the non-PSC subgroup however because the administration of PSC was not randomised it was not possible to draw a clear conclusion as the choice of PSC might have been influenced by the evidence of clinically recognized combination of factor that favoured better survival (Manegold et al., 2005). Table 1.6 shows a list of second line clinical trials that have been conducted since 2002 and their clinical outcomes. The highest response rate (32.5%) was observed in the trial conducted by Janne et al where 96 patients received pemetrexed plus cisplatin regimen after a pemetrexed naive first line therapy. There is a role for second-line therapy in the management of MPM hence the need for randomised SLTs.

Table 1.6 Second-line chemotherapy trials in MPM patients in order of year conducted

Treatment regimens	No. of patients	Previous treatment	Response Rate (%)	Median time to progression (months)	Median survival (months)	Author (s)
Picoplatin (ZD0473)	47	pemetrexed-naive	12	2.5	6.7	(Giaccone et al., 2002)
Raltitrexed + Oxaliplatin	15	pemetrexed-naive	20	6.2	10.1	(Fizazi, 2003)
Raltitrexed + Oxaliplatin	14	pemetrexed-naive	0	1.9	3.2	(Porta et al., 2005)
Pemetrexed	91	pemetrexed-naive	5.5	Not reported	4.1	(Jänne et al., 2006)
Pemetrexed + Cisplatin	96	pemetrexed-naive	32.5	Not reported	7.6	
Irinotecan, Cisplatin, Mitomycin-C	13	pemetrexed-naive	20	7.3	7.3	(Fennell et al., 2007)
Pemetrexed +/- Carboplatin	17	Platinum/ pemetrexed	Not reported	Not reported	Not reported	(Serke & Bauer, 2007)
Pemetrexed	28	pemetrexed-naive	21	4.9	9.8	(Sørensen et al., 2007)
Pemetrexed + Carboplatin	11	pemetrexed-naive	18	7.4	9.1	
Pemetrexed	396	pemetrexed-naive	12.1	4.9	Not reported	(Taylor et al., 2008)
Pemetrexed	123	pemetrexed-naive	19.2	3.8	8.6	(Jassem et al., 2008)
Pemetrexed +/- Carboplatin	18	pemetrexed based chemotherapy	11	33	3.5	(Giovanni L Ceresoli et al., 2011)
Oxaliplatin +/- gemcitbine	29	pemetrexed based chemotherapy	7	2.2	5.6	(Xanthopoulos et al., 2008)
Gemcitabine + vinorelbine	30	pemetrexed based chemotherapy	10	2.8	10.9	(Zucali et al., 2008)
Vinorelbine	63	pemetrexed-naive	16	Not reported	9.6	(Stebbing et al., 2009)
Docetaxel + gemcitabine	37	pemetrexed-naive	19	7	16.2	(Tourkantonis et al., 2011)
Vinorelbine	59	pemetrexed based chemotherapy	15.2	2.3	6.2	(Zucali et al., 2014)

Vinorelbine +/- gemcitabine	56	pemetrexed based chemotherapy	1	1.7	5.4	(Zauderer et al., 2014)

1.1.6.3.3 Targeted therapy

This will be discussed in Chapter 2 under section 2.5 of the molecular biology of mesothelioma.

CHAPTER TWO

HALLMARKS OF MALIGNANT PLEURAL MESOTHELIOMA

Chapter 2 Molecular Biology of Mesothelioma

The hallmarks of cancer cells as described by Hanahan and Weinberg (2011) is their ability to sustain proliferative signals, evade growth suppressors, activate invasion and metastasis, enable immortal replication, induce angiogenesis, resist programmed cell death, avoid immune destruction and deregulate cellular energetic (Hanahan & Weinberg, 2011). These characteristics also hold true for malignant pleural mesothelioma (MPM) cells. Although the molecular mechanism of asbestos-induced mesothelioma remains elusive, over the past decades several chromosomal aberrations have been described to exist in MPM cells. Frequent losses have been shown to be limited to chromosome arms 1p, 3p, 4q, 6q, 9p, 13q, 14q and 22q and gains are found in chromosome arms 1q, 5p, 7p, 8q and 17q. Although these chromosomal aberrations are not specific to MPM as they are observed in other tumour types; some could be used to distinguish malignant mesothelioma from benign mesothelial proliferations (Didier et al., 2012). Unlike other solid tumours, mutations of the common oncogenes frequently mutated in solid tumours such as those of the Ras family are rare in mesothelioma.

The extended latency period between exposure to asbestos fibres and the diagnosis of MPM suggests that mesothelial cells undergo multiple genetic alterations to become malignant. Several cytogenetic studies have demonstrated the complexity of these changes and a number of mutated genes in addition to aberrant expression have been revealed to be recurrent in malignant mesothelioma. Inhaled asbestos also induces cytokines and growth factors which stimulate receptor tyrosine kinases and results in the downstream signalling of cell survival pathways (Musti et al., 2006).

2.1. Tumour Suppressor genes

2.1.1. CDKN2A/ARF inactivation

The *cyclin-dependent kinase inhibitor 2A (CDKN2A)/ alternative reading frame (ARF)* gene is known to be the most frequently inactivated tumour suppressor gene (TSG) in 70% of malignant mesothelioma (Musti et al., 2006). This gene is located at chromosome 9p21.3. *CDKN2A* encodes $p16^{INK4a}$ with exon 1α, 2 and 3, whilst ARF encodes $p14^{ARF}$ (mouse $p19^{ARF}$) with exon 1β, 2 and 3 with an alternative open reading frame. This indicates that these proteins have unique first exons but share exons 2 and 3 translated from the alternative reading frame (Ruas & Peters, 1998). $p14^{ARF}$ regulates p53 by binding to and promoting the degradation of the human ortholog of mouse double minute 2 (HDM2) resulting to the

stabilization of p53, while $p16^{INK4a}$ controls the cell cycle by inhibiting the cyclin-dependent kinase 4 (CDK4)/cyclin D-retinoblastoma protein (RB) pathway. RB is a tumour suppressor protein that limits cell proliferation by regulating entry into the S-phase of the cell cycle. p53 is also a tumour suppressor protein that is activated in response to cellular stress therefore initiating gene transcription which leads to cell cycle arrest and apoptosis. The homozygous deletion of *CDKN2A/ARF* signifies the inactivation of two major tumour suppressing pathways RB and p53 in the cell resulting in aberrant tumour growth (Brown et al., 2011).

Homozygous deletion of $p16^{INK4a}$ was observed in 85% of 34 mesothelioma cell lines and 22% of 23 primary tumours by Cheng et al (1994) via southern blotting and PCR analysis (Cheng et al., 1994). An immunohistochemical study of the $p16^{INK4a}$ protein in 12 primary mesotheliomas and 15 mesothelioma cell lines further demonstrated the absence of $p16^{INK4a}$ expression in all specimens (Kratzke et al., 1995). The co-deletion of $p16^{INK4a}$ and $p15^{INK4B}$ (a neighbouring tumour suppressor gene located on chromosome 9p21 and encoded by *CDKN2b*) was also reported in 72% of mesotheliomas implicating other TSGs as targets of the frequent chromosome 9p deletion in mesothelioma (Xio et al., 1995). Homozygous deletions of the locus was found to be present in primary mesothelioma tissues or mesothelioma cells from pleural effusion of approximately 70% of cases when analysed with fluorescence in situ hybridization (FISH). When regrouped into subtypes, the sarcomatoid subtype showed approximately 100% homozygous deletion and the epithelioid and biphasic showed ~70% and ~89% respectively (Illei et al., 2003; Chiosea et al., 2008; Onofre et al., 2008; Takeda et al., 2012; Wu et al., 2013; Matsumoto et al., 2013). Subsequent studies are summarised in Table 1 in Appendix A. For a better understanding of the role of these genes, knockout mice for $p19^{ARF}$ but expressing $p16^{INK4a}$ and vice versa were studied and the results revealed that the deficiency of both genes exhibited increased development of spontaneous and carcinogen-induced cancers (Serrano et al., 1996; Kamijo et al., 1997; Sharpless et al., 2001). Recently, a knockout mice model for $p19^{ARF}$ but expressing $p16^{INK4a}$ was developed to further enunciate the significant role of this gene in the pathogenesis of mesothelioma. In addition, the authors in a later study examined the role of $p16^{INK4a}$ and $p19^{ARF}$ in the development of malignant mesothelioma associated with asbestos exposure. Mice deficient for one of the genes ($p16^{INK4a\ (+/-)}$ or $p19^{ARF\ (+/-)}$) and those with double deficiency ($p16^{INK4a\ (+/-)}$/ $p19^{ARF\ (+/-)}$) were exposed to asbestos. The latter displayed significant ($p<0.0001$) accelerated asbestos-induced mesothelioma in comparison to mice with single deficiency (Altomare et al., 2009, 2011). p53 was seen to be functional in the absence of $p19^{ARF}$ showing that the loss of $p19^{ARF}$ facilitates mesothelioma progression via p53-independent

pathway(s). These studies showed $p16^{INK4a}$ and $p19^{ARF}$ do not have redundant roles in mesothelioma and that their absence increases tumorigenesis caused by asbestos exposure (Altomare et al., 2009, 2011)

2.1.2. Neurofibromatosis type 2 (NF2) gene inactivation

The *NF2* gene is an autosomal dominantly inherited tumour predisposing syndrome characterised by the development of tumours of the nervous system such as spinal schwannomas, bilateral vestibular schwannomas of the eighth cranial nerve, cranial meningiomas and ependymomas (Evans, 2009). It is located on chromosome 22q12 and encodes Merlin (Moesin-ezrin-radixin-like protein), a 595 amino acid tumour suppressor protein that shows a significant homology to the highly conserved family of proteins (FERM proteins) that have been hypothesized to connect the components of the plasma membranes and cytoskeleton (Bianchi et al., 1995; Sekido et al., 1995). Merlin functions as a growth inhibitor by accumulating in the nucleus where it binds and suppresses the activity of the E3 ligase CRLA ^{DCAF1}. Although loss of Merlin exhibits a pro-mitogenic effect, this effect is hindered when a merlin-insensitive mutant of *DCAF1* is expressed or *DCAF1* is depleted (Li et al., 2010). In 1995, a study observed somatic mutations in 41% (7/17) mesothelioma cell lines when analysed by southern blotting and single-strand conformation polymorphism (SSCP). The mutations observed were large deletions (~10 – 50 kilobases) in the NF2 gene, nonsense mutations at codons 57 and 341 and a 10-base pair microdeletion from nucleotide 1004 – 1013 resulting in a frameshift mutation (Sekido et al., 1995). Another study in the same year investigated the function if the NF2 gene in MM. cDNAs from 15 mesothelioma cell lines and genomic DNAs from 7 matched primary tumours were analysed for mutations within the NF2 coding region. 53% (8/15) of the cell lines showed abnormal single-strand conformation polymorphism patterns. NF2 mutations were also confirmed in 6 of the 7 matched primary tumours (Bianchi et al., 1995). The findings from these studies and other supporting evidence (summarized Table 2 in appendix A) suggests that the NF2 is not exclusive to nervous system neoplasm but could also play an important role in the pathogenesis of mesothelioma. Recently, few studies have emerged to bolster past findings. Andujar and colleagues reported NF2 gene mutations in 32.2% (13/34), deletions in 29.4% (10/34) and point mutations in 11.8% (4/34) of MPM cases but no mutations in non-small cell lung cancer (NSCLC) patients irrespective of asbestos exposure (Andujar et al., 2013). The frequent inactivation of the NF2 gene previously observed in MPM led to the use of genetically-engineered NF2 knockout mice to examine the role of NF2 inactivation in the

pathogenesis of mesothelioma. NF2 (+/-) knockout mice designed to mimic human NF2 syndrome were treated with asbestos to induce malignant mesothelioma. The asbestos-exposed NF2 (+/-) knockout mice exhibited significant increase in tumour development in comparison to asbestos-treated wild type littermates. Biallelic inactivation due to loss of the wild type NF2 allele was observed in all nine asbestos-induced malignant mesotheliomas from knockout mice but seen in only 50% of mesotheliomas arising from asbestos-induced wild type mice. Tumours from NF (+/-) mice showed homologous deletions of the *CDKN2A/ARF* locus and adjacent *CDKN2B* tumour suppressor gene, a common phenomenon in human MM (Altomare et al., 2005). In another mouse model, the pleural cavity of conditional knockout mice for NF2, Ink2a/Arf and p53 were directly injected with adenoviruses encoding the site-specific recombinase Cre (Adeno-Cre) in order to limit the inactivation of the conditional TSGs. Conditional NF2;Ink4a/Arf mice exhibited more invasive, aggressive mesothelioma and longer median survival time (30 weeks) in comparison to conditional NF2:p53 mice with shorter survival (20 weeks) (Jongsma et al., 2008).

Re-expression of merlin in NF2 deficient tumour cells has been shown to inhibit cell proliferation, G_1 phase arrest, dephosphorylation of pRB, decrease in cyclin D1 expression and CDK4 kinase activity (Xiao et al., 2005) . Another study on the re-expression of merlin in NF2 deficient mesothelioma cells has also shown significant decrease in cell motility and invasiveness by negatively regulating focal adhesion kinases (FAK). Over-expression of merlin also mitigates FAK tyrosine phosphorylation resulting in decreased phosphorylation at the critical FAK autophosphorylation site, tyrosine 397. The inhibition of FAK phosphorylation at Tyr397 subsequently impaired the binding of FAK to Src family kinases and p85 subunit of PI3K (Poulikakos et al., 2006). A recent study showed that p53 was down regulated while MDM2 was upregulated in merlin deficient human primary schwannoma cells. However, the reintroduction of merlin into the cells enhanced p53 expression and activity. Subsequent inhibition of the p53/MDM2 complex with Nutlin-3, a drug which increases the stability of p53 led to decrease in tumour growth and cell survival (Ammoun et al., 2014). Shapiro et al (2014) recently reported marked sensitivity of merlin negative cell lines to the focal adhesion kinase (FAK) inhibitor, VS-4718. Merlin-negative mesothelioma cells (Mero 41) with weak cell-cell adhesion were observed to show enhanced sensitivity to FAK inhibition suggesting their dependence on cell-ECM-induced FAK signalling. These results and those of Soria et al 2012 (abstract EORTC) provided the rationale for the ongoing clinical trial of the FAK inhibitor (Defactinib) as second-line therapy in merlin-negative

mesothelioma patients (ClinicalTrials.gov NCT01870609). In ovarian cancer cell lines – CAL-62, COV318 and CAOV-4, expression of wild-type merlin did not limit their sensitivity to VS-4718 suggesting that sensitivity to FAK inhibitors exert their cytotoxic effect via a different molecular mechanism (Shapiro et al., 2014).

2.1.3. BRCA1 associated protein-1 (BAP1)

The BAP1 tumour suppressor gene is located on chromosome 3p21 and encodes a nuclear ubiquitin carboxy-terminal hydrolase (UCH), a class of deubiquitinating enzymes that regulates deubiquitination in cell cycle processes and DNA damage response as reviewed by Eletr and Wilkinson (2011). BAP1 was found to regulate cell proliferation by deubiquitinating host cell factor-1 (HCF-1), a protein involved in chromatin modification and transcriptional processes (Machida et al., 2009; Eletr & Wilkinson, 2011). It is however suggested to be a tumour suppressor gene that plays a role in cell proliferation and growth inhibition but commonly (30-84%) deleted in several cancers including cancers of the lung, breast, uveal melanoma and malignant pleura mesothelioma (Jensen & Rauscher, 1999; Ventii et al., 2008; Harbour et al., 2010; Kato et al., 2016). Recently it was identified as an important tumour suppressor gene that is frequently inactivated in mesothelioma from a study designed to identify driver genes in MPM (Bueno et al., 2016). Bott et al (2011) carried out an integrated genomic analysis of 53 MPM tumour samples and discovered that the three common deletions observed were at 22q (NF2), 9p21 (CDKN2A) and 3p21 harbouring BAP1. The BAP1 gene showed the highest frequency of non-synonymous mutations in 23% (12/53) of the initial MPM samples analysed and 18% (12/68) in an additional cohort of MPM tumour samples. High rates of somatic mutations were also observed in 24% (6/25) MPM cell lines within the same study. Thirty-two different BAP1 mutations were identified which included 13 frameshifting, 8 at or near splice sites, 6 nonsense and 5 missense mutations. In total BAP1 alterations (loss, mutation or both) were identified in 42% of MPM cases. Immunohistochemistry was further used to confirm the association between BAP1 mutation and absence of BAP1 protein in tissue samples (Bott et al., 2011). Germline BAP1 mutations have also been identified in unrelated families with high incidence of mesothelioma. Testa et al (2011) carried out an array-comparative genomic hybridisation experiment on two MPM tumours from individuals with familial cluster of MPM and no prior asbestos exposure. Somatic alterations were observed indicating biallelic inactivation of the gene. Apart from mesothelioma, individuals with BAP1 mutation also developed uveal melanoma and cutaneous melanoma (Testa et al., 2011). Similarly, Weisner et al (2011) and

other researchers identified germline BAP1 mutations in two families characterised by atypical melanocytic tumour, cutaneous melanoma, uveal melanoma and other cancer types. These findings together with those from previous studies suggest the existence of a BAP1-related cancer susceptibility syndrome whose underlying mechanism in tumour development remains unclear (Testa et al., 2011). Lack of BAP1 activity has been associated with the pathogenesis of epithelioid mesothelioma. In a clinical study by Landanyi et al (2012), 20% (24/121) of MPM tumours harboured BAP1 somatic mutations. There was no significant difference observed in clinical features or survival in MPM patients with or without BAP1 mutation (Ladanyi et al., 2012). In contrast, Arzt et al (2014) reported shorter overall survival in 40% of 123 MPM tissue samples with wild type BAP1 with high protein expression. BAP1 mutation has been previously associated with long survival in familial cases and correlates with improved survival observed in BAP1 negative MPM in the study (Arzt et al., 2014). Since germline mutation of BAP1 has been identified as a predisposing factor in the development of mesothelioma and other malignancies while somatic mutations have been implicated in transcriptional changes in the pathogenesis of mesothelioma, a clear understanding of the exact spectrum of BAP1 mutation might be useful in the prevention or treatment of mesothelioma in individuals who are genetically predisposed. The interaction of key genes in the development of mesothelioma is represented in Figure 2.1.

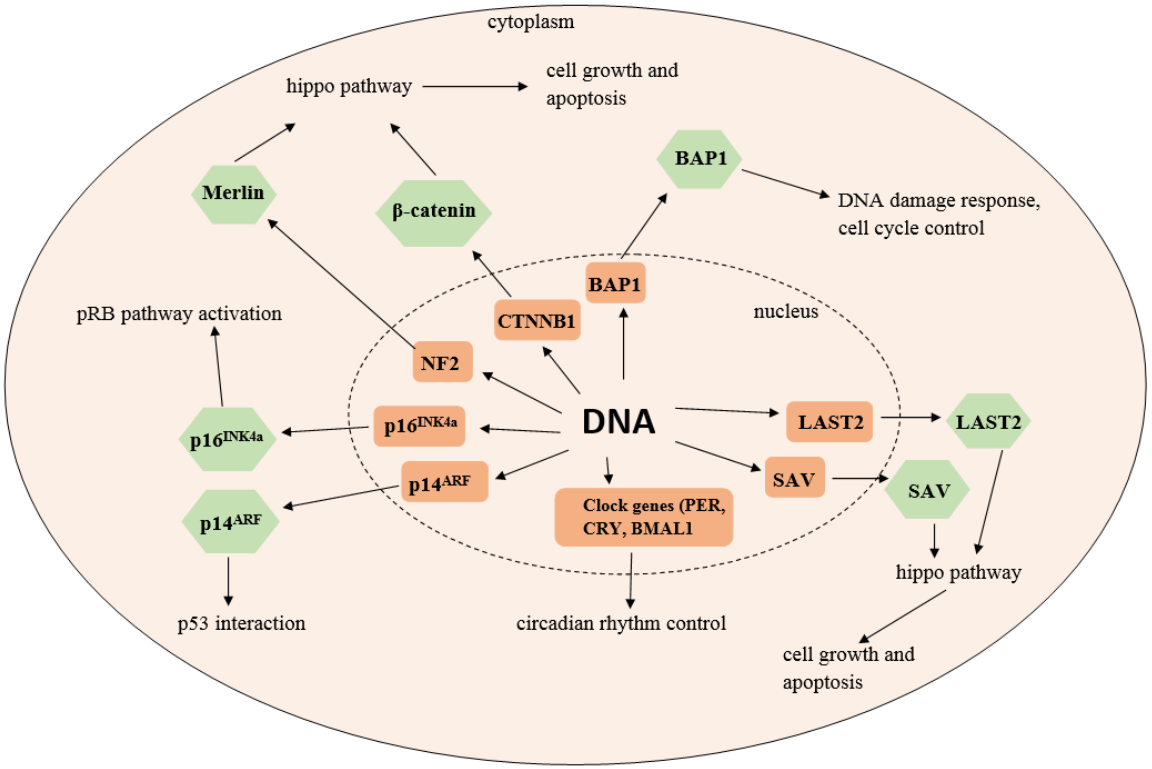


Figure 2.1 The interaction of key genes and their proteins in the development of mesothelioma. $p16^{INK4a}$ and $p14^{ARF}$ activates the pRB pathway and modulates p53 respectively. Merlin, β-catenin, LATS and SAV play important roles in the hippo pathway and subsequently cell growth and apoptosis. BAP-1 plays an important role in DNA damage response and cell cycle control. The clock genes (PER, CRY and BMAL1) regulates the circadian rhythm control. Adapted from (De Assis et al., 2014).

2.1.4. Epigenetic modulation in mesothelioma tumorigenesis

The genesis of MPM is also associated with epigenetic errors which lead to modifications of gene expression via the inactivation of tumour suppressors and other growth regulatory genes. Differences in the methylation profile of MPM when compared to normal pleura or other tumours suggests the presence of aberrant clusters of CpG dinucleotides ("CpG islands") methylation that is precise and may be involved in the carcinogenesis of mesothelioma as shown by global epigenetic analysis. The first study compared DNA methylation of 803 cancer associated genes in 18 normal pleura and 158 mesothelioma specimens. DNA methylation profile was able to distinguish non-malignant pleura from mesothelioma and was a significant predictor of shorter survival (Christensen et al., 2009). In another study comparing the methylation profile between MPM and lung adenocarcinomas, results showed that 11% of heterozygously deleted genes were affected by DNA methylation and or trimethylation of H3 lysine 27 (H3K27me3) in mesothelioma. Of these genes, *MAPK13, KAZALD1* and *TMEM30B* were specifically only methylated in mesothelioma and could be useful potential diagnostic markers. In addition, a subset of MPM cases with low levels of DNA methylation had longer survival (Goto et al., 2009). Several studies have also identified other genes that have prognostic implications. Hypermethylation of *LZTS1, SLC6A20, HIC-1, TMS1* or combination of RAR*β* with either *DAPK* or *RASSF1A* has been associated with significantly shorter overall survival (Suzuki et al., 2005; Fischer et al., 2006; Tsou et al., 2007). Christensen et al (2008) have also studied the methylation status in MPM with a directed approach by analysing specific pathway genes. Promoter hypermethylation of six cell cycle pathway genes; *APC, CCND2, CDKN2A, CDKN2B, RASSF1* and *HPPBP1* was significantly associated with increased asbestos burden (Christensen et al., 2008). Promoter methylation of the WNT inhibitory factor-1 (*WIF-1*) and *SFRP* 1, 2 and 4 was observed in 96% of mesothelioma tissues by methylation-specific polymerase chain reaction (Kohno et al., 2010). The biosynthetic rate-limiting enzyme for arginine; argininosuccinate synthetase - 1 (ASS1) has also been reported to be reduced or absent in 63% of mesothelioma tumours as a result of hypermethylation of the AS promoter site. In addition, arginine depletion in the AS-negative cells resulted in marked apoptosis of tumour cells through the upregulation of BAX indicating the need to explore arginine deprivation therapy in MPM patients (Szlosarek et al., 2005).

Based on the epigenetic findings in MPM, several histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors (HDACi) have been tested *in vitro* and are being

evaluated clinically. The largest study of an HDAC inhibitor in MPM was a phase III trial that tested vorinostat hydroxamate. Results from the study concluded that there was no survival benefit from the drug when compared to best supportive care in patients who relapsed after first-line chemotherapy (Krug et al., 2015). Another attempt on an HDAC inhibitor was a phase II trial of valproate and doxorubicin combination in 45 MPM relapsing patients. The response rate was 16% (7 partial responses) and the best rate of disease control was 36% indicating that the combination was well tolerated (Scherpereel et al., 2011). An *in vitro* study also indicated that tumour growth suppression was achieved when vaporate is combined with standard of care chemotherapy regime, cisplatin plus pemetrexed (Vandermeers et al., 2009). Recently, a more promising result was achieved in the ADAM study, a multicentre phase II randomized clinical trial evaluating the arginine-lowering agent pegylated arginine deiminase (ADI-PEG20) in ASS1-deficient MPM patients. Results from this first biomarker-driven study of ADI-PEG20 in MPM patient showed that arginine deprivation using ADI-PEG20 significantly improves progression free survival in mesothelioma patient with deficient ASS1 enzyme (Szlosarek et al., 2013; 2017). More studies are underway to assess the combination of ADI-PEG20 with pemetrexed and cisplatin in MPM patients with low/no ASS1 [NCT02709512] and those whose tumours require arginine [NCT02029690].

2.1.5 The role of tumour stroma in mesothelioma tumorigenesis

The tumour microenvironment is functionally crucial for tumour development and progression. It is made up of multiple components such as the extracellular matrix (ECM), surrounding stromal cells and infiltrating cells, and signalling molecules. The stroma, ECM and infiltrating cells interact with tumour cells and have the ability to stimulate or inhibit tumour development (Dunn et al., 2004; Kumar & Weaver, 2009). Understanding the role of the immune system in the pathogenesis of cancer has led to the use of immunotherapy, a therapeutic modality that boasts the human immune system against cancer cells. In MPM, studies have shown that antitumour immune responses have an influence on prognosis. High expression of CD8+ tumour-infiltrating lymphocytes (TILs) was strongly associated with better prognosis in MPM patients treated with induction chemotherapy and surgery (Anraku et al., 2008; Yamada et al., 2010). In epithelioid mesothelioma patients, increased chronic inflammation in the stroma was an independent predictor of prolonged survival which further highlights the importance of investigating the stromal components of tumours (Suzuki et al.,

2011). Immunosuppressive cytokines and regulatory T cells (Tregs) have also been reported to infiltrate the tumour microenvironment therefore inhibiting antitumour immune function and promoting mesothelioma tumour growth (Hegmans et al., 2006). A recent comprehensive analysis of immune responses in the tumour and tumour-associated stroma of 230 epithelioid mesothelioma patients, demonstrated that tumour CD20-expressing lymphocytes, IL-7 receptor, high $CD163^+$ tumour-associated macrophages/low $CD8^+$, and low $CD163^+$/ high $CD20^+$ were significant prognostic indicators of epithelioid mesothelioma. The findings of these authors shed more light on the need for immunomodulatory therapies in the management of epithelioid mesothelioma (Ujiie et al., 2015).

Immunotherapy has been evaluated in mesothelioma in both pre-clinical and clinical settings. A phase II study examined the feasibility of intrapleural interleukin-2 (IL-2) administration in 22 MPM patients and reported response rates of about 50% and an overall median survival time of 18 months (Astoul et al., 1998). The combined infusion of intrapleurally infused human activated macrophages and γ-interferon was well tolerated in MPM patients but showed limited antitumour effect (Monnet et al., 2002). The evaluation of intrapleural interferon-β gene transfer (IFN-β) using an adenoviral vector (Ad. IFN-β) reported antitumour immune responses in 70% of patients and stable disease in 40% of MPM patients and patients with metastatic pleural effusions (Sterman et al., 2007). Table 2.1 is a list of other ongoing clinical trials using immunotherapy for the treatment of MPM. Future success in immunotherapy also requires the identification of biomarkers that would determine patients' response to specific treatment options, identify target antigens and overcome the mechanisms of resistance and inhibition within the complex tumour microenvironment. (Dozier et al., 2017).

Table 2.1 Clinical trials using immunotherapy for the treatment of malignant pleural mesothelioma.

Agent	Phase	Comments	Representative clinical trials
Check point inhibitors			
- *PD-1 inhibition*			
Nivolumab	Phase II	Monotherapy in patients with recurrent/relapsed mesothelioma.	NCT03063450, NCT02497508
Pembrolizumab (MK-3475)	Phase II/III	First-line therapy or adjuvant therapy in patients with advanced mesothelioma.	NCT02959463, NCT02399371, NCT02784171, NCT02991482
- *PD-L1 Inhibition*			
Durvalumab (MEDI4736)	Phase II	First-line combination therapy for unresectable mesothelioma.	NCT02899195
Immunotoxin			
SS1P	Phase II/III	MSLN-targeted immunotoxin plus chemotherapy to decrease immunogenicity.	NCT01362790, NCT01445392
LMB-100	Phase I	MSLN-targeted immunotoxin for patients with advanced MPM.	NCT02798536
Oncolytic virus			
GL-ONC1 vaccinia	Phase I	Neoadjuvant GL-ONC1 oncolytic virus, with or without eculizumab	NCT02714374
Measles virus	Phase I	Dose-escalation study of intrapleural measles virus therapy	NCT01503177

Vaccine therapy			
Autologous DC	Phase I/II	First-line therapy or adjuvant therapy in patients with advanced mesothelioma	NCT02151448, NCT02395679, NCT02649829
WT-1	Phase I/II	Adjuvant therapy following multi-modality therapy	NCT01265433, NCT01890980
Adaptive cell therapy			
T-cell receptor (TCR)	Phase I/II	TCR targeting WT-1 in NSCLC and MPM	NCT02408016
CAR T-cell	Phase I/II	CAR T-cell targeting MSLN	NCT02580747, NCT01583686, NCT02414269, NCT01355965
Combination therapy			
Combined checkpoint blockade	Phase II	Combined checkpoint blockade for unresectable MPM	NCT02588131, NCT03075527, NCT02592551

CAR, chimeric antigen receptor; DC, dendritic cell; MSLN, mesothelin, NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; WT1, Wilms tumour. (Adapted from Dozier et al., 2017)

2.2. Growth Factors and growth factor receptors

2.2.1. The Epidermal growth factor family

The protein tyrosine kinases of the Epidermal Growth factor family are one of the most extensively studied cell signalling molecules in cancer biology. The discovery of the epidermal growth factor (EGF) was pioneered by Cohen whilst studying nerve growth factor in mouse sub-maxillary glands (Cohen, 1960; Cohen & Carpenter, 1975). Cohen et al (1982) isolated a solubilized 170 kDa polypeptide containing both EGF binding activity and protein kinase activity from A-431 cells identified as the EGF receptor (EGFR) (Cohen et al., 1982). EGFR shares a structural homology with the mammalian v-erb-B transforming protein from the avian erythroblastosis virus (AEV) from which the ERBB gene symbol is derived. AEV has a truncated EGFR that lacks the extracellular EGF binding domain which plays a significant role in influencing tyrosine kinase activity (Downward et al., 1984). The human epidermal growth factor receptor (HER) family consists of four structurally related members (ErbB1 (EGFR/HER1), ErbB2 (HER2/NEU), ErbB3 (HER2) and ErbB4 (HER4). These are ubiquitously expressed in the majority of normal cells. EGFR consists of 1186 amino acids with three regions; the extracellular ligand binding region, a single hydrophobic transmembrane region and an intracellular domain with tyrosine kinase activity (Figure 2.2) The extracellular region is further divided into four parts: domains I and III are leucine-rich segments which are responsible for ligand binding; domains II and IV are cysteine-rich residues which participate in disulphide bond formation. Domain II also plays a role in homo- and hetero-dimerization with other EGF receptors (Ullrich et al., 1984; Carpenter & Cohen, 1990). Apart from the epidermal growth factor, 10 other EGF-like polypeptide ligands also interact with EGF receptors. These ligands are epigen (EPG), transforming growth factor-α (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding epidermal growth factor-like growth factor (HB-EGF), epiregulin (EPR), neuregulin-1 (Nrg-1) neuregulin-2 (Nrg-2), neuregulin-3 (Nrg-3) and neuregulin-4 (Nrg-4). Seven of these ligands binds to EGFR/HER 1, two bind to HER3, seven binds to HER4 but none to HER2 (Figure 2.2). The inactive ligand-binding domain I of HER2 impedes homodimer formation in normal physiological conditions making it a favoured dimerization partner for other EGFR members. However functional HER2 homodimers have been identified in HER2 overexpressing breast cancers (Tzahar et al., 1996; Graus-Porta et al., 1997; Ghosh et al., 2011).

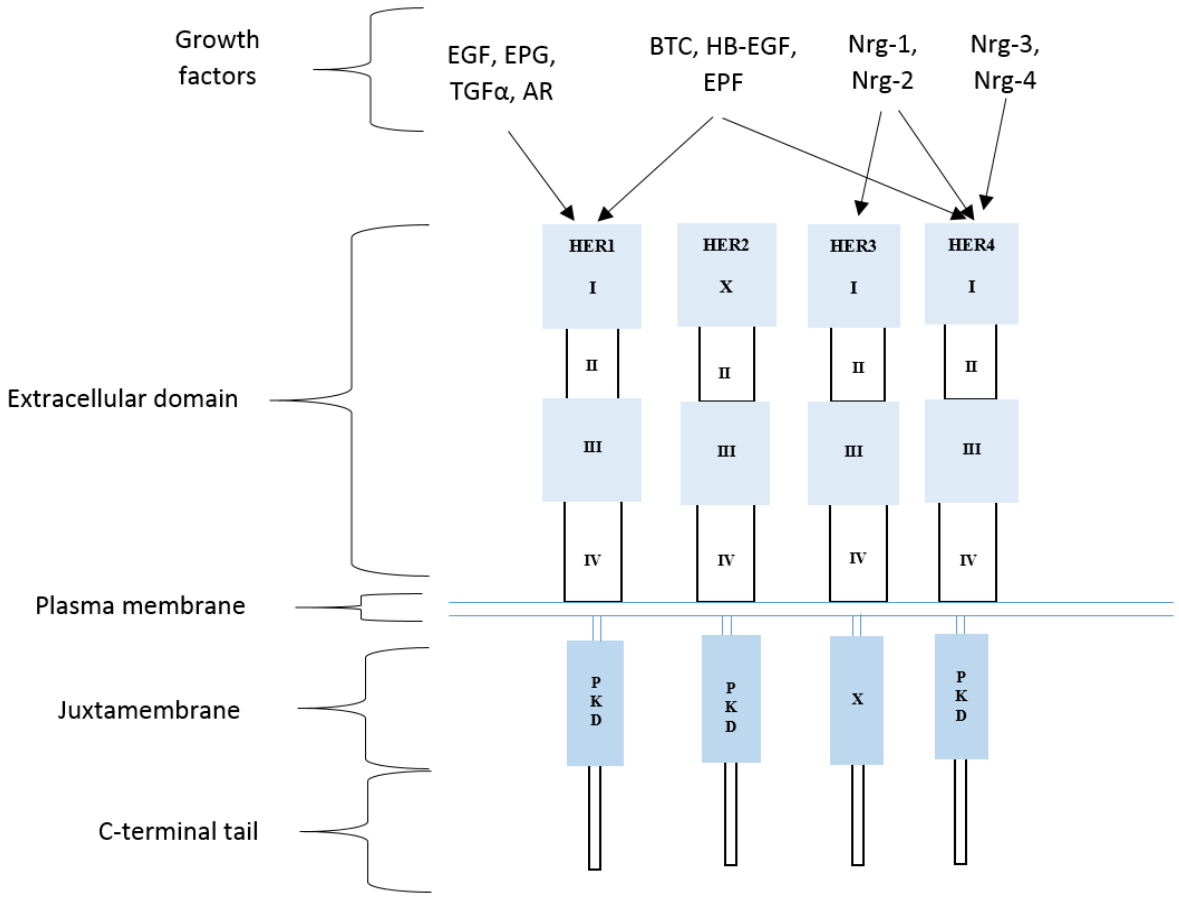


Figure 2.2 The EGFR/HER structure and ligands. The EGFR/HER extracellular domain has four domains labelled I – IV. Domains I and III are the ligand binding regions. HER2 ligand-binding domain and HER3 protein kinase domain (PKD) are inactive hence denoted 'X' (Adapted from Roskoski 2014).

Like other receptor tyrosine kinases (RTK), upon ligand binding, EGFR monomers form homodimers or heterodimers by dimerizing with another EGFR or other receptors of the HER family. The juxtaposed protein kinase domain catalyses the phosphorylation of tyrosine residues thus, resulting in protein kinase activation. The kinase domains also triggers the phosphorylation of additional tyrosine residues in the c-terminal end of the receptor that serves as docking sites for adaptor proteins including GRB2 (Growth Factor Receptor-Bound Protein-2), PLC-Gamma (Phospholipase-C-Gamma), SHC (Src Homology-2 Domain containing transforming protein), STATs (Signal transducer and activator of transcription) and other proteins with a phosphotyrosine binding domain (PTB). Subsequently downstream signalling cascades are initiated resulting in cell proliferation, invasion, metastasis and anti-apoptosis (Schlessinger, 2000; Bogdan & Klämbt, 2001). EGFR is ubiquitously expressed in healthy cells that originate from the three germ cell layers (endoderm, mesoderm and

ectoderm) particularly those of epithelial origin e.g. hair follicles, skin, liver, mammary glands, lung, and gastrointestinal tract; as well as malignant cells. EGFR also mediates normal physiological functions such as migration, proliferation, differentiation and cell survival (Wells, 1999; Herbst & Shin, 2002; Sibilia et al., 2007). Amplification of ErbB1 has been reported in breast, bladder, head and neck, kidney, prostate, non-small cell lung cancers and mesothelioma (Salomon et al., 1995; Blume-Jensen & Hunter, 2001; Yarden & Sliwkowski, 2001; Agarwal et al., 2011). A well described variant is the EGFRvIII associated with a truncated extracellular domain resulting in a constitutively active EGF receptor. This frequent alteration has been reported in glioblastoma, breast, lung, ovarian and gastric carcinomas (Garcia de Palazzo et al., 1993; Moscatello et al., 1995; Wikstrand et al., 1995; Takehana et al., 2003; Al-Kuraya et al., 2004; Marquez et al., 2004).

ErbB2 is expressed in a range of normal tissues and overexpressed in breast (Owens et al., 2004; Bose et al., 2013), oesophageal (Chan et al., 2012; Gonzaga et al., 2012), glioblastoma (Mineo et al., 2007), lung (Hirsch & Langer, 2004; Tomizawa et al., 2011), gastric, ovary, colon, bladder, salivary duct, pancreatic, cervix and endometrial cancers (Lesnikova et al., 2009; Bang et al., 2010; Fleischmann et al., 2011; Li et al., 2011; Chou et al., 2013; Anglesio et al., 2013; Buza et al., 2013; Nardi et al., 2013). In addition, HER2 mutations have been identified in a subset of breast, lung, ovary and colon cancers. HER2 does not bind to any known ligands and its ectodomain exists in a constitutively active state making it a preferred dimerization partner or co-receptor. HER2 possesses a strong catalytic kinase activity whilst that of HER3 is inactive, hence ligand-activated HER3 favourably binds to HER2 resulting in the activation of HER2 kinase domain activity and subsequent activation of the downstream PI3K/AKT signalling (Yan et al., 2014).

The major downstream signalling pathways stimulated by the trans-phosphorylation of the EGFR family kinases include the RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT/MTOR pathway, the phospholipase C (PLCγ) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway (Figure 2.3). These signalling networks are interconnected and overlapping resulting in alterations in the protein function and activation of gene transcription (Henson & Gibson, 2006).

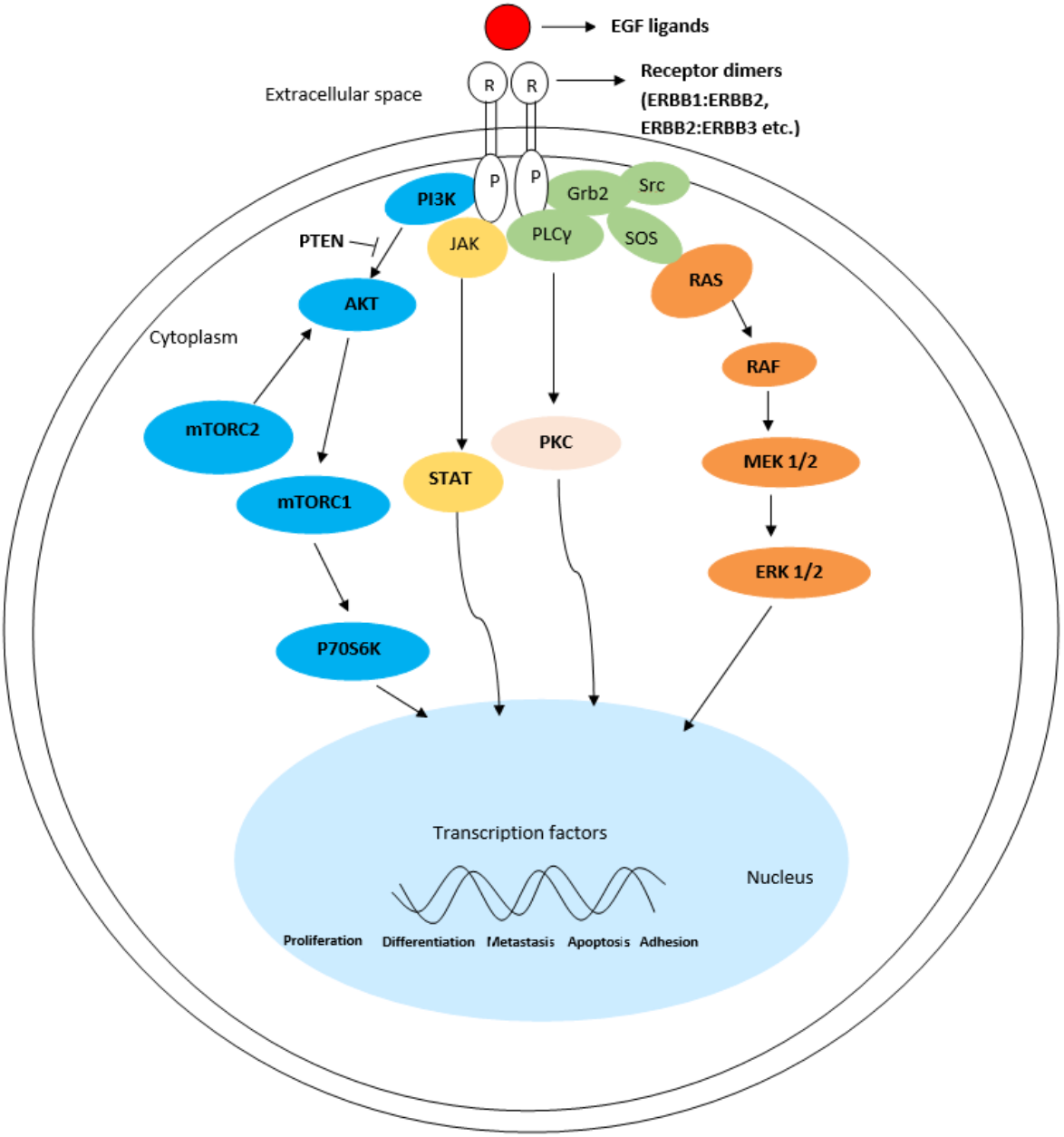


Figure 2.3 EGFR signalling pathways. Binding of the EGF ligands stimulates EGF receptors resulting in formation of homo- or hetero-dimers. PI3K, PLCγ, Grb2 and JAK bind to phosphoryrosine residues in the C-terminal tail of the receptors to initiate a network of signalling pathways. Adapted from (Henson & Gibson, 2006; Roskoski, 2014).

2.2.1.1. The EGFR family and MPM

This will be discussed is Chapter 7

2.2.1.2. Therapies targeting EGFR/HER1 and HER2

Monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors are the two classes of EGFR and HER2 inhibitors in clinical use (Table 3 in appendix A). Monoclonal antibodies block ligand binding to the extracellular domain of the EGF receptors and hence impede tyrosine kinase phosphorylation (Figure 2.4). Anti-EGFR monoclonal antibodies that have been developed include Cetuximab/Erbitux and Panitumumab/Vectibix. Cetuximab is effective for the treatment of wild type KRAS colorectal cancer (in combination with cytotoxic therapies) and head and neck cancers (in combination with radiation therapy or cytotoxic chemotherapy). Panitumumab is currently used as a second-line treatment for metastatic colorectal cancer after cytotoxic therapies. Anti-ERBB2 approved mAbs include Trastuzumab/Herceptin (used to treat HER2 positive breast, gastric and oesophageal cancers) and Pertuzumab/Omnitarg (used in combination with trastuzumab and docetaxel for the treatment of chemo-naïve HER2 positive metastatic breast cancers). Recently, Ado-trastuzumab, an antibody-drug (trastuzumab) conjugate, was approved to improve the efficacy of trastuzumab for HER2 positive metastatic breast cancers previously treated with Trastuzumab (Roskoski, 2004, 2014; Henson & Gibson, 2006). Besides monoclonal antibodies, small molecule tyrosine kinase inhibitors (TKIs) have also been developed. Gefitinib (Iressa) and Erlotinib (Traceva) selectively targets EGFR by binding the adenosine triphosphate (ATP) region in the catalytic domain (protein kinase domain) subsequently inhibiting auto-phosphorylation and downstream signalling. Lapatinib (Tykerb) reversibly blocks the ATP binding site on the kinase domain of HER2 and EGFR. Afatinib (Gilotril) irreversible blocks the kinase domain of HER2 and other HER family members and also possesses increased potency against resistant tumours (Bridges, 1999). There are other EGFR and HER2 experimental agents in different phases of clinical trial for the treatment of solid tumours.

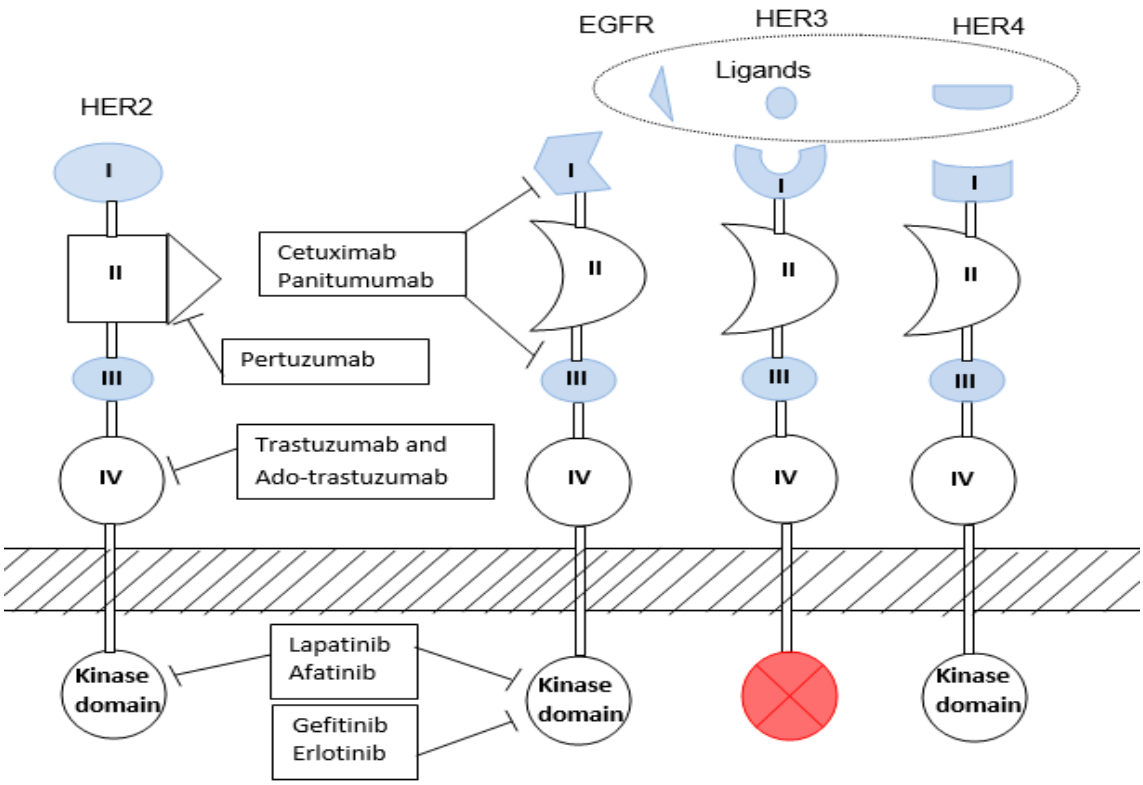


Figure 2.4 EGFR/HER family receptors and their inhibitors approved in clinical practice. Domain I of EGFR, HER3 and HER4 become activated upon ligand binding. Domain I of HER2 has no known ligand. The kinase domain of HER3 (red) has no catalytic activity. Cetuximab and Panitumumab blocks ligand binding to domain I and III of EGFR (HER1). Trastuzumab and its new derivative binds to the domain IV of HER2 preventing HER2 signalling. Gefitinib and Erlotinib inhibits the cytoplasmic kinase domain of EGFR while Lapatinib and Afatinib inhibit the kinase domains of both EGFR and HER2 Adapted from (Yarden & Pines, 2012).

2.2.1.3. EGFR related 'driver' oncogenes

EGFR mutations are commonly observed in non-small cell lung cancers. 90% of EGFR mutations include somatic mutations within exons 18-21 of the tyrosine kinase domain, in-frame deletions of exon 19 (around amino acids L746 to A750) and point mutation of L858R in exon 21. Mutations involving exons 18-21 were found to be higher in NSCLCs of East Asian populations (30%) when compared to those in the United States (10%) and predominant in patients with adenocarcinomas, females and non-smokers (Shigematsu et al 2005; Pao and Miller, 2005). EGFR gene amplification has been observed in approximately 15% of adenocarcinomas and 30% of squamous cell carcinomas while HER2 amplification occurs in 6% of adenocarcinomas and 2% of squamous cell carcinomas; the alterations of either gene is deficient in small-cell cancers (Herbst et al., 2008). Several studies have provided evidence that suggest that the presence of activating mutations in NSCLC patients is associated with response to EGFR tyrosine kinase inhibitors Gefitinib and Erlotinib. In 2004, three research groups compared the tumours of NSCLC patients sensitive to gefitinib with non-responders. Each group observed that the majority of the gefitinib-resistant tumours lacked EGFR gene mutations. Similar findings were also observed in patients who were sensitive to erlotinib (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004; Tsao et al., 2005). Activating mutations in the tyrosine kinase domain of EGFR disrupts auto-inhibitory interactions that regulate stability of the dormant resting state of the receptor hence locking it in a constitutively active state. The alterations in the kinase domain strongly favours inhibitor binding making the L858R mutant more susceptible to tyrosine kinase inhibitors than the wild type EGFR (Yun et al., 2007). A common mechanism of acquired resistance to EGFR-TKIs in NSCLC is the T790M gatekeeper mutation, a single amino acid substitution from threonine to methionine at position 790 in the kinase domain of wild type EGFR which subsequently confers resistance to both mutant and wild type EGFRs. (Pao et al., 2005). It is termed a 'gatekeeper' gene because it directly controls the cell cycle and its mutation results in uncontrolled cell growth.

Common EGFR mutations usually found in NSCLC are rarely found in MPM. A study conducted by Cortese et al (2006) on 66 mesothelioma patients did not identify any polymorphisms or point mutations in the specimens. In addition, simultaneous screen of 99 lung adenocarcinomas revealed that 19% harboured either an in-frame deletion in exon 19 or a L858R mutation (Cortese et al., 2006). Analysis of exons 18-21 of EGFR in 16 MPM patients using denaturing high performance liquid chromatography (DHPLC) and real-time PCR did not identify any mutations in the EGFR gene (Destro et al., 2006). In addition,

Okuda et al (2008) reported also no mutation in the EGFR gene of 25 MPM patients using a TaqMan PCR assay (Okuda et al., 2008). Recently, a study in 38 Japanese patients revealed five EGFR missense mutations using PCR. Two were novel (N816K and G875E), two had been previously observed in NSCLC (T725M, Q787Q) and T787T had been previously identified by Foster et al (2009) in peritoneal mesothelioma (Foster et al., 2010; Enomoto et al., 2012). The identification of novel EGFR genes in the Japanese MPM patients suggests the need for larger multi-institutional studies in order to identify patients that might benefit from anti- EGFR therapy.

Alterations in some genes downstream of the EGFR pathway have been shown to predict unfavourable responses to EGFR TKIs. For instance, KRAS, a GTPase that activates the RAS/RAF/MAPK pathway is commonly mutated in approximately 30% of adenocarcinomas and prevalent in non-Asian populations unlike EGFR mutations. In normal cells, guanine nucleotide exchange factors (GEFs) activate the RAS proteins in order to exchange guanosine diphosphate (GDP) for guanine triphosphate (GTP). GTPase-activating protein (GAPs) subsequently catalyses the hydrolysis of the bound GTP to return it to its dormant state. When KRAS is transformed, the activity of GAPs is impaired resulting in the accumulation of RAS in its constitutively-active GTP-bound state thereby sustaining the RAS signalling cascade (Aviel-Ronen et al., 2006; Karachaliou et al., 2013). In 2008, a meta-analysis studies reported that response to EGFR TKIs and anti-EGFR mAbs were unlikely in the presence of KRAS mutations in NSCLC and metastatic colorectal cancer (mCRC) (Linardou et al., 2008). The findings in mCRCs were further substantiated in another systematic review of 22 studies of 2188 mCRC patients treated with Cetuximab. The study revealed that KRAS mutations in mCRC patients was indicative of an adverse response to Cetuximab when compared with wild-type KRAS patients (14% vs 39% in wild-type KRAS) (Qiu et al., 2010). A recently published large systematic review that included 41 trials (6939 NSCLC patients) reported that KRAS mutations are associated with poor prognosis exclusively in patients with adenocarcinomas, early stage NSCLC and of Asian origin. Heterogeneity was however observed in the collective 41 studies, advanced NSCLCs and in the PCR-direct nucleotide sequencing method (Meng et al., 2013). The prognostic significance of KRAS was in keeping with results of a meta-analysis of 28 studies previously published in 2005 (Mascaux et al., 2005).

Three published studies have failed to identify the presence of KRAS in MPM cell lines and tissue samples (Metcalf et al., 1992; Ni et al., 2000; Kitamura et al., 2002) resulting in a distorted perspective of the presence of these mutations in MPM patients. Recently,

Mezzapelle et al (2013) were the first to observe five KRAS mutations in codons 12 and 13 of 77 MPM patients using a mutant-enriched PCR (ME-PCR) technology. Unlike previous studies, large sample number and a sensitive method of 0.1% sensitivity was used. This might explain in part the discrepancies observed in the results. There was significant difference in the overall survival of patients with or without KRAS mutation although all patients with KRAS mutation had prior asbestos exposure (Mezzapelle et al., 2013). Another recent study by Shukuya et al (2014) identified one KRAS mutation (G12D) in 42 MPM patient samples using pyrosequencing and qPCR methods. Poor survival was observed in the patient with KRAS mutation although based on the small sample size there is a need for the study of genetic alterations in a larger study (Shukuya et al., 2014). Both of these studies are from patients with different ethnicity and based on EGFR mutations observed in NSCLC, it is possible to expect higher KRAS frequency amongst patients from East Asian populations. These findings underline the importance of molecular characterisation to enable individualized treatments in MPM patients.

The BRAF gene encodes a serine/threonine protein kinase that is a downstream effector of KRAS in the RAS/RAF/MAPK pathway. Activating mutations are common within the kinase domain with glutamate substituting valine at codon 600 (V600). Other non-V600E mutations such as G468A and L596R accounts for approximately 50% of BRAF mutations in lung cancer (Davies et al., 2002; Cardarella et al., 2013). In colorectal cancers, BRAF mutation is associated with adverse overall survival and progression-free survival in addition to resistance to anti-EGFR mAbs (De Roock et al., 2010). A study analysed 53 tumours and 6 6 cell lines from MPM patients using a sensitive PCR-restriction fragment length polymorphism method (PCR-RFLP) but did not identify any BRAF mutations (Dote et al., 2004) In contrast, Mezzapelle et al (2013) recently identified three BRAF mutations in 77 MPM samples analysed. The mutation observed was the classic V600E mutation occurring in two epithelioid and one biphasic MPM tumour with no prior asbestos exposure (Mezzapelle et al., 2013). The identification of BRAF mutation in MPM patients might infer a possible role of new targeted therapies such as Vemurafenib in the treatment of mesotheliomas with mutant BRAF.

2.2.2. The Vascular Endothelial Growth Factor

Angiogenesis (neovascularization) is a fundamental process in tumour growth, development and metastasis which ensures sufficient oxygen and nutrient supply to malignant cells via the creation of new blood vessels, from the pre-existing vascular network. The role of angiogenesis and its potential therapeutic benefit in cancer was first proposed by Folkman in 1971 after a series of experiments, that provided evidence that solid tumours are 'angiogenesis-dependent' (Folkman, 1971; Folkman & Klagsbrun, 1987). 'Angiogenic switch' during tumour development arises when endogenous inducers of angiogenesis overpower endogenous inhibitors therefore altering the balance of angiogenic mediators and stimulating angiogenesis (Hanahan & Folkman, 1996). The most studied and major drivers of normal and tumour angiogenesis are the members of the vascular endothelial growth factor family which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). Other molecule families that are active in angiogenesis include: platelet derived growth factor family (PDGF), basic fibroblast growth factor family (bFGF), ANG and tyrosine kinase endothelial family (TIE), the cadherin family, the notch- and delta-like ligand family (DLL_4), the ephrin family (EPH), the semaphorin family (SEMA) and several others.

VEGF-A is also referred to as VEGF, exists in multiple isoforms through alternative splicing and is the most important in angiogenesis. VEGF derived from tumours plays a critical role in the recruitment and formation of a vascular system that supports tumour growth. It is also known to play multiple roles in stimulating the proliferation, migration, and invasion of endothelial cells, organisation of the endothelial cells into functional tubular structures, recruiting circulating endothelial cells and progenitor cells to sites of neovascularization and also inducing vascular permeability (Cébe-Suarez et al., 2006; Shibuya & Claesson-Welsh, 2006). VEGF-B and PlGF are mainly involved in embryology however, VEGF-C & D also induce angiogenesis.

The VEGF family ligands signal through three different tyrosine receptor kinases: VEGFR1 (Flt-1), VEGFR2 (Flk1/KDR), VEGFR3 (Flt-4) and co-receptors such as neuropilins (NRPs) and heparin sulphate proteoglycan (HSPGs). The tyrosine kinase receptors are structurally similar and regulate diverse downstream activities. Each one contains a seven member immunoglobulin-like (Ig) extracellular domain, a single transmembrane region, a juxtamembrane segment, a split intracellular protein-tyrosine kinase domain interrupted by a large kinase insert (70-100 amino acid residues) and a carboxyterminal tail (Figure 2.5). On the other hand, the co-receptors lack VEGF-induced catalytic functions (Ferrara, 2004; Hoeben et al., 2004; Olsson et al., 2006; Sullivan & Brekken, 2010). The expression of

VEGFs and their receptors was initially associated with endothelial cells but studies have shown that they are also expressed in several tumours and their expression is related to clinical variables such as survival, disease progression etc. as reviewed by (Goel & Mercurio, 2013).

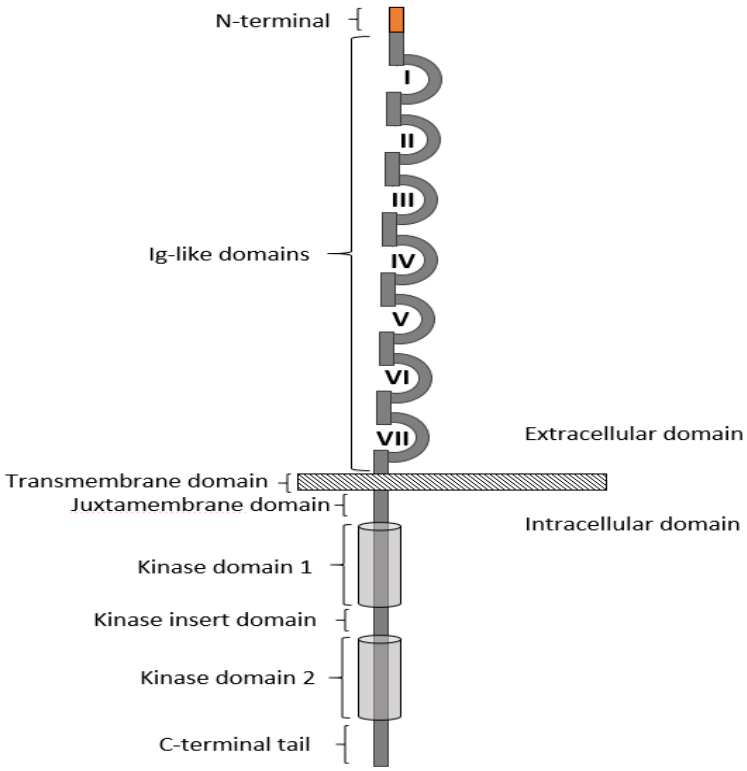


Figure 2.5 Structure of the VEGF receptor. This diagram shows the basic structure of a VEGFR which includes: seven immunoglobulin-like (Ig-like) ligand binding loops, a single transmembrane region, a juxtamembrane segment, a split intracellular protein-tyrosine kinase domain interrupted by a large kinase insert and a carboxyterminal tail. In VEGFR1, ligand binding is dependent on Ig-like loops 2 and 3, VEGFR2 ligand binding also depends on similar loops but with 10-times less affinity than VEGFR1. VEGFR3 ligand binding is dependent on loops 1 and 2. Adapted from (Holmes et al., 2007)

VEGFR1 (flt-1: fms-like tyrosyl kinase-1) is mainly expressed in endothelial cells during embryonic development. It has a high binding affinity for VEGF-A, -B and PGF but its weak tyrosine kinase activity makes it difficult to evaluate the basal levels of VEGFR1 auto-phosphorylation in cells, hence its role remains less well defined (Peters et al., 1993). It was observed that VEGFR1-null mice do not survive early stage embryogenesis as a result of

disorganised vascular network and an increase in the number of endothelial cells. However, mice that lack the tyrosine kinase domain of VEGFR1 but preserve the ligand-binding extracellular domains and transmembrane segments remain viable, emphasizing the significant role of ligand sequestration in the function of VEGFR1 (Fong et al., 1995; Hiratsuka et al., 1998; Kearney et al., 2002). VEGR1 has been shown to form a heterodimer with VEGFR2 which has stronger signalling properties. The splice variant of this receptor is the soluble VEGFR1 (sVEGFR1), both VEGFR1 and sVEGFR1 bind VEGF-A with higher affinity than VEGFR-2 and can prevent the activation of VEGFR-2. Upregulation of sVEGFR1 is commonly linked to hypertension and pre-eclampsia in expectant mothers (Clark et al., 1998; Huang, 2001; Robinson et al., 2006). Although the mechanism of action of VEGFR1 is yet to be elucidated, there is growing evidence that supports its involvement in haematopoiesis, recruitment of bone marrow-derived progenitor cells and migration of monocytes hence increasing tumour growth and metastasis. It has the ability to act as a positive or negative regulator of angiogenesis under different biological conditions (Barleon et al., 1996; Hiratsuka et al., 2001; Gerber et al., 2002; Hattori et al., 2002; Luttun et al., 2002). VEGFR1 is expressed in cancers such as bladder, breast, lung, brain, melanoma, oesophageal, ovarian, pancreatic, prostate and mesothelioma (Sato et al., 1998; Marschall et al., 2000; Strizzi et al., 2001; Straume & Akslen, 2003; Mylona et al., 2007; Gockel et al., 2008; Carrillo de Santa Pau et al., 2009).

VEGFR2 (Flk-1/KDR; Fetal liver kinase-1/Kinase Domain-containing Receptor) as its name denotes, has a strong kinase activity and binds all VEGF-A isoforms however, proteolytically cleaved forms of VEGF-C and VEGF-D can also activate it. It has a molecular weight of 210-230 kDa and is the major positive signal transducer of VEGF-induced endothelial cell migration, survival, proliferation and enhanced vascular permeability in physiological or pathological angiogenesis (Waltenberger et al., 1994; Bernatchez et al., 1999; Gille et al., 2001; Takahashi et al., 2001). In adults, VEGFR2 is mainly expressed on vascular endothelial cells but also detectable in megakaryocytes, neuronal cells and haematopoietic stem cells (Katoh et al., 1995). VEGFR2 is the predominant and most studied VEGF receptor that controls VEGF signalling in endothelial cells and initiates VEGF-mediated angiogenesis. VEGFR2 knockout mice were reported to be embryonic lethal as a result of severe defects in the development of endothelial and haematopoietic cells. There was absence of organized blood vessels and blood islands in the developing embryo (Shalaby et al., 1995). VEGFR2 expression has been reported in several tumour types including lung cancer and mesothelioma (Strizzi et al., 2001; Carrillo de Santa Pau et al., 2009; Miettinen et al., 2012).

Since VEGFR2 has been identified as the major player in tumour angiogenesis, it is the main focus in this brief review and the signal transduction pathway of this receptor is described below.

VEGFR3 (Flt4) preferentially binds to VEGF-C and VEGF-D and plays a significant role in the modification of primary vascular networks in embryos and promotes angiogenesis and lymphangiogenesis in adults (Pajusola et al., 1992; Galland et al., 1993; Kaipainen et al., 1995). It is usually present in all endothelial cells during development but becomes restricted to lymphatic vessels in adults. It is also the only VEGF receptor for which mutations have been described. Studies on VEGFR3 knock out mice revealed the development of cardiovascular failure at embryonic day 9.5. Large vessels displayed abnormal structure and organisation with defective lumens hence resulting in the accumulation of fluid within the pericardial cavity (Dumont et al., 1998). These finding suggest a role for VEGFR3 in the development of the cardiovascular system. Missense mutations within the catalytic loop of the VEGFR3 kinase domain is also linked to primary lymphoedema (Milroy disease) an indication of its role in the lymphatic vasculature in adults (Karkkainen et al., 2000). VEGF-C and VEGF-D are also able to undergo proteolytic processing in other to bind to VEGFR2. VEGFR3 is capable of forming homodimers or heterodimers with VEGFR2 in response to processed VEGF-C. VEGFR3 has been shown to be expressed in various cancer cells including mesothelioma (Filho et al., 2007). The major difference in the receptor tyrosine kinases is found in the fifth Ig extracellular domain of VEGFR3 which is proteolytically cleaved and the polypeptides remain linked by a disulphide bridge (Pajusola et al., 1994). Studies have also shown that the binding of specific ligand results in phosphorylation of selective tyrosine residues and the stimulation of distinct cellular function. The signal transduction pathway of VEGFR1 remains to be elucidated but has been implicated as a negative regulator of angiogenesis by binding VEGF and preventing the activation of VEGFR. Nonetheless there are also reports which shows that VEGFR1 could promote VEGFR2 activity (Rahimi et al., 2000; Carmeliet et al., 2001; Zeng et al., 2001). VEGFR2 can also limit the activity of VEGFR3 by binding to proteolytically cleaved VEGF-C and VEGF-D.

2.2.2.1. VEGFR2 signalling

Low oxygen tension (hypoxia) is a major regulator of VEGF production under both physiological and pathological conditions. During hypoxia, the hypoxia-inducible factor 1 (HIF-1) accumulates and allows the binding of dimerized HIF-1 to hypoxia-responsive element (HRE) on the promoter region of VEGF resulting in the transcription of multiple genes (Levy et al., 1995; Liu et al., 1995). Other stimuli such as growth factors and inactivation of tumour suppressor genes can also induce hypoxia and VEGF (Van Meir et al., 1994; Maher & Kaelin, 1997; Déry et al., 2005).

The binding of covalently linked VEGF dimer to the receptor, induces homo- or hetero-dimerization and further results in autophosphorylation and activation of a complex intracellular signalling cascade. The auto-phosphorylation occurs in trans such that one kinase of the dimer initiates the phosphorylation of tyrosine residues in the second and the latter catalyses the phosphorylation of tyrosine residues in the first. Within the activation loop of the kinase domain, autophosphorylation of tyrosine residues stimulates catalytic activity while autophosphorylation at other sites generates docking sites for Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains (Roskoski, 2007; Stuttfeld & Ballmer-Hofer, 2009). Studies have shown that tyrosine residues Y801, Y951, Y996, Y1054, Y1059, Y1175 and Y1214 are phosphorylated in the intracellular domain of VEGFR2 upon ligand binding. Y1054 and Y1059 have been identified as autophosphorylation sites that are vital to the catalytic activity of the receptor. Phosphorylation of Y1175 recruits several signalling proteins including PLCγ and adaptor proteins SHB and SCK (Warner et al., 2000; Holmqvist et al., 2003). Y1214 and Y951 serves as a docking site for adaptor protein NCK and signalling adaptor protein VRAP/TSAd (VEGFR-receptor associated protein/T-cell-specific adaptor molecule) respectively (Figure 2.6) The docking of these proteins stimulates the activation of the MAPK, protein kinase C and AKT resulting in cell proliferation and survival. Cell migration is also induced via the activation of focal adhesion kinase, PI3K and matric metalloproteinases (MMPs). Phosphorylation of Y1214 activates the Cdc42 and p38 MAPK pathway which subsequently regulates cell motility. Activation of VEGFR2 also results in the synthesis of prostaglandins via a pathway requiring increased intracellular calcium and via ERK1/2 mediated phosphorylation of cytosolic phospholipase A_2 (Wheeler-Jones et al., 1997; Gerber et al., 1998; Gliki et al., 2001; Gately & Li, 2004; Lamalice et al., 2004, 2006; Matsumoto et al., 2005).

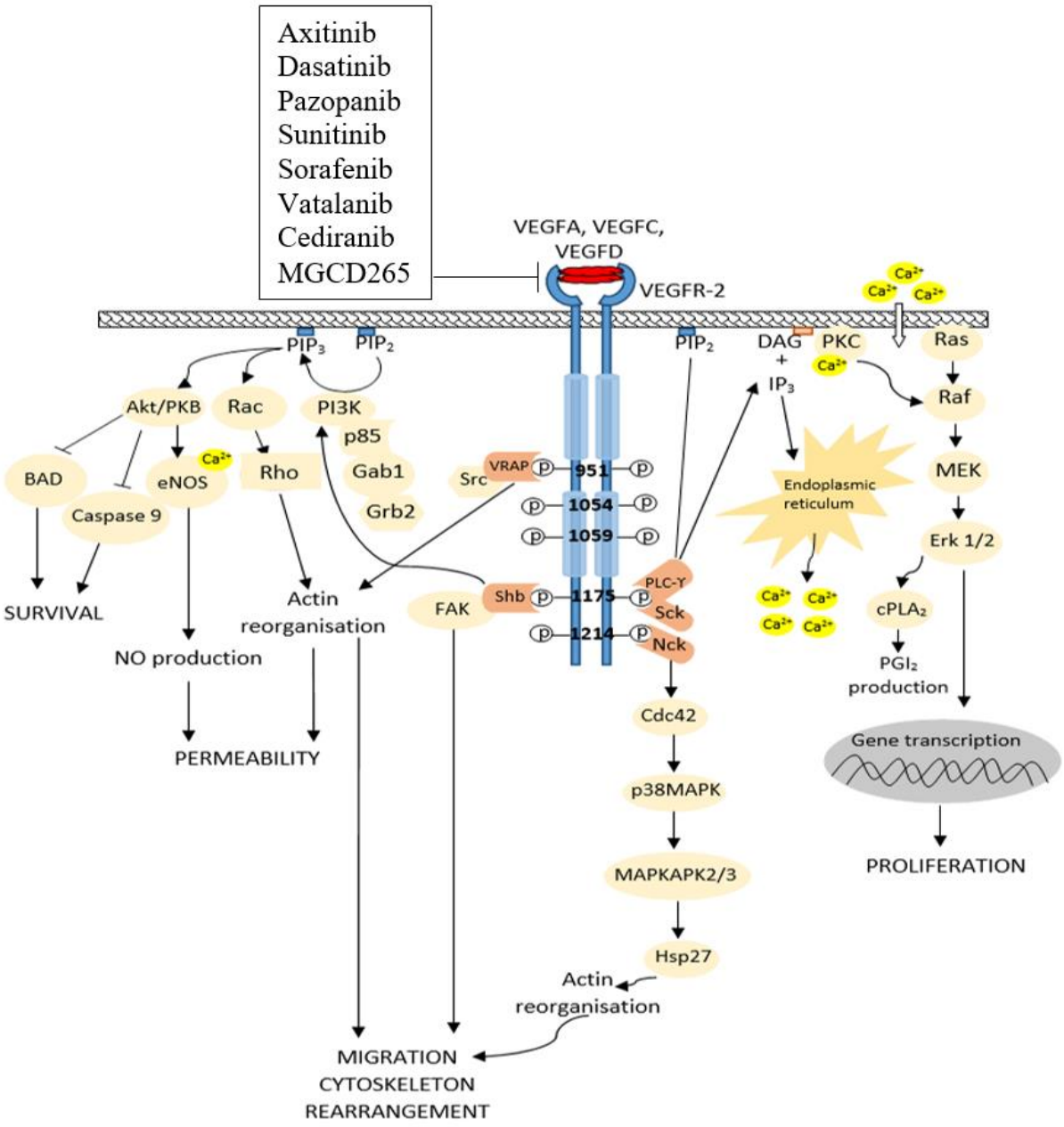


Figure 2.6 VEGFR2 and its intracellular signalling cascade. Upon ligand binding, the receptor dimerizes and phosphorylation of specific intracellular tyrosine residues commence. Adaptor proteins such as SHB, Grb2, SCK, NCK etc bind to specific phosphorylated tyrosine residues and recruit several intracellular proteins. Influx of extracellular calcium (yellow circles) is important for the activation of prostaglandins. Tyrosine kinase inhibitors that inhibit VEGFR-2 and other related proteins are also listed. Adapted from (Olsson et al., 2006; Holmes et al., 2007)

2.2.2.2. VEFGR and other receptors

Apart from multiprotein complexes with co-receptors and additional cellular protein such as neuropilins, heparan sulfate, cadherins and integrins; VEGF receptors and their co-receptors (NRPS) also interact and influence the function of other growth factor receptors. VEGFR2

has been reported to form a complex with the hepatocyte growth factor receptor (c-MET) (section 2.2.3) in response to VEGF stimulation in glioblastoma cells hence regulating MET signalling. VEGFR2 has the ability to activate the ligand-binding function of several integrins in tumour cells via the PI3K-AKT pathway. NRP1 also interacts with MET receptor and stimulates invasion of malignant pancreatic cells as well as the survival and proliferation of glioma by upregulating HGF (Hu et al., 2007; Matsushita et al., 2007; Lu et al., 2012). NRPs have also been reported to bind other growth factors including bFGF, transforming growth factor-β (TGF-β) and PDGF but the involvement of VEGF and the signalling responses generated from these interactions is yet to be determined (West et al., 2005; Banerjee et al., 2006; Glinka & Prud'homme, 2008).

2.2.2.3. VEGFR and MPM

This will be discussed in Chapter 6

2.2.2.4. VEGFR therapies

Over the years, several monoclonal antibodies and small molecules that target the VEGF pathway have been developed and studied as single agents and in combination with chemotherapy. VEGF inhibitors can be grouped into three categories that include neutralizing antibodies to VEGF or VEGFRs, soluble VEGF receptors or receptor hybrids and tyrosine kinase inhibitors. Two monoclonal antibodies have been developed for angiogenic inhibition. Bevacizumab (Avastin), a humanized IgG1 monoclonal antibody against VEGF-A was the first FDA approved therapy to inhibit angiogenesis. It prevents the binding of VEGF-A to its receptors hence interrupting signal transduction. It is effective in many tumours types and is currently approved for the treatment of glioblastoma, NSCLC, metastatic renal cell carcinoma, ovarian cancer and metastatic colorectal carcinoma. Recently it was approved in combination with IFN-α to treat patients with relapsed or stage IV and medically or surgically unresectable renal cell carcinoma (Falk et al., 2015; Majid et al., 2015). Ramucirumab (IMC-1121B, LY3009806) is a recombinant human monoclonal antibody that binds exclusively to VEGFR-2. It binds to the extracellular domain of VEGFR2 as a receptor antagonist and blocks the binding of VEGF to VEGFR2 thus inhibiting downstream effects. It was only recently (2014) approved as a single agent or in combination with a chemotherapy drug (paclitaxel) for the treatment of patients with advanced gastric or gastro-oesophageal junction adenocarcinoma. Subsequently, FDA approved it in combination with docetaxel for the treatment of metastatic NSCLC (Aprile et al., 2014; Poole & Vaidya, 2014).

IMC-18F1 is a fully humanized monoclonal antibody that inhibits VEGFR1 but still in preclinical stage (Wu et al., 2006).

The use of peptide-antibody fusion drugs to block angiogenesis led to the development of Aflibercept (VEGF-Trap, AVE0005). The fusion uses the peptide as a decoy protein to bind the ligand of interest and they are thought to have increased affinity for target ligands than monoclonal antibodies. Aflibercept is a recombinant fusion protein that consists of VEGF binding portions from the extracellular Ig domains of VEGFR1 and 2 used to the antibody Fc fragment of IgG1. It has a high affinity for all isoforms of VEGF-A, VEGF-B and PIGF therefore preventing them from binding and activating their cognate receptors. It was approved in 2012 in combination with 5-flurouracil, leucovorin and irinotecan for the treatment of metastatic colorectal cancer. It is also being tested in other solid tumours (Ciombor et al., 2013; Tarallo & De Falco, 2015).

Small molecule inhibitors of VEGFR tyrosine kinases are another therapeutic approach for inhibiting angiogenesis. Several of these molecules are at different stages of clinical development. The VEGFR tyrosine kinase inhibitors currently approved for use in clinics for solid tumours include sorafenib, imatinib and sunitinib. These inhibitors are multitarget drugs that compete with ATP for binding within the intracellular domain of the receptor tyrosine kinase. Sorafenib was the first approved anti-angiogenic tyrosine kinase inhibitor as a single agent for the treatment of metastatic renal cell carcinoma and unresectable hepatocellular carcinoma (HCC). Subsequently, several anti-angiogenic inhibitors have been approved by the FDA for cancer therapy: Axitinib, Pazopanib, Sunitinib, Vandetanib, Regorafenib, and Lenvatinib (see details in Table 2.2) (Niu & Chen, 2010; Tarallo & De Falco, 2015)

Table 2.2 Approved anti-angiogenic tyrosine kinase inhibitors

Drugs/Company	Year approved (FDA)	Target	Indication
Sorafenib (Bayer/Onyx)	2005	VEGFRs, PDGFRs, FGFR1, KIT, RAF	Metastatic renal cell carcinoma Hepatocellular carcinoma Thyroid cancer (differentiated)
Sunitinib (Pfizer)	2006	VEGFRs, PDGFRs, KIT	Metastatic renal cell carcinoma Gastrointestinal stromal tumour Unresectable pancreatic neuroendocrine tumours
Pazopanib (GlaxoSmithKline)	2009	VEGFRs, PDGFRs, KIT	Unresectable renal cell carcinoma Advanced soft tissue sarcoma
Vandetinib (Astrazeneca)	2011	VEGFRs, EGFR, RET	Late-stage Medullary carcinoma of thyroid
Axitinib (Pfizer)	2012	VEGFRs, PDGFRs, KIT	Metastatic renal cell carcinoma
Regorafenib (Bayer/Onyx)	2013	VEGFRs, TIR2, PDGFRs, RET, KIT, FGFRs	Metastatic colorectal cancer Gastrointestinal stromal tumour
Lenvatinib (Eisai Co.)	2015	VEGFR-2&3	Thyroid cancer (differentiated)

2.2.3. The Hepatocyte growth factor and its receptor (c-MET)

The mature form of Hepatocyte Growth Factor (HGF)/scatter factor (SF) is a heterodimer comprising of a 69 kDa α-chain and a 34 kDa β-chain linked by a disulphide bond, that is cleaved after the secretion and proteolytic conversion of the 92 kDa inactive pro-HGF in the extracellular environment. It was initially discovered in the 1980s in the serum of partially hepatectomized rats and identified as a mitogen that stimulates the growth of rat hepatocytes in primary cultures. It was identified as both a motility factor and a scatter factor for hepatocytes in addition to being a high affinity ligand for MET (Nakamura et al., 1984, 1987; Stoker et al., 1987; Naldini et al., 1991; Naldini et al., 1991; Weidner et al., 1991). HGF is a large multi-domain protein that is closely related to plasminogen, a blood protease precursor responsible for the lysis of blood clots. The α-chain contains an amino-terminal domain (N), four tandem repeats of kringle domains (K1-K4) and a serine protease-like C-terminal β-chain that lacks enzymatic activity due to mutations (Figure 2.7). Studies have shown that the inactive pro-HGF and mature HGF possess similar binding affinity to c-Met but only the mature form is able to activate c-Met (Hartmann et al., 1992; Lokker et al., 1992). The α-chain region which consist of the amino-terminal domain and the K1 domain has a high affinity for the Ig3 and Ig4 domains of c-Met in a manner independent of HGF maturation. The SPH domain in the β-chain region has low affinity binding to the semaphorin domain of c-Met; nonetheless both interactions are essential for receptor dimerization and activation (Stamos et al., 2004; Holmes et al., 2007; Basilico et al., 2008).

The HGF/SF receptor c-MET was first identified in a human osteosarcoma tumour cell line (HOS) exposed to a carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Exposure to MMNG produced a gene rearrangement resulting in a novel fusion protein between the translocated promoter region (TPR) on chromosome 1 and the MET kinase domain on chromosome 7. The TPR-MET fusion protein consisted of an N-terminal leucine-zipper protein-protein interaction domain and a C-terminal kinase expressed from tpr and c-met respectively. This fusion protein was also found to possess a constitutively active kinase activity with transforming abilities and the isolation of TPR-MET cDNA led to the identification of the full-length Met receptor and receptor tyrosine kinase properties (Park et al., 1986; Park et al., 1987). The c-MET proto-oncogene is located on chromosome 7q21-q31 and spans more than 120 kb in length which consists of 21 exons partitioned by 20 introns (Liu, 1998). MET is initially produced in epithelial cells as a single chain precursor of 170 kDa and subsequently undergoes co-translational glycosylation before cleaving to produce a mature 190 kDa transmembrane receptor. The mature form of c-Met is also a disulphide-

linked heterodimer which consists of an extracellular α-chain (50 kDa) linked to a longer membrane-spanning β-chain (145 kDa) that contains part of the ectodomain, a transmembrane helix and a cytoplasmic region (Park et al., 1987; Giordano et al., 1989). The extracellular region of c-Met adopts a seven-bladed β-propeller structure (which consists of the α & β semaphorin domains) that lies above a cysteine-rich Plexin, Semaphorin, Integrin (PSI) domain and four immunoglobulin-like regions in Plexin and Transcription factors (IPT) (Gherardi et al., 2003). The PSI and IPT domains are similar to those found in plexins, semaphorins and integrins (Bork et al., 1999). The intracellular region is composed of the juxtamembrane, a catalytic tyrosine kinase domain and a carboxy-terminal docking site essential for downstream signalling (Figure 2.7) (Ponzetto et al., 1994).

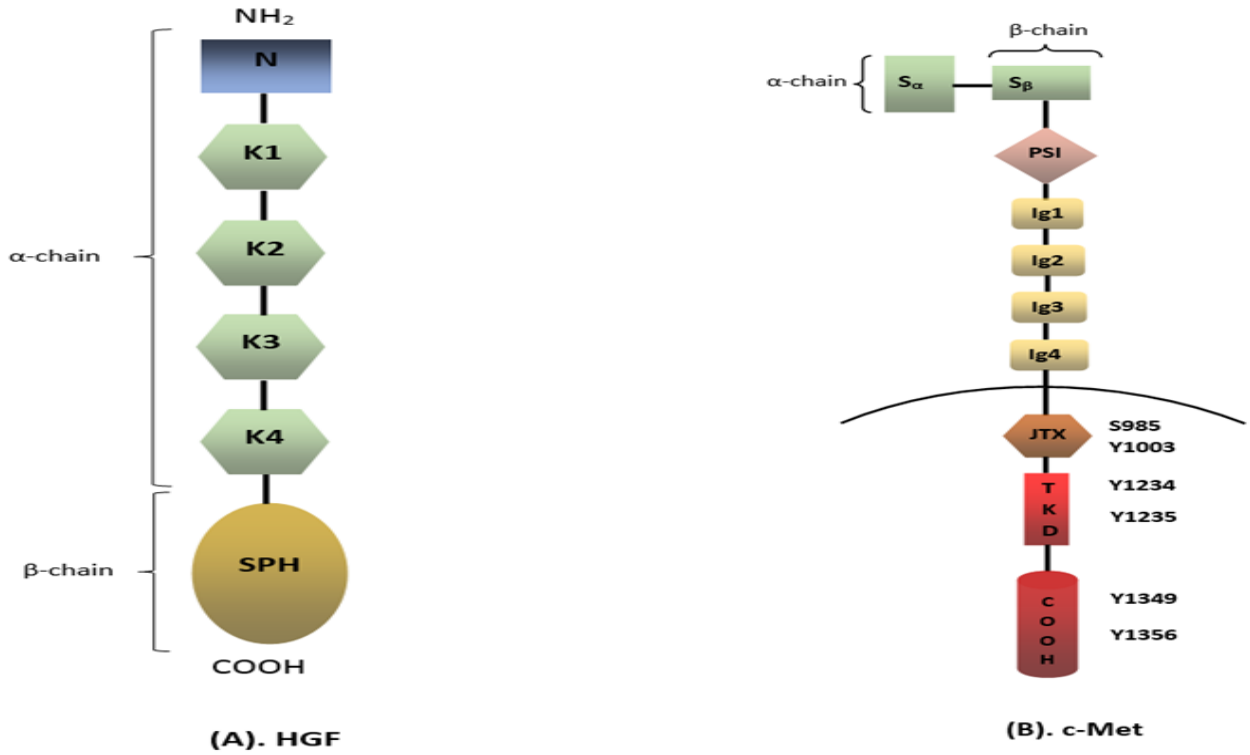


Figure 2.7 A schematic diagram of HGF and c-MET. A.) Structure of HGF shows the functional domains of the mature hepatocyte growth factor heterodimer. The α-chain consists of an amino-terminal domain (N) (blue), four tandem repeats of kringle domains (K1-4) (green) and the β-chain contains the serine protease homology (SPH) domain. B.) Mature form of the c-Met receptor is a heterodimer made of α and β subunits. The extracellular region consists of the sema domain (Sα and Sβ) (green), a PSI domain (peach), and four IPT domains (yellow). The intracellular region contains a juxamembrane domain (orange) with two regulatory sites (S985 and Y1003), a kinase domain (red) with phosphorylation sites (Y1234 and Y1235) and a carboxy-terminal (COOH) with phosphorylation sites (Y1349 and Y1356). Adapted from (Birchmeier et al., 2003; Trusolino et al., 2010)

In normal physiological condition, c-MET is predominantly expressed in cells of epithelial origin but has also been seen in endothelial cells, melanocytes, hematopoietic progenitors, neuronal cells etc. while HGF/SF is mainly expressed in mesenchymal cells (Jeffers et al., 1996). When HGF is secreted from mesenchymal cells, it binds to expressed c-Met on the surface of epithelial cells and activates it via paracrine signalling. The ligand-receptor pairing permits communication between epithelial and mesenchymal cells therefore inducing several biological processes that involve interactions between these two groups of cells that are critical for normal development. In embryogenesis, c-MET is required for liver and placental development as well as migration of myogenic precursor cells while in adult tissues, c-MET is essential for tissue remodelling and morphogenic differentiation via epithelial-mesenchymal transition (EMT) interactions. HGF/c-Met signalling plays a critical role in invasive growth and other complex morphogenetic processes such as proliferation, migration, and tubulogenesis required for tissue organisation, embryonic development, muscle development, nervous system formation and angiogenesis (Kawaida et al., 1994; Schmidt et al., 1995; Ebens et al., 1996; Nakamura et al., 2000; Chmielowiec et al., 2007). These attributes of c-Met are also exploited by cancer cells to promote metastasis, inhibit apoptosis and induce resistance.

Aberrant c-Met expression has been documented in numerous human epithelial cancer including glioma, breast, pancreas, lung cancers and mesothelioma (Furukawa et al., 1995; Tuck et al., 1996; Koochekpour et al., 1997; Tolnay et al., 1998; Navab et al., 2009; Kentsis et al., 2012; Xie et al., 2012). In cancers, c-Met is up-regulated and constitutively active resulting in tumour cell metastasis, hyperproliferation and tumour angiogenesis. The invasive mechanism occurs when c-Met activated cells dissociates and invades the basement membrane. The degradation of the basement membrane allows the cells to reach the bloodstream and extravasated blood allows the cells to migrate and colonize other sites (Migliore & Giordano, 2008).

2.2.3.1. HGF/c-MET signalling

In non-malignant cells c-Met activation is a tightly regulated process. The binding of HGF to c-MET induces a cascade of downstream signalling resulting in mitogenesis, motogenesis, morphogenesis and angiogenesis. c-MET is dimerized upon ligand binding leading to the autophosphorylation of the tyrosine residues Y1234 and Y1235 in the kinase domain therefore inducing kinase activity. Studies have shown that when Y1234 or Y1235 is substituted with phenylalanine (an essential amino acid), the c-Met kinase activity is significantly reduced (Longati et al., 1994). Conformational change within the receptor also leads to the phosphorylation of tyrosine residues Y1349 and Y1356 in the carboxy-terminal tail which serves as a multi-substrate docking site for adaptor proteins and signalling molecules. Mutations in Y1349 and Y1356 have been shown to result in the loss of the biological function of c-Met (Ponzetto et al., 1994). The adaptor proteins recruited to the docking site include substrates such as the Src homology 2 domain-containing (Shc) adaptor, the non-receptor tyrosine kinase Src, the growth factor receptor-bound protein 2 (Grb2) adaptor, the p85 subunit of phosphatidylinositol 3′ kinase (PI3K), phospholipase Cγ (PLCγ), Src homology-2 containing inositol 5-phosphastase 1 (SHIP1), Tyrosine phosphatase SHP2, Grb2-associated binding protein (GAB1) and signal transducer and activator of transcription 3 (STAT3) (Figure 2.8) (Ponzetto et al., 1993, 1994; Fixman et al., 1996). Most of these adaptor proteins contain Src-homology 2 (SH2) binding domains that may not allow some adaptor proteins bind to the docking site simultaneously due to steric effects (Stefan et al., 2001). GAB1 is a universal docking protein that once phosphorylated by c-Met, forms an extra binding site for other docking molecules. It is able to bind directly to c-Met through its 13-amino acid c-Met-binding domain (MBD) or indirectly via the GRB2 adaptor protein. GRB2 can only bind through its SH2 domain to the pY1356 region of c-Met and GAB1 binds to the SH3 domain of GRB2 to become activated. GAB1 signalling following EGFR activation (section 2.2.1) is transient compared to the prolonged and sustained Gab1 phosphorylation after c-Met activation indicating its unique interaction with c-Met in relation to protein recruitment and phosphorylation kinetics which is essential for stimulating morphogenesis (Maroun et al., 1999; Lock et al., 2000). The intracellular downstream signal transduction pathways activated after c-MET phosphorylation include the ERK/MAPK pathway, the AKT/PKB pathway and the signal transducer and activator or transcription proteins (STATs).

Activation of the RAS/RAF/MAPK pathway occurs via different mechanisms; it can be activated directly through the binding of the GRB2-SOS complex to the c-MET docking site

or indirectly via the SHC adaptor protein (Ponzetto et al., 1994; Pelicci et al., 1995). Another possible mechanism of RAS-MAPK activation is the dephosphorylation of the p120 -Ras-GAP binding site on GAB1 by the tyrosine phosphatase SHP2, to promote the activation of Ras (Maroun et al., 2000; Montagner et al., 2005). Ras is regulated by GEPs (Guanine exchange proteins) and GAPs (GTPase-activating proteins). SOS (son of sevenless), a Ras specific guanine nucleotide exchange factor is shuttled to the plasma membrane where it activates Ras. This sequence of events leads to the activation of v-raf murine sarcoma viral oncogene homology B1 kinases (RAF) which subsequently activates MEK1/2 (MAPK/ERK kinase-1 and 2) and finally ERK1/2. Active ERK is translocated to the nucleus where it activates transcription factors (Elk1 and Ets) and adhesion molecules responsible for cell cycle progression, proliferation and motility (Paumelle et al., 2002). Phosphorylated Ras also activates the Rac1 and CDC42 pathways that are responsible for the regulation of cytoskeleton which subsequently promotes cell polarity and motility. The Rac1 pathway also activates the MEKK/JNK (MAP/ERK kinase kinases/c-Jun Kinase) and MEK/p38 pathways that contributes to cell survival and differentiation following the activation of transcription factors c-Jun and c-Fos (Xiao et al., 2001).

The PI3K/AKT signalling pathway is activated following the binding of the p85 subunit of PI3K to phosphorylated c-Met or through the protein adaptor GAB1, which then signals through AKT/protein kinase B. The Akt axis primarily regulates cell survival by suppressing apoptosis through the inactivation of the proapoptotic protein BAD (Bcl-2-associated death promoter) and activation of the E3 ubiquitin-protein ligase MDM2 resulting in the degradation of tumour suppressor gene p53 (Ponzetto et al., 1994). The mammalian target of rapamycin (mTOR) which is downstream of Akt is activated following the inhibition of positive cell cycle regulators (Myc and cyclin d1) via the inactivation of glycogen synthase kinase 3β (GSK3β) by Akt thus stimulating protein synthesis and survival (Graziani et al., 1991; Xiao et al., 2001; Jung et al., 2012). STAT3 is activated by binding to the trans-phosphorylated docking site of c-Met. On activation, STAT3 dissociates from the receptors, becomes homodimerized via their SH2 domains and eventually gets translocated to the nucleus. In the nucleus, STAT3 dimers function as transcription factors that regulate the expression of several genes involved in cell invasion (Zhang et al., 2002).

The termination of c-Met signalling is predominantly controlled through internalization and degradation of the receptor. The phosphorylated Y1003 residue on the juxtamembrane (JM) region of c-Met is recognized and bound by the phosphotyrosine-binding module of casitas B-lineage lymphoma (CBL), an E3 ubiquitin ligase resulting in monoubiquitylation of c-Met

at multiple sites. This results in the delivery of activated c-Met to the endosomal compartments (characterized by a bilayered clathrin coat) followed by c-Met localization to the internal membranes of multivesicular bodies. The multivesicular bodies subsequently fuses with the lysosomes and c-Met undergoes proteolytic degradation via the ubiquitin-proteosomal pathway (Peschard et al., 2001; Peschard & Park, 2003).

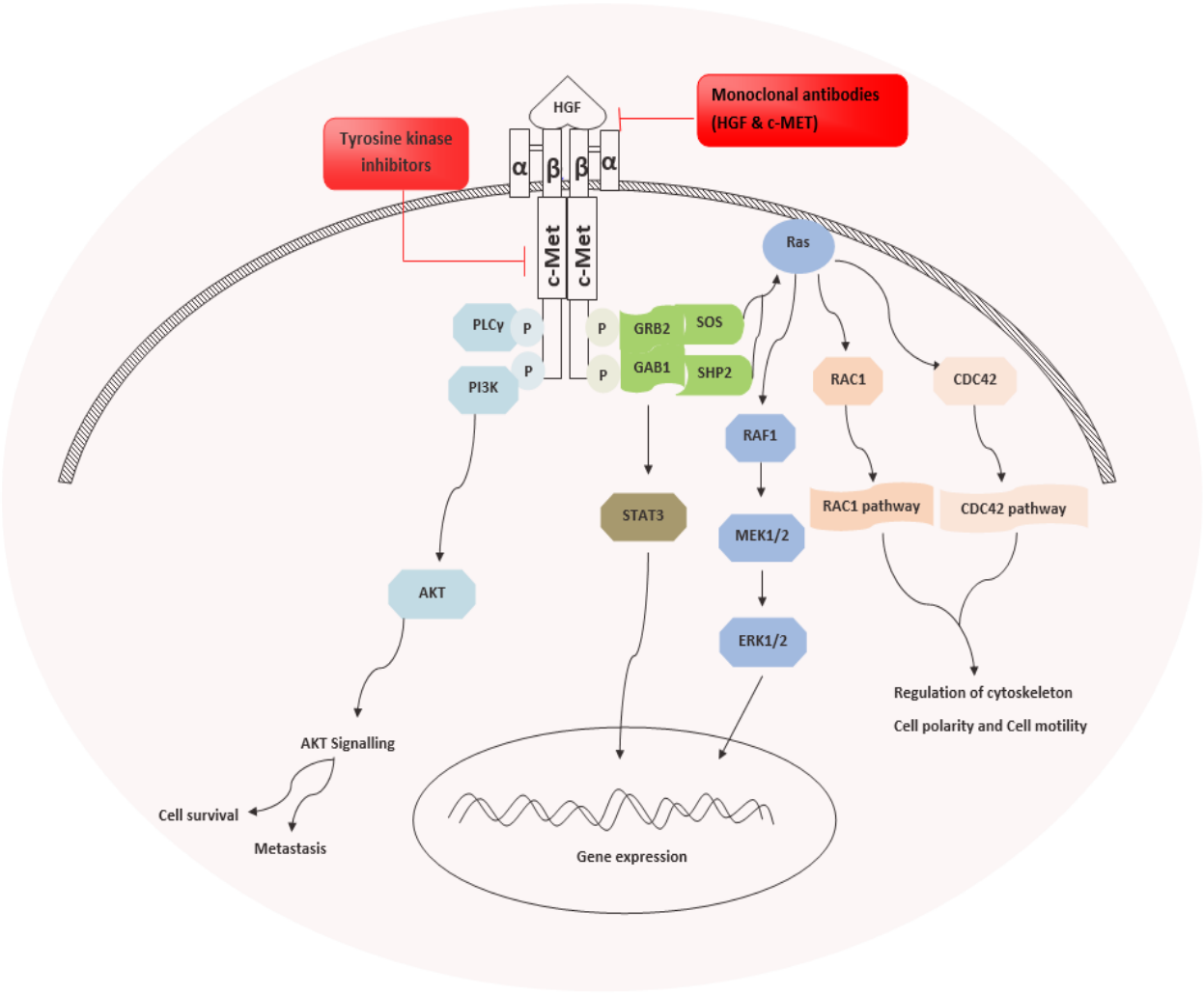


Figure 2.8 c-MET signalling pathway. Binding of HGF induces the dimerization and phosphorylation of c-MET. Adaptor protein binds to the phosphorylated kinase residues and stimulates the intracellular signalling cascades. Adapted from (Peters & Adjei, 2012; Pérez-Ramírez et al., 2015)

Phosphorylation of another negative regulatory site, serine residue 985 by protein kinase C (PKC) or Ca^{2+}/camodulin-dependent kinase on the JM region of c-Met also inhibits c-Met activation (Gandino et al., 1994). c-Met signalling can also be attenuated by protein tyrosine phosphatases (PTPs) including protein-tyrosine phosphatase 1B (PTP1B or PTPN1), T-cell

phosphatase (TCPTP/PTPN2) and density enhanced protein tyrosine phosphatase-1 (DEP-1) (Maroun & Rowlands, 2014). In summary, under normal physiological conditions, c-MET signalling is controlled by ligand activation, paracrine ligand delivery and ligand-activated receptor degradation.

In cancer, c-MET is activated either by a ligand-dependent or a ligand-independent mechanism. Ligand-dependent activation may occur in a paracrine or autocrine fashion. The latter occurs when a cells expresses both HGF and c-Met and activates itself while paracrine signalling occurs when the surrounding stroma secretes high levels of HGF recognised by c-Met expressing cells hence resulting in constitutive activation within the cells. Majority of ligand-dependent activation are paracrine. This model of activation was first demonstrated in a strain of human HGF (designated hHGF-Tg) transgenic mice with a severe combined immunodeficiency (SCID) background. The presence of ectopically expressed hHGF significantly enhanced the growth of heterotopic subcutaneous xenografts derived from human c-Met expressing cells but not human melanoma (M14-Mel) xenografts with insignificant c-Met levels. This shows that hHGF specifically activates c-Met and the model has since been useful in the evaluation of therapeutic agents (Zhang et al., 2005). Overexpression of HGF has been reported in plasma and tumours of cancers of various origins and has been associated with inferior outcome and resistance to therapy. Co-expression of HGF and c-Met has also been observed in breast carcinoma, AML, melanoma and osteosarcoma. Spontaneous metastasis to the bone, brain and kidney was observed as a result of autocrine c-Met activation when NSCLC cells that co-expressed c-Met and HGF were orthotopically transplanted in nude rats (Navab et al., 2009).

Ligand-independent activation is commonly observed in cells that overexpress c-Met leading to spontaneous receptor dimerization and activation. c-Met overexpression could be as a result of gene amplification caused by chromosomal duplication or transcriptional mechanisms by oncogenes such as RAS and ETS (Furlan et al., 2008). The most common cause of aberrant c-Met expression in cancers is gene amplification. In an in vitro model, c-Met overexpression induced malignant transformation of primary human osteoblasts into osteosarcoma cells possibly due to ligand-independent clustering resulting in receptor dimerization and activation (Patanè et al., 2006). c-MET can also be mediated by environmental stimuli such as hypoxia. Under hypoxic conditions, c-MET is induced and results in a 3-fold increase which is sufficient to activate and result in a dramatic amplification of signal transduction and subsequently inducing invasive growth (Pennacchietti et al., 2003).

2.2.3.2. c-MET mutations and cross talks

Activating point mutations of the MET gene are less frequently reported than MET amplification and have been observed in sporadic and hereditary hepatocellular carcinomas, human renal carcinomas and several other cancers (Park et al., 1999; Lee et al., 2000; Jagadeeswaran et al., 2006; Ma et al., 2008; Graziano et al., 2011). MET mutations can be found in the extracellular domain, juxtamembrane domains and kinase domain and are similar to other cancer-stimulating mutations that occur in other receptor tyrosine kinases such as EGFR, RET and KIT. So far, over 20 different somatic or germline c-MET mutations have been reported by sequencing DNA from patients' samples. The majority of these mutations are missense mutations and are associated with tumour progression (Danilkovitch-Miagkova & Zbar, 2002). Mutation of the MET gene in mouse models resulted in the development of tumours including sarcomas, carcinomas and lymphomas. In the mammary epithelium, the expression of the oncogenic mutant phenotype induced aggressive mammary carcinoma with similar features to basal-like breast carcinomas (Graveel et al., 2004; Ponzo et al., 2009). Di Renzo et al demonstrated that the frequency of neoplastic cells carrying c-MET gene activating mutations increases from 2% in primary tumours to 50% in metastatic colonies indicating the involvement of aberrant MET in tumour progression and metastasis (Di Renzo et al., 2000). In lung cancer, mutations have been identified in all three domain resulting in HGF-independent MET activation and alterations in cell motility and migration (Pérez-Ramírez et al., 2015).

Crosstalk of MET with other growth factor receptors such as EGFR, ERBB2 or insulin-like growth factor 1 receptor (IGF1-R) have been identified in different experimental models (Khoury et al., 2005; Bauer et al., 2006) and has emerged as a major mechanism for cancer progression and resistance to tyrosine kinase inhibitors. MET amplification in lung cancer cells with acquired resistance EGFR inhibitors has been reported to activate the EGFR3-PI3K-AKT signalling axis. However, combined inhibition of EGFR and MET restored the suppression of cell growth (Engelman et al., 2007; Turke et al., 2010; Zhang et al., 2010). c-MET has also been reported to crosstalk with cell surface receptors (CD44 and integrins), developmental signalling pathways (WNT-β-catenin and TGFβ-bone morphogenetic protein (BMP) , tetraspanins and tumour suppressors (INK4A and ARF) (Sharp et al., 2002; Sridhar & Miranti, 2006; Orian-Rousseau et al., 2007; Klaus & Birchmeier, 2008; Gherardi et al., 2012).

2.2.3.3. c-MET/HGF and MPM

This will be discussed in chapter 8

2.2.3.4. c-MET Therapies

Since the discovery of MET three decades ago, there has been significant progress in the development of MET-HGF inhibitors as targeted therapies. The inhibitors can be categorised as: HGF activation inhibitors (prevents the binding of inactive HGF into the active form), HGF inhibitors (binds to HGF and blocks its cleavage to the MET receptor), MET antagonists (binds the receptor extracellular domain) and MET TKIs (targets MET intracellularly). Inhibitors in these categories are either monoclonal antibodies or small molecule inhibitors. The small molecule inhibitors are further subdivided into ATP competitive and non-ATP competitive inhibitors. ATP-competitive inhibitors (examples are cabozantinib, crizotinib and foretinib) are potent but because of the similarity in the structure of the ATP pocket of MET and other kinases, they are less specific and can inhibit other kinases at concentrations required for inhibiting MET. Non-ATP competitive inhibitors (examples are tivantinib, savolitinib, AMG 337 and INC 280) are more specific since they interact with the allosteric sites of the receptor resulting in a change in the conformation of the active site which prevents the binding of HGF to MET. HGF inhibitors include rilotumumab (AMG102), ficlatuzumab (AV-299) and TAK701. Monoclonal antibodies that binds MET include onartuzumab (MetMab), CE-355621, DN-30 and LA480 (Cao et al., 2001; Parr et al., 2010; Underiner et al., 2010; Eathiraj et al., 2011; Rickert et al., 2011; Gherardi et al., 2012; Cui, 2014). There is currently no FDA approved selective c-MET agent in clinics but a few have progressed to more advanced stages in clinical development as monotherapy or in combination with other agents and are listed in Table 2.3.

Table 2.3 c-MET inhibitor in advanced stage clinical development

Drugs/Company	Stage	Target	Indication
Crizotinib (Pfizer)	Approved	c-MET, ALK	ALK-positive NSCLC
Tivantinib (Arqule Inc.)	Phase III	c-MET	Advanced NSCLC HCC?
Cabozantinib (Exelis)	Approved	c-MET, VEGFR-2	Medullary thyroid cancer
Onartuzumab (Genentech)	Phase III	c-MET	Advanced NSCLC HER2-negative gastric cancer
Ficlatuzumab (AVEO pharmaceuticals)	Phase II	HGF	Advanced NSCLC

2.3. The PI3K/PTEN/AKT/mTOR pathway

The signalling network of phosphoinositide-3-kinase (PI3K), protein kinase B (AKT/PKB) and mammalian target of rapamycin (mTOR) regulates most hallmarks of cancer including proliferation, survival, cell cycle, genomic stability, metabolism and motility (Hanahan & Weinberg, 2011). It also influences aspects of the tumour microenvironment such as recruitment of inflammatory cells and angiogenesis (Beagle & Fruman, 2011; Graupera & Potente, 2013; Hirsch et al., 2014). PI3K/AKT/mTOR signals downstream of cell surface receptor such as EGFR, VEGFR, PDGFR, HGF (c-MET), bFGFR and Insulin growth factor receptor (IGFR) as mentioned in sections 2.2.1, 2.2.2, 2.2.3 and Figure 2.8 above. PI3K, a heterodimeric dual lipid/protein kinase which consists of separate catalytic (p110) and regulatory (p85) subunits encoded by distinct genes, has been divided into three classes (I, II & III) based on their structure and substrate specificity. The four class 1 enzymes namely:

PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ are the most studied and widely implicated in the pathogenesis of cancers (Domin & Waterfield, 1997; Cidado & Park, 2012; Fruman & Rommel, 2014). This group of enzymes catalyses the formation of phosphatidylinositol (3,4,5) triphosphate (PIP_3) in the phospholipid bilayer of the cell membrane in response to stimulation by activated receptor tyrosine kinases, RAS GTPase, G-protein coupled receptors (GPCRs) thereby initiating pathway signalling (Stoyanov et al., 1995; Vanhaesebroeck & Waterfield, 1999). PTEN, negatively regulates PI3K by dephosphorylating PIP_3 back to its inactive lipid state (PIP_2) (Cidado & Park, 2012). Activated PI3K recruits phosphoinositide dependent kinase (PDK1) and AKT that binds to PIP_3 in the cell membrane. PDK1 initially phosphorylates AKT at threonine 308 (Thr308) and further phosphorylates it at serine 473 (Ser473) for full activation (Hennessy et al., 2005; Engelman et al., 2006). Phosphorylated AKT translocates to the cytosol and nucleus where it stimulates growth pathways, inhibits apoptotic proteins and transcription factors (BIM, BAX, BAD, Caspase 9, FOXO, NFκB) and transition through restriction point in the G1 phase of the cell cycle (Liang & Slingerland; Mitsiades et al., 2004). pAKT also phosphorylates and inactivates tuberous sclerosis complex 2 (TSC2), a tumour suppressor protein which together with TSC1 forms the tuberous sclerosis complex (Potter et al., 2002).

One of the major downstream effectors of AKT and a central element of the pathway is the serine/threonine kinase mTOR (mammalian target of rapamycin). mTOR exists in two functionally and structurally distinct complexes and each complex consists of mTOR, domain-containing mTOR-interacting protein (DEPTOR) and mLST8/GβL (mammalian lethal with SEC13 protein 8/G protein beta subunit-like protein). Structurally, mTOR has up to 20 tandem HEAT (Huntington-elongation factor 1A-protein phosphatase 2A subunit-TOR) repeats at the N-terminal which mediates protein-protein interactions. This is followed by a FAT domain, FRB domain, a kinase domain, an auto-inhibitory (repressor domain or RD) and a FAT Carboxy-terminal (FATC) domain (Figure 2.10). (Gingras et al., 2001; Perry & Kleckner, 2003; Zhou & Huang, 2010). The exact mechanism of the effect of the FKBP12-rapamycin complex on mTOR's intrinsic kinase activity is yet to be understood. mTOR complex 1 (mTORC1) in addition to mTOR, Deptor and mLST8 contains the regulatory associated protein of mTOR (Raptor) and proline-rich AKT1 substrate 40 (PRAS40) while mTOR complex 2 (mTORC2) consists of a complex of mLST8, mTOR, rapamycin insensitive companion of mTOR (Rictor), S1N1, Deptor and PROTOR/PRR5 proteins (Figure 2.10) (Sarbassov et al., 2004; Jacinto et al., 2006; Pearce et al., 2007; Wang et al., 2008; Peterson et al., 2009).

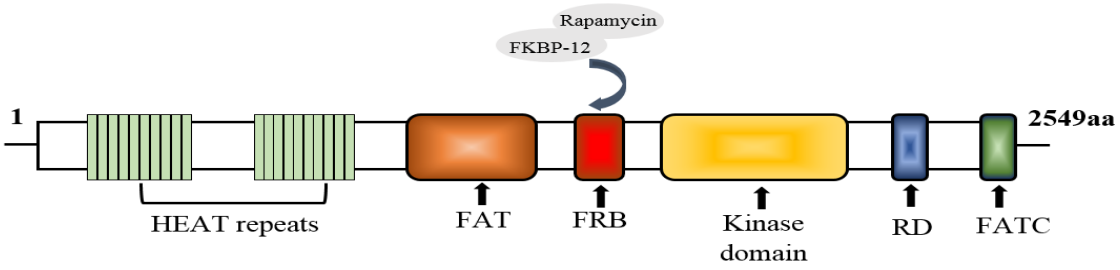


Figure 2.9 Domain structure of mTOR. The N-terminus contains up to 20 tandem HEAT repeats followed by a FAT domain, a FRB domain, a kinase domain, a repressor domain (RD) and a FATC domain in the C-terminus. The FRB domain serves as a docking site for the FKBP12-rapamycin complex interrupting mTOR:Raptor interaction while the FAT and FATC domain modulate mTOR kinase activity Adapted from (Zhou & Huang, 2010)

Inhibiting the TSC1/TSC2 dimer activates GTP-binding protein Rheb (Ras homolog enriched in brain) which binds to and upregulates mTORC1. mTORC1 phosphorylates p70S6K (70 kDa ribosomal protein S6 kinase) and 4E-BP1 (eIF4E-binding protein), resulting in increased ribosome biogenesis and translation of cell growth and division proteins (Hay & Sonenberg, 2004; Sengupta et al., 2010). Activation of p70S6K can also result in a negative feedback mechanism by phosphorylating and degrading IRS-1 (insulin receptor substrate 1) and impairing its association with the insulin receptor hence preventing the activation of the PI3K/AKT/mTOR pathway after insulin stimulation (Harrington et al., 2004). mTORC2 is suggested to be activated by growth factors and has been shown to be resistant to rapamycin. However, it was recently observed that long-term treatment with rapamycin dissociates the mTORC2 complex in certain cell lines (Sarbassov et al., 2006). mTORC2 was initially reported to control actin cytoskeleton organisation through the phosphorylation of PKCα and focal adhesion proteins (paxillin) and regulate small GTPases Rac and Rho (Schmidt et al., 1997; Sarbassov et al., 2004; Jacinto et al., 2004; Liu et al., 2008). Another important finding is the identification of mTOR2 as the kinase that directly phosphorylates AKT on the hydrophobic motif site Ser473 which is required for the full activation of AKT. Active AKT subsequently regulates cellular processes such as cell growth, apoptosis, proliferation, cell cycle and glucose metabolism (Sarbassov et al., 2005; Hresko & Mueckler, 2005; Manning & Cantley, 2007).

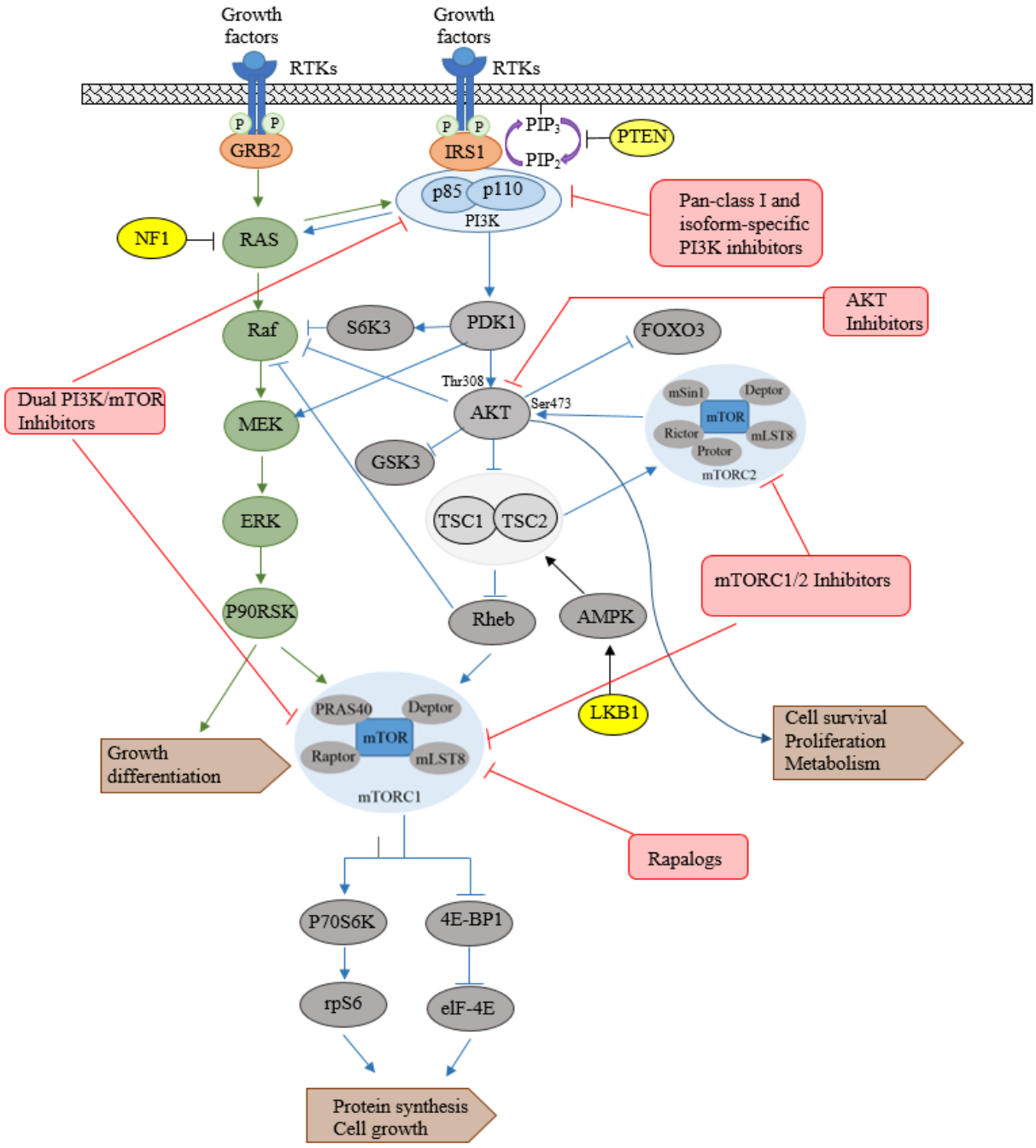


Figure 2.10 Overview of the PI3K/AKT/mTOR pathway and drug targets. The pathway shows the canonical PI3K pathway (blue and grey), pathway inhibitors (red boxes) and cross talks with MAPK pathway (green) and tumour suppressors (yellow). Adapted from (Saini et al., 2013; Dienstmann et al., 2014; Polivka Jr. & Janku, 2014)

2.3.1. PI3K/AKT/mTOR pathway and mesothelioma

This will be discussed in chapter 9

2.3.2. Cross talks with other signalling molecules

The PI3K and MAPK pathways are interlinked and functionally co-regulate similar transcription factors associated with cell cycle and survival. Both pathways interact at multiple points which results to cross-activation, cross-inhibition as well as pathway convergence (Mendoza et al., 2011).

When cross-activation occurs, a member of one pathway positively regulates an upstream component of another pathway leading to an increase in the activity of that pathway. For example, RAS-GTP can directly bind and allosterically activate PI3K; activated ERK and RSK, two effector molecules downstream of RAS can further promote mTOR activity by phosphorylating TSC2 at sites distinct from those phosphorylated by AKT (Kodaki et al., 1994; Suire et al., 2002). Cross-inhibition is observed when a member of one pathway negatively regulates an upstream component of another pathway therefore inhibiting the other pathway's signalling ability. Strong IGF1 stimulation induces a cross-inhibition between AKT and RAF such that AKT negatively regulates ERK activation by phosphorylating inhibitory sites of RAF in its amino-terminus (Zimmermann & Moelling, 1999; Guan et al., 2000). Pathway convergence take place when two or more signalling pathways act directly on the same complex or protein and both pathways positively or negatively regulates the complex or protein. For example, when ERK, RSK, AKT and p70S6K are activated, they phosphorylate the same substrates such as c-Myc transcription factors, FOXO3A, BAD and GSK3 to induce cell survival, proliferation and motility (Yang et al., 2008). These interactions are potential mechanism of therapy resistance to agents that target a single step in a specific signalling pathway and can be observed from the limited efficacy of PI3K/mTOR inhibitors alone in cancer.

The PI3K/mTOR pathway is constitutively activated but aberrant activation can arise from irregular upstream signalling, somatic mutations, amplification or epigenetic alterations of several components of the pathway. Loss of PTEN is a common means of over-activating PI3K signalling and can occur through mutation, deletion or epigenetic silencing. The frequency of PTEN loss has been reported to be between 8-62% in mesothelioma (Opitz et al., 2008).

2.3.3. PI3K/mTOR inhibitors in cancer therapy

The PI3K pathway is a much desired target for therapeutic intervention because of its crucial role in protein synthesis resulting in cell growth and development. The pathway can be targeted by a variety of approaches such as (i) directly targeting PI3K isoforms, (ii) targeting

kinases that result in the activation of AKT, PI3K and PDK-1, (iii) directly targeting Akt isoforms, (iv) inhibiting downstream effectors of Akt such as mTOR or (v) combination of targets within the PI3K pathway and other intracellular proteins that interact with the PI3K/Akt/mTOR pathway. There currently over 30 drugs in clinical trials for cancer therapy but despite the possibilities of targeting the PI3K pathway, responses of solid tumours to monotherapy with PI3K inhibitors have been somewhat disappointing and drug resistance mechanisms have rapidly emerged. Resistance mechanisms to PI3K inhibitors consist of the incomplete inhibition of the PI3K pathway, reactivation of the PI3K pathway or activation of complementary pro-survival pathways (Garrett et al., 2011; Dienstmann et al., 2014; Brown & Toker, 2015).

The two earliest first generation pan-PI3K inhibitors; LY294002 and wortmannin were shown to be active in several in vitro models but due to poor solubility, instability and high toxicity they have been restricted to preclinical studies. Compounds with improved pharmacokinetic properties have been developed and are being evaluated in clinical trials for several malignancies. Third generation compounds (dual PI3K/mTOR inhibitors) have also been developed with the potential advantage to inhibit not only all PI3K class I isoforms but also mTORC1 and mTORC2 and ultimately overcome feedback and cross talk within the pathway (Martini et al., 2013; Porta et al., 2014).

One of the first generation of Akt inhibitors is perifosine; it exhibits tumour activity by preventing the translocation of Akt to the cell membrane. Despite its success in preclinical models, it failed as a single agent in clinical trials and is being investigated in combination with other therapeutic agents (Richardson et al., 2012). First generation of mTOR inhibitors; analogs of rapamycin also known as ‘rapalogs’ are the first agents to be clinically approved. These inhibitors bind to the FK506-binding protein (FKBP-12) to form a complex that subsequently binds to mTOR and inhibits mTORC1and its downstream substrates. mTORC2 is rapamycin insensitive therefore cannot bind to the FKBP12-rapamycin complex. However studies have shown that prolonged treatment with rapamycin can inhibit mTORC2 and that it is tissue specific (Gao et al., 2003; Sarbassov et al., 2006). Rapamycin analogs have been approved by the FDA for the treatment of pancreatic neuroendocrine tumours, breast cancer and renal cell carcinoma. Despite their successes, these agents have modest single agent activity overall which can be attributed to its limited activity against mTORC2 resulting in the feedback activation of other pathways such as Akt and ERK (O’Reilly et al., 2006; Hudes et al., 2007; Carracedo et al., 2008; Motzer et al., 2008; Hess et al., 2009; Yao et al., 2011; Baselga et al., 2012). In contrast to rapalogs, catalytic mTOR inhibitors (e.g. AZD2014) have

been developed which directly inhibits the kinase activity of mTOR regardless of its complex and emerging clinical data suggest they may have single agent activity higher than those of rapalogs (Banerji, 2012; Bendell et al., 2015). Table 2.4 is a list of PI3K/Akt/mTOR pathway inhibitors that have progressed in clinical development.

Table 2.4 PI3K/Akt/mTOR pathway inhibitors that are in clinical trials

Drugs/Company	Stage	Target	Indication
NVPBEZ235 (Novartis)	Phase II	PI3K/mTOR	Advanced solid tumours
GDC-0980 (Genentech)	Phase II	PI3K/mTOR	Advanced solid tumours
PF-05212384 (Pfizer)	Phase I/II	PI3K/mTOR	Advanced solid tumours
SAR245409 (Sanofi/Exelis)	Phase II	PI3K/mTOR	Advanced solid tumours, CLL
BAY80-6946 (Bayer)	Phase II	Pan-class I PI3K	Advanced solid tumours
Buparlisib (Norvatis)	Phase IV	Pan-class I PI3K	Advanced solid tumours
Pictilisib (Genentech)	Phase II	Pan-class I PI3K	Breast cancer, NSCLC
PX-866 (Oncothyreon)	Phase II	Pan-class I PI3K	Advanced BRAF-mutant cancers, NSCLC, prostate cancer
BYL719 (Novartis)	Phase II	PI3K, p110α	Advanced solid tumours
Idelalisib (Gilead/Callistoga)	Phase III	PI3K, p110δ	CLL, Lymphomas
Perifosine	Phase I/II	AKT	Advanced solid tumours, multiple myeloma
MK2206 (Merck)	Phase II	AKT	Advanced solid tumours
GDC-0068 (Genentech)	Phase II	AKT	Advanced solid tumours
GSK2110183 (GlaxoSmithKline)	Phase II	AKT	Advanced solid tumours, CLL
GSK2141795 (GlaxoSmithKline)	Phase II	AKT	Advanced solid tumours
AZD5363 (AstraZeneca)	Phase I/II	AKT	Advanced solid tumours
AZD2014 (AstraZeneca)	Phase II	mTORC1/2	Advanced solid tumours
CC-223 (Celgene)	Phase I/II	mTORC1/2	Breast cancer, glioblastoma etc.

2.4. Metabolites of arachidonic acid pathway

Arachidonic acid is a polyunsaturated fatty acid found in the phospholipid layer of the cell membrane. It is released by the phospholipase A2 enzyme and metabolized by Cyclooxygenase (COX), Lipoxygenase enzymes and Cytochrome P450s (CYP) resulting in the production of eicosanoids such as prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETE) and hydroperoxyeicosatetraenoic acids (HPETE) which have been shown to play a role in inflammation and carcinogenesis.

2.4.1. Cyclooxygenases

Epidemiological studies linking the use of aspirin to reduced risk of colorectal cancer led to exploration into the role of cyclooxygenases in cancer. Cyclooxygenase enzymes, also known as prostaglandin endoperoxide H synthases (PGHS), are a family of myeloperoxidases found in the luminal surface of the endoplasmic reticulum and the outer membrane of the nuclear envelope. They are the key regulatory enzymes that catalyse the rate limiting step of the biosynthesis of prostaglandins from arachidonic acid. Cyclooxygenases exist as homodimers with identical subunits of approximately 70 kDa. Each subunit consists of three domains: an epidermal growth factor domain responsible for dimerization, a membrane-binding domain with four amphipathic helices which interlocks the protein to one leaflet of the membrane and a large globular C-terminal catalytic domain responsible for substrate and inhibitor binding (Chandrasekharan & Simmons, 2004; Blobaum & Marnett, 2007). There are three COX isoforms. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are glycoproteins that share ~ 60% amino acid sequence homology but encoded by distinct genes. COX-1 is ubiquitously and constitutively expressed in mammalian tissues and cells. It is encoded by the Ptgs-1 gene on chromosome 9 and plays an important role in tissue homeostasis by regulating other cellular processes such as proliferation, angiogenesis, cell-to-cell signalling and cytoprotection. COX-2 is primarily an inducible isoform activated in response to growth factors, cytokines, mitogens and carcinogens. It is encoded by the Ptgs-2 gene on chromosome 1 and generally expressed in some mammalian tissues at very low levels. COX-3 was recently identified as a splice variant of COX-1. It is expressed in the brain and spinal cord but its function remains unclear (Cao & Prescott, 2002; Simmons et al., 2004; Sobolewski et al., 2010). The two structural differences between COX-1 and COX-2 with pharmacological importance are (i) the active site of COX-2 is larger because of the substitution of isoleucine 523 in COX-1 for a valine in COX-2 which reveals a hydrophobic

pocket that allows the binding of selective COX-2 inhibitors, (ii) COX-1 exhibits negative allosterism at low concentrations of arachidonic acid, allowing COX-2 to compete more effectively for new arachidonic acid released within the cell (Smith et al., 2000). COX-2 is overexpressed in various cancers such as lung, breast, colon, pancreas, bladder, glioma and mesothelioma (Eberhart et al., 1994; Wolff et al., 1998; Tucker et al., 1999; Marrogi et al., 2000; O'Kane et al., 2005). Cyclooxygenases are bifunctional enzymes possessing cyclooxygenase (bis-dioxygenase) and peroxidase activities that are interconnected for the conversion of arachidonic acid to prostaglandin H_2 and subsequently prostaglandins and thromboxane (Blobaum & Marnett, 2007).

2.4.1.1. Prostanoid biosynthesis

The initial step in the formation of prostaglandins is the release of arachidonic acid from membrane-bound phospholipids by the Phospholipase A2 enzyme. When arachidonic acid is released, oxygenated arachidonic acid produces prostaglandin G_2 (PGG2), a cyclopentane hydroperoxy endoperoxide. The peroxidase activity subsequently reduces PGG2 to prostaglandin H_2 (PGH2). PGH2 forms the root prostaglandin which specialised prostaglandin synthases/isomerases convert to prostaglandins D_2. E_2, $F_{2\alpha}$, I (prostacyclin) and thromboxane A_2 via isomerization and oxidation or reduction reactions (Figure 2.11) (van der Donk et al., 2002). These prostaglandin isomers are involved in numerous physiological and pathophysiological processes such as fever, inflammation, algesia, mitogenesis, parturition, ovulation, renal function, thrombosis, vasodilation and vasoconstriction. The effects of prostanoids are mediated by binding to specific cell membrane spanning G-protein-coupled receptors in an autocrine or paracrine manner (Chandrasekharan & Simmons, 2004). PGE2 activates four EP receptors (EP1-4) and has been identified as the principal prostanoid that promotes cell growth and survival in several cancers.

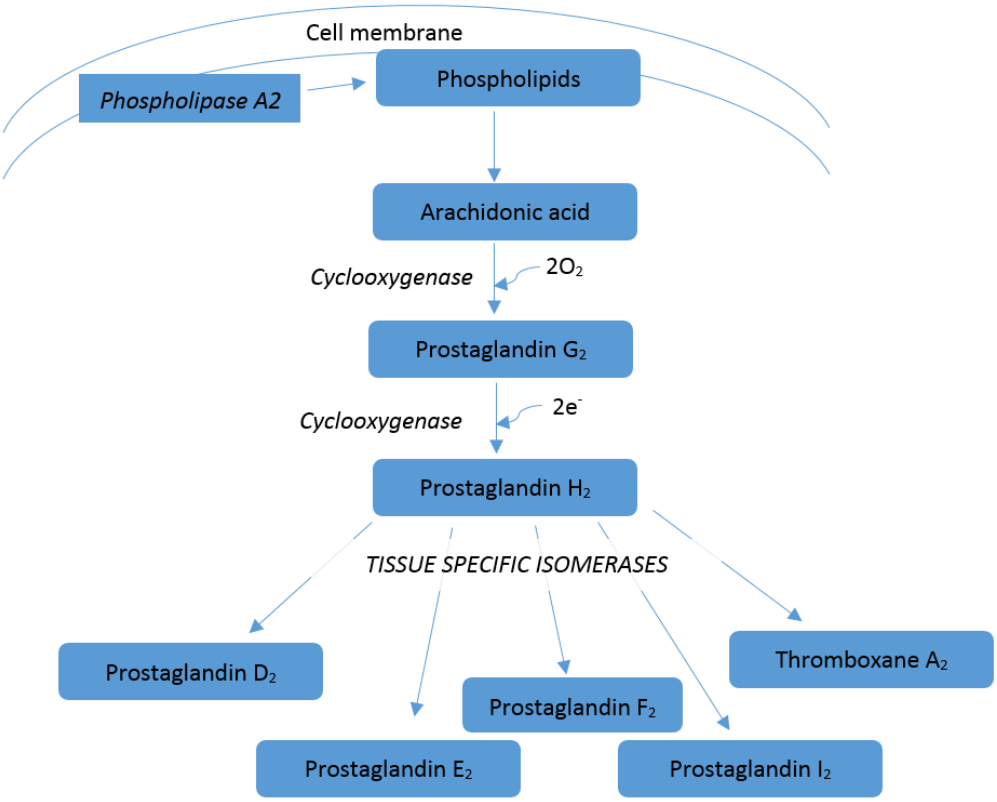


Figure 2.11 Synthesis of prostanoids from arachidonic acid. Arachidonic acid is released by phospholipase A2 followed by the oxygenation of free arachidonic acid by COX-2 to produce PGG2. PGG2 is reduced in the peroxidase active site of COX-2 to produce PGH2. PGH2 is the major precursor which tissue specific isomerases act to produce prostaglandins and thromboxane. Adapted from (Smith, 1989).

2.4.1.2. COX-2/Prostaglandin E2 pathway and Cancer

PGE2 is the most widely produced prostaglandin, frequently up regulated in several cancers, often associated with poor prognosis and exhibits versatile biological effects. A plausible reason for abundant PGE2 could be the support role of inducible microsomal or membrane-associated perinuclear PGE synthase-1 (mPGES-1) and the ability of PGE2 to bind to four receptors (EP1-4) present in different organs of the body (Samuelsson et al., 2007; Sugimoto & Narumiya, 2007). The COX-2/PGE2 pathway has been shown to drive the hallmarks of cancer via a series of cell signalling pathways as reviewed by (Wang & Dubois, 2006). Briefly, COX-2/PGE2 induces cell proliferation, migration and invasion via multiple signalling cascades including the PI3K/AKT-peroxisome proliferator-activated receptor-δ (PPARδ), Ras-MAPK/ERK and glycogen synthase kinase-3β (GSK3β)-β-catenin signalling pathways in colorectal cancer and non-small cell lung cancer cells (Castellone et al., 2005;

Wang et al., 2005; Krysan et al., 2005; Wang & Dubois, 2006; Yang et al., 2006). Treatment of colon cancer cells with PGE2 resulted in increased BCL2 expression via the MAPK pathway indicating a possible mechanism for the evasion of apoptosis by cancer cells (Sheng et al., 1998). Significant crosstalk have been shown to exist between COX-2/PGE2 and the EGFR pathway. Exposure to PGE2 can initiate a positive feedback mechanism in which EGFR activation results in increased expression of COX-2 and enhanced prostanoid synthesis. A few studies have also reported the transactivation of EGFR by PGE2 receptors via an intracellular mechanism which could be triggered by EGF-like ligand. Recently, Han et al (2006) also reported a possible crosstalk between COX-2/PGE2/EP1 and EGFR/c-MET signalling pathways in human hepatocellular cancer resulting in increased cell invasion (Rama Pai et al., 2002; Buchanan, 2003; Han et al., 2015).

PGE2 also upregulates the expression of proangiogenic factors VEGF and bFGF by stimulating the ERK2/JNK1 signalling pathway in endothelial cells. EP2/EP4 receptors have also been reported to regulate the induction of VEGF in cancer cells. VEGF and bFGF (basic fibroblast growth factor) can induce COX-2 via a positive feedback loop resulting in the increased production of PGE2 and subsequently amplification of the regulation of VEGF and bFGF. PGE2 can directly act on endothelial, epithelial and/or immune cells to promote angiogenic factors. (Kage et al., 1999; Pai et al., 2001; Spinella et al., 2004). Several studies have documented the role of COX-2 in inflammation and cancer pathogenesis emphasizing the link between the two processes. Since the initial hypothesis of the link between inflammation and cancer by Virchow in 1863, there has been increasing body of evidence that support the notion that many malignancies arise from areas of infection and inflammation. A study of 77952 asthma patients revealed a significant increase in lung cancer risk resulting from localized chronic inflammation in both men and women. Similar trends have been observed in other tumour types as reviewed by Fitzpatrick 2001 (Vesterinen et al., 1993; Coussens & Werb, 2001; Fitzpatrick, 2001).

2.4.1.3. COX-2 and Mesothelioma

This will be discussed in Chapter 5

2.4.2. Lipoxygenases

Lipoxygenases are a structurally related family of non-heme iron dioxygenases that catalyses the conversion of polyunsaturated fatty acids into biologically active leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs). These enzymes act by inserting molecular oxygen into polyunsaturated fatty acids at specific carbon regions. There are four distinct isoforms of LOX that have been identified namely: 5-, 8-, 12-, and 15-lipoxygenase. They are named based on the carbon site that the enzyme acts on its substrate (Steele et al., 1999; Funk, 2001). Mammalian lipoxygenases metabolize arachidonic acid to biologically active hydroperoxyeicosatetraenoic acids (HPETEs) which are further reduced to corresponding 5-, 8-, 12- and 15- hydroxyeicosatetraenoic acids (HETEs) (Figure 2.12). 5-LOX and 12-LOX have been implicated in the pathogenesis of several cancer and the functions of 8-LOX and 15-LOX remains to be fully elucidated.

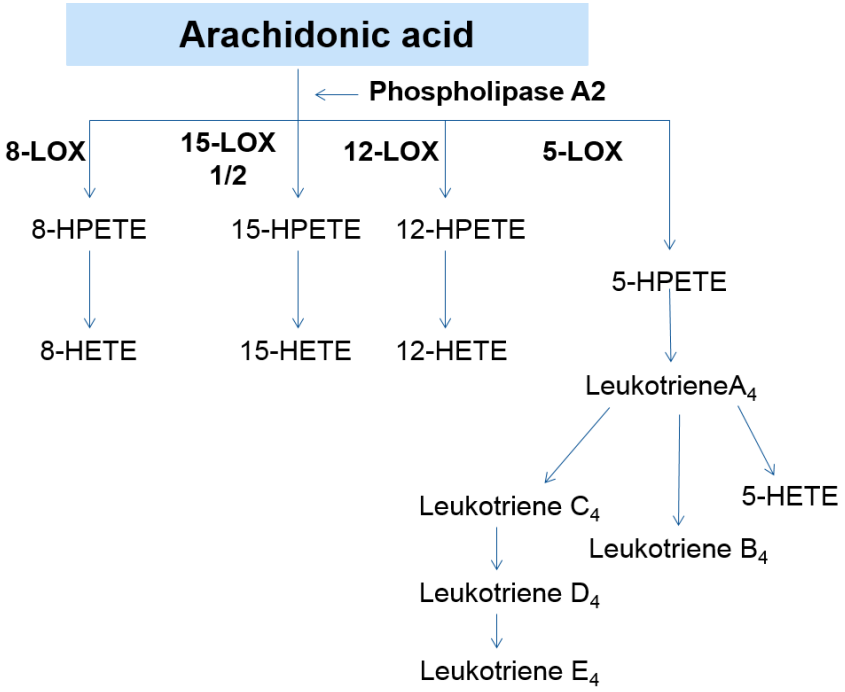


Figure 2.12 Synthesis of leukotrienes from arachidonic acid by lipoxygenases. Arachidonic acid is converted into intermediary 5-HPETE by 5-LOX and further metabolized to leukotrienes. Adapted from (Dingzhi Wang & DuBois, 2010)

2.4.2.1. Leukotriene biosynthesis

Unlike other lipoxygenases, 5-LOX has two distinct enzymatic abilities. Following the incorporation of molecular oxygen into arachidonic acid (oxygenase activity) to produce 5-HPETE, it further metabolizes HPETE by the removal of the hydroxyl moiety to form unstable epoxide leukotriene A_4 in the presence a helper protein known as five lipoxygenase activating protein (FLAP) (Pidgeon et al., 2007). FLAP is an 18 kDa membrane-bound protein that mediates the catalytic activity of 5-LOX by facilitating the delivery of arachidonic acid to 5-LOX (Peters-Golden & Brock, 2003). LTA_4 can be further transformed by three possible mechanisms. It can be converted to 5-HETE, hydrolysed by leukotriene A4 hydrolase to form LTB_4 (a potent chemotactic factor) or conjugated with glutathione to form cysteinyl leukotriene (CysLT), LTC_4 (a bronchoconstrictor). The peptide region of LTC_4 is subject to extracellular metabolism subsequently forming LTD_4 and LTE_4. Leukotrienes are predominantly expressed by inflammatory cells such as neutrophils, macrophages and mast cells (Abramovitz et al., 1993; Funk, 2001). However, epithelial and endothelial cells can also stimulate LTB_4, LTC_4 and LTD_4 at inflammatory sites. Epithelial and endothelial cells express LTA4 hydrolase which acts on LTA_4 release from immune cells (particularly neutrophils) via transcellular metabolism. Arachidonic acid secreted from epithelial cells can also be synthesized by leukocytes to produce leukotrienes. The transcellular biosynthesis between immune cells and epithelial or endothelial cells results in the accumulation of leukotrienes which further amplifies inflammatory responses (Folco & Murphy, 2006; Zarini et al., 2009). Leukotrienes exert their biological effects via the activation of transcription factors of the PPAR family or by interact in an autocrine or paracrine manner with specific transmembrane G-protein-coupled receptors (GPCR). Four distinct GPCRs have been characterized (BLT1, BLT2, CysLT1 and CysLT2). BLT1 and BLT2 are high and low-affinity receptors respectively that bind to LTB_4 resulting in the stimulation of leukocyte functions. The cysteinyl leukotriene receptors $CysLT_1$ and $CysLT_2$ bind to LTC_4 and LTD_4 to mediate their actions. CysLT1 has increased affinity for LTD_4 and CysLT2 has a reduced equal affinity for both CysLTs. BLT1 and CysLT1 are physiologically expressed in leukocytes while BLT2 and CysLT2 are expressed in several cell types (Metters, 1995; Toda et al., 2002).

2.4.2.2. Lipoxygenases and Cancer

Several studies have reported the possible association of 5-LOX and 12-LOX with carcinogenesis. Both lipoxygenases have been reported to be absent in normal epithelia but

constitutively expressed in epithelial cancers such as lung, oesophageal, breast, prostate, bone, pancreas, brain, colon and mesothelioma (Boado et al., 1992; Natarajan et al., 1997; Hong et al., 1999; Gupta et al., 2001; Romano et al., 2001; Hennig et al., 2002; Winer et al., 2002; Yoshimura et al., 2003, 2004; Öhd et al., 2003; Li et al., 2005; Barresi et al., 2007). LTB_4 has been shown to stimulate proliferation and promote survival by coupling with the BLT1 receptor to activate the MAPK pathway in colon cancer cell lines. In pancreatic cancer cell lines, LTB_4 induces cell proliferation via both the MAPK and PI3K-AKT pathways. Interaction between LTD_4 and CysLT1 has also been shown to activate multiple parallel pathways in non-transformed human intestinal epithelial cell lines and colorectal cancer cell lines (Ohd et al., 2000; Paruchuri et al., 2002; Tong et al., 2005; Mezhybovska et al., 2006; Ihara et al., 2007).

12-HETE has been shown to activate the NF-kB, ERK and p38 MAPK pathways in different cancer models by binding to G-protein coupled receptors resulting in cell proliferation and survival. Significant number of evidence has also emerged over the years for the role of 12-HETE in regulating adhesion, invasion and motility in tumour cells by activating protein kinase C (Honn et al., 1994; Ding et al., 2001; Kandouz et al., 2003; Pidgeon et al., 2007).

2.4.2.3. LOXs and mesothelioma

This will be discussed in chapter 5

2.4.3. Cyclooxygenase, lipoxygenase and growth factor cross talks

COX-2, 5-LOX, 12-LOX and several growth factors have been shown to be co-expressed and upregulated in various cancer cell lines and tumours such as lung, prostate, breast and colon (Honn 1998). These arachidonic acid metabolizing enzymes stimulate proliferation and are proangiogenic. Based on the similarity in their mechanism of action and substrate requirement, blocking one enzymatic pathway could likely activate the other resulting in the reduced efficacy of COX-2 inhibitors (Romano & Claria, 2003). Several studies have provided evidence that support the hypothesis of a shunt in the arachidonic acid pathway and that combined inhibition of eicosanoids has enhanced therapeutic effect *in vitro* studies (Tucker et al., 1999; Chen et al., 2004; Hoque et al., 2005; Ye et al., 2005; Cianchi et al., 2006; Zhi et al., 2006). However, there is currently no published study demonstrating the modulation and co-inhibition of eicosanoids in mesothelioma.

COX and LOX enzymes have also been reported to interact with other growth factor receptors that stimulate proliferation and inhibit apoptosis. Metabolites of the arachidonic

acid pathway can directly act on epithelial, endothelial and/or immune cells to stimulate angiogenic factors such as VEGF, FGF2 and chemokines. Therefore, prostaglandins and leukotrienes play an important role in mediating cross talks between surrounding stromal cells and epithelial cells in the tumour microenvironment (Wang and Dubois 2010). The independent activation of the individual pathways provide a rationale for combined inhibition in the treatment of mesothelioma.

2.5. Targeted therapy in MPM

Since the approval of the first line of chemotherapy in 2005, clinical trials of receptor kinase inhibitors as targeted therapy in mesothelioma patients have been discouraging. The negative results can be attributed to lack of specific biomarkers for response and multiple pathways being upregulated in mesothelioma. Common mutations are rare in this malignancy but could be acquired following treatment with standard chemotherapy. There is a lack of complementary genomic and proteomics studies in order to identify biomarkers to stratify patients for appropriate clinical trials. The use of targeted therapy has led to clinically significant improvement in survival in the treatment of other cancers in recent years and could be useful in the treatment of mesothelioma. The advent of the next generation sequencing is another important tool to be harnessed in order to identify driver mutations in MPM. Very few reports of the proteomic analysis of MPM are available. So far very few clinical trials have been conducted to analyse the use of targeted therapy and relevant biomarkers. Table 2.5 is a list of ongoing clinical trials on targeted therapy in MPM. There is a need for more studies on the expression pattern and frequency of potential biomarkers and the effect of available selective therapeutic target for a better understanding of mesothelioma.

Table 2.5 Some ongoing clinical trials for targeted therapy in MPM

Chemotherapy regimens	Target	No. of patients	Phase	Previous treatment	Status	Investigator/ Trial number
Ganetespib + Pemetrexed + Cisplatin/ carboplatin	HSP90	6	I/II	Chemotherapy-naive	Recruiting	Prof. Dean Fennell (NCT01590160)
ADI-PEG 20 + Pemetrexed + Cisplatin	Arginosuccinate synthase (ASS)	88	I	Chemotherapy-naive	Recruiting	Dr Peter Szlosarek (NCT02029690)
Cixutumumab (IMC-A12)	Insulin-like growth factor 1 receptor (IGF-1R)	20	II	Pemetrexed + Cisplatin/ Carboplatin	Ongoing	Dr Raffit Hassan (NCT01160458)
Dasatinib	BCR-ABL, SRC family	60	I	Chemotherapy-naive	Ongoing	M.D. Anderson Cancer centre (NCT00652574)
Defactinib	FAK	35	II	Chemotherapy-naive	Recruiting	Verastem, Inc. (NCT02004028)
VS-5584 + Defactinib	PI3K/mTOR	56	I	Pemetrexed + Cisplatin/ Carboplatin	Ongoing	Verastem Inc. (NCT02372227)
Tazemetostat	EZH2	67	II	Pemetrexed + Cisplatin/ Carboplatin	Recruiting	Epizyme, Inc. (NCT02860286)
Cetuximab + Pemetrexed + Cisplatin/ carboplatin	EGFR	18	II	Chemotherapy-naive	Recruiting	University Hospital, Ghent (NCT00996567)
Tivantinib + Pemetrexed + Carboplatin	c-MET	35	I/II	Chemotherapy-naive	Recruiting	Dr Paolo Zucali (NCT02049060)

CHAPTER THREE

PRINCIPLES OF LABORATORY TECHNIQUES

Chapter 3 Principles of Laboratory Techniques

3.0 Translational research

Over the past three decades, translational research has been a major focus in the field of medicine. It involves harnessing knowledge obtained from conducting basic research in the laboratory and transforming its significant findings into clinical practice. It is also termed the 'bench to bedside approach' or the interface between basic science and clinical practice. Following the advent of comprehensive molecular technologies, a multidisciplinary approach has been adopted in order to understand the pathophysiology of diseases especially cancer. The molecular mechanisms of novel targeted inhibitors can also be studied using different platforms. Several assays have been developed to understand the effect of drugs on cell viability and proteomics is an important tool in the identification of biomarkers and understanding the mechanism of action of inhibitors.

3.1. Cellular assays

The effect of inhibitors on cells can be monitored in cell culture using different methods. The methods can be broadly classified as assays of cell proliferation and cytotoxicity assays. The choice of a cellular screening assay requires the understanding of the endpoint being measured, correlation with cell viability and the limitations of the assay. Likewise, the choice of a biologically representative cell line and appropriate conditions are vital for providing relevant results. Irrespective of the model system of choice, important factors in setting up a cell based assay include ease of use and the establishment of consistent and reproducible procedure. Cytotoxicity assays are useful to assess markers that indicate the number of dead cells while cell proliferation or viability assays indicate the number of live cells. However, during most cytotoxicity events, cell viability measures are inversely proportional to cytotoxicity measures and vice versa (Niles et al 2008).

3.1.1. Cytotoxicity assays

Cell death usually results in the shutdown of metabolism leading to loss of membrane integrity and the release of cytoplasmic contents into the cell culture medium. Two of the most definitive methods to assess cell death are; the measurement of leakage of cellular components from compromised cells and the uptake of extracellular substances such as trypan blue due to loss of membrane integrity. Lactate dehydrogenase is a stable cytoplasmic enzyme that is present in nearly all living cells. It catalyzes the conversion of lactate to pyruvic acid and back to lactate. LDH activity can be measured by subjecting the sample to a

coupled enzymatic reagent containing lactate, NAD^+, disporase and a redox dye such as reazurin which results in a change in the absorbance or a shift in the fluorescence profile of the sample. These are available as commercial kits but a major disadvantage is the negative impact of serum supplemented medium on the kits hence, a cell-free medium only control has to be included in every assay.

3.1.2. Cell proliferation assays

Cell proliferation assays measures the number of viable cells proliferating in a cell-based medium. Several assays can be used to estimate cell viability based on cellular metabolism and these assays include clonogenic assays, DNA synthesis assays and mitochondrial activity assays.

3.1.2.1. Clonogenic assays

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay that measures the ability of a single cell to form a colony consisting of 50 or more cells. It essentially measures the ability of every cell to undergo unlimited division. It is a preferred method for analysing cell reproductive death after ionizing radiation and can also be used for measuring the effectiveness of cytotoxic agents. Cells are seeded in appropriate media and culture plates before and after treatment and the colonies are counted after 7-21 days (Franken et al 2006). The long duration of the assay and the counting of individual colonies makes this assay time consuming and laborious hence not practical for high-throughput screening assays.

3.1.2.2. DNA Synthesis assays

DNA replication is an important step that precedes cell division and the biochemical pathway correlates well with DNA synthesis which is specific for cell division. Therefore these assays measure DNA synthesis as a surrogate marker for cell proliferation. Direct measurement involves the addition of pre-labelled DNA precursors to cells which are then absorbed by the dividing cells and incorporated into their genomic DNA. The precursors can be quantified such that the amount of precursors integrated into the DNA is directly proportional to the multiplying cells. An example is the incorporation of thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) into the DNA of replicating cells. Cultured cells are incubated with BrdU for 1-24 hours depending on how rapidly the cells divide, during the incubation period BrdU is incorporated into the DNA. The incorporated BrdU can be detected by using anti-BrdU

antibodies available in commercial immunodetection kits. Quantification is carried out by chemiluminescent or colorimetric assays (Madhavan, 2007).

3.1.2.3. Mitochondrial activity assays

In viable cells, mitochondrial activity is constant such that an increase or decrease in the number of viable cells is directly proportional to the mitochondrial activity. This assay is based on the reduction of tetrazolium salts to coloured formazan product by metabolically active cells. NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells are known to carry out this conversion and the formazan product can be quantified using a microplate reader. Mitochondrial activity assays can be used to measure cell proliferation and the cytotoxic effect of a drug. The most widely used tetrazolium salts are the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) salts. MTT is a yellow water soluble tetrazole and its use as the first homogeneous cell viability assay for high throughput screening was first described in 1983 by Mosmann (Mosmann, 1983) (Figure 3.1). In the MTT assay, the formazan product of the tetrazolium salt is deposited as an insoluble precipitate near the cell surface of the cells and in the culture medium. Before absorbance readings can be taken, the formazan product must be solubilized in order to stabilize the colour, avoid evaporation and reduce interference by culture medium components. The common method of solubilisation is the addition of a solubilisation solution (40% SDS, 40%DMF, pH6.7) to the culture medium.

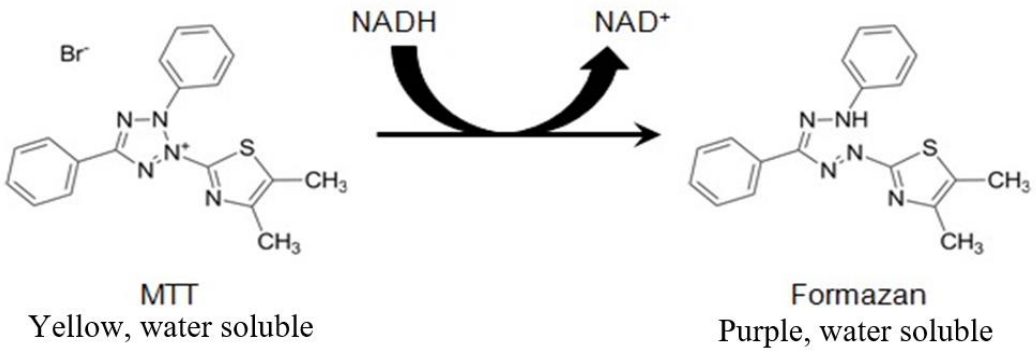


Figure 3.1 Chemical reaction illustrating the conversion of MTT to formazan. Adapted from (Riss et al., 2004).

The MTS assay is based on a recently developed tetrazolium reagent that can be reduced by metabolically active cells to generate formazan products that are soluble in culture medium. This method eliminates the additional step in the MTT assay because the addition of a solubilisation solution is not required. MTS requires combination with an electron coupling reagent such as phenazine methyl sulfate (PMS) or pheazine ethyl sulfate (PES) which allows easy penetration and exit in viable cells where it can be converted to soluble formazan products (Figure 3.2). Formazan is a water soluble product that can be quantified spectrophotometrically at a wavelength of 490 nm.

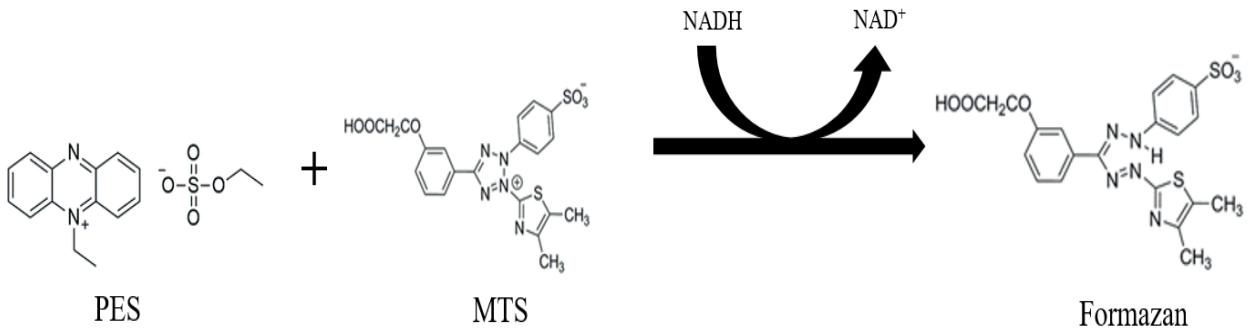


Figure 3.2 Chemical reaction illustrating the conversion of MTS to formazan. Adapted from (Riss et al., 2004).

The MTS reagent has several advantages over the use of the MTT reagent. It is easy to use and usually supplied as a single solution if PES is used as the coupling agent due to its good stability. It is faster because it eliminates the solubilisation steps in the MTT assay and it is also safer because no volatile organic solvent is required for solubility. Since MTS is not cytotoxic, plates can be returned to the incubator for multiple readings at different time intervals. The effect of drugs on cell lines can be evaluated with either cytotoxic or cell proliferation assays. To assess the effect of drugs that act on specific targets, cell proliferation assays are better than cytotoxicity assays because the drugs might not induce cell death if they are cytostatic. Cytotoxic assays based on altered cell permeability can underestimate the effect of cytotoxic agents due to intracellular damage (Sumantran, 2011). For instance, some cells could be irreversibly damaged and committed to die but their plasma membrane might be intact. Clonogenic assays are impractical as high throughput assays because they require counting of individual colonies making them time consuming and laborious. Multiple assays are better carried out in 96-well microplates because the effect of several drugs can be assessed simultaneously and multiple dilutions of an inhibitor or multiple cell lines can also

be assessed at the same time. Less volume of media and drugs are required making them cost effective. Absorbance readings can be carried out using a standard microplate reader.

3.2. *In vitro* migration assays

Cell migration is the direct movement of cells on basal membranes within the body. Tumour cell migration has major clinical relevance as it may result in tumour metastasis which is indicative of poor prognosis and a major challenge in the treatment of cancer. Migration assays can be used for screening the effect of therapeutic substances or genetic modifications in mammalian cells. There are several types of migration assays including: the boyden chamber assay, the cell exclusion zone assay, the fence assay, the microcarrier bead assay, the spheroid migration assay, the microfluidic chamber assay and the *in vitro* scratch assay. Most of these methods involve complex equipment and are expensive. Metastasis is a multistage process that occurs over several months or years therefore it is difficult to identify a method that encapsulates all the essential steps in metastasis. The common, easy, low cost but well developed method to measure cell migration *in vitro* is the use of the *in vitro* scratch assay (Kramer et al., 2013).

3.2.1. *In vitro* scratch assay

This is a simple, straightforward and inexpensive method to study cell migration *in vitro*. This method is based on the principle that when an artificial gap is made on a confluent cell monolayer using a plastic pipette tip, the cells on the edge of the created gap move toward the opening to close the gap until new cell to cell contact is formed again (Figure 3.3). The movement of the cells can be monitored microscopically as the cells travel from the intact zone into the gap. Cell migration can be calculated by measuring the decrease of the gap at different time points until the gap is closed. The major advantages of this assay are its ease of use, low cost and the ability to visualise cell movement in real time using time-lapse microscopy. It is often the method of choice to analyse cell migration *in vitro* because it is easy to set up, no specialized equipment is required and all materials are readily available in cell culture laboratories (Liang et al., 2007; Kramer et al., 2013).

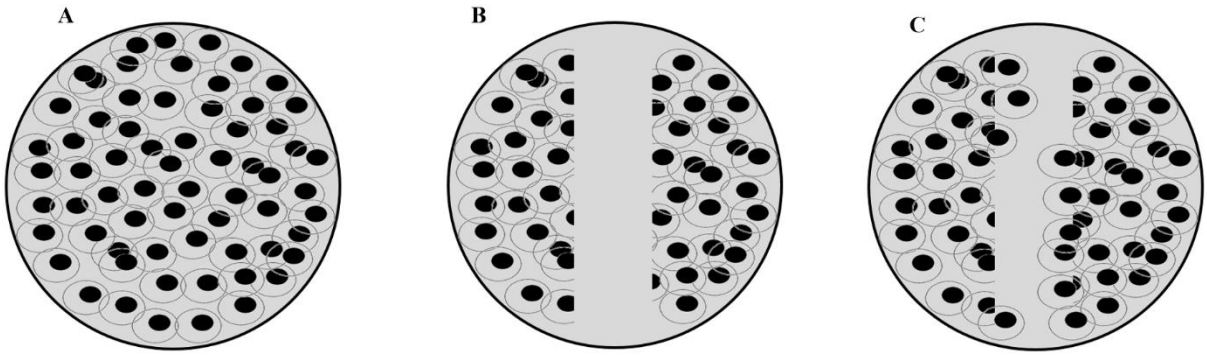


Figure 3.3 A diagrammatic representation of scratch assay. (A) represents a confluent culture, (B) the scratch on the surface, and (C) the healing scratch.

3.3. Biomarker Discovery

There is a need to identify prognostic and predictive biomarkers in mesothelioma which are quick, easy to use and cheap for routine labs. These biomarkers can also assist in the identification of possible therapeutic targets. Proteomics is a promising tool in the identification of new protein biomarkers because it allows a large study of the functions and structures of protein using high resolution protein separation and identification techniques (Anderson & Anderson, 1998). The successful completion of the draft sequence of the human genome led to the use of functional genomics and proteomics in the identification of genes associated with cancer and their protein products (Ardekani et al., 2008). These technologies have the potential to identify markers in the early detection, classification and prognosis of tumours by analysing thousands of genes and proteins simultaneously (Simpson & Dorow, 2001). However it is a clear fact that mRNA expression data alone does not predict the levels of the corresponding protein in a cell (Simpson & Dorow, 2001; Hardiman, 2004). Very little information is known about the localisation of corresponding proteins, post-translational modifications and other modifications by external agents because the difference in the stability of mRNAs and difference in the efficiency of translation results in the generation of new proteins (Simpson & Dorow, 2001; Hardiman, 2004). Studies have shown that proteins are generally more stable in clinical samples compared to mRNA levels. A 48hour post mortem study on the human brain by Anderson and Anderson (1998) showed that mRNA levels can decrease almost 200-fold while proteins in the same samples show little or no decrease. Proteins are involved in almost all biological functions and are also involved in normal body metabolic activities, disease processes and drug effect therefore RNA

expression studies should support proteomics to give a complete picture of cell alterations in malignant transformation (Figure 3.4) (Anderson & Anderson, 1998; Simpson et al., 2001).

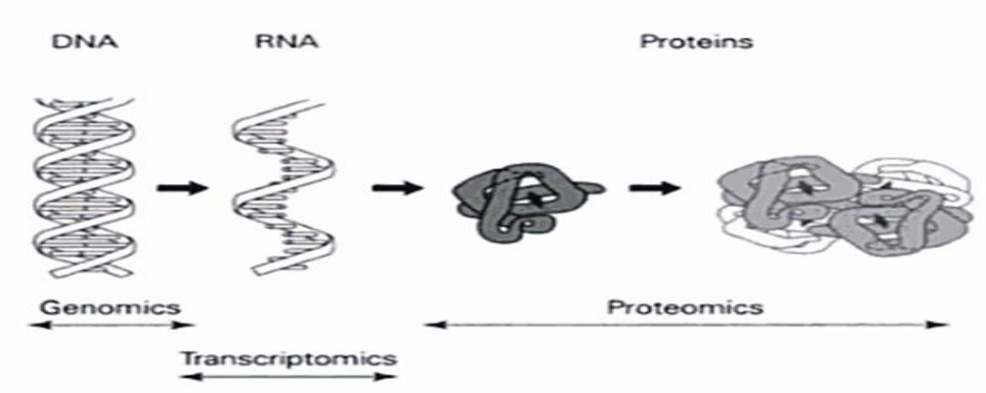


Figure 3.4 Showing the relationship between the 'omics. Genomics deals with the understanding of the complete DNA sequence of an organism's genome. Transcriptomics studies the full set of RNA transcripts produced from the genome, Proteomics studies the total set of proteins in the genome of a cell and other expressed subsets from the transcriptome. (Adapted from Turner et al., 2005)

Several proteomic techniques have been used in cancer research in the identification of novel biomarkers. These techniques include: Two-dimensional gel electrophoresis, with, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, liquid chromatography with electrospray ionisation mass spectrometry, and antibody microarrays (Smith et al., 2006). For the identification of specific proteins or biomarkers in tissue samples and complex cell mixtures, methods such as immunohistochemistry and western blotting are commonly used.

3.3.1. Immunohistochemistry

Immunohistochemistry is a semi-quantitative method used to visualize the distribution and localization of specific antigens in tissues samples based on antigen-antibody interaction (Figure 3.5). It is a routine and essential tool in the diagnosis of disease, biological research and drug development. Formalin fixed paraffin embedded clinical tissue samples are mounted onto a glass slide. Formalin preserves the morphologic features and induces alterations in the 3-dimensional structures of protein without causing any irreversible reduction or loss of antigens in the paraffin sections. Masked antigen sites and methylene bridges formed during formalin fixation are reversed by heating the tissue sections in a citrate or EDTA based

buffered solution. This process is known as antigen retrieval. Blocking buffers such as normal serum, non-fat dry milk, BSA or gelatine are used to reduce background staining caused by nonspecific binding of antibodies. For direct immunohistochemistry, a labelled primary antibody is applied and reacts directly with the antigen in the tissue sections. Although this method uses just one antibody making it simple and quick, its sensitivity is lowered due to low signal amplification in contrast to indirect methods. For indirect immunohistochemistry which is commonly used, an unlabelled primary antibody is applied which binds to the target antigen then a labelled secondary antibody that reacts to the primary antibody is added. The secondary antibody is conjugated to a fluorescent or enzyme reporter such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) which forms coloured insoluble precipitates that can be visualized under a light microscope when a chromogen substrate such as DAB (3,30-diaminobenzidine tetrahydrochloride) is added. Counterstains such as haematoxylin, Hoechst stain or DAPI are then applied to provide contrast that helps distinguish the primary stain.

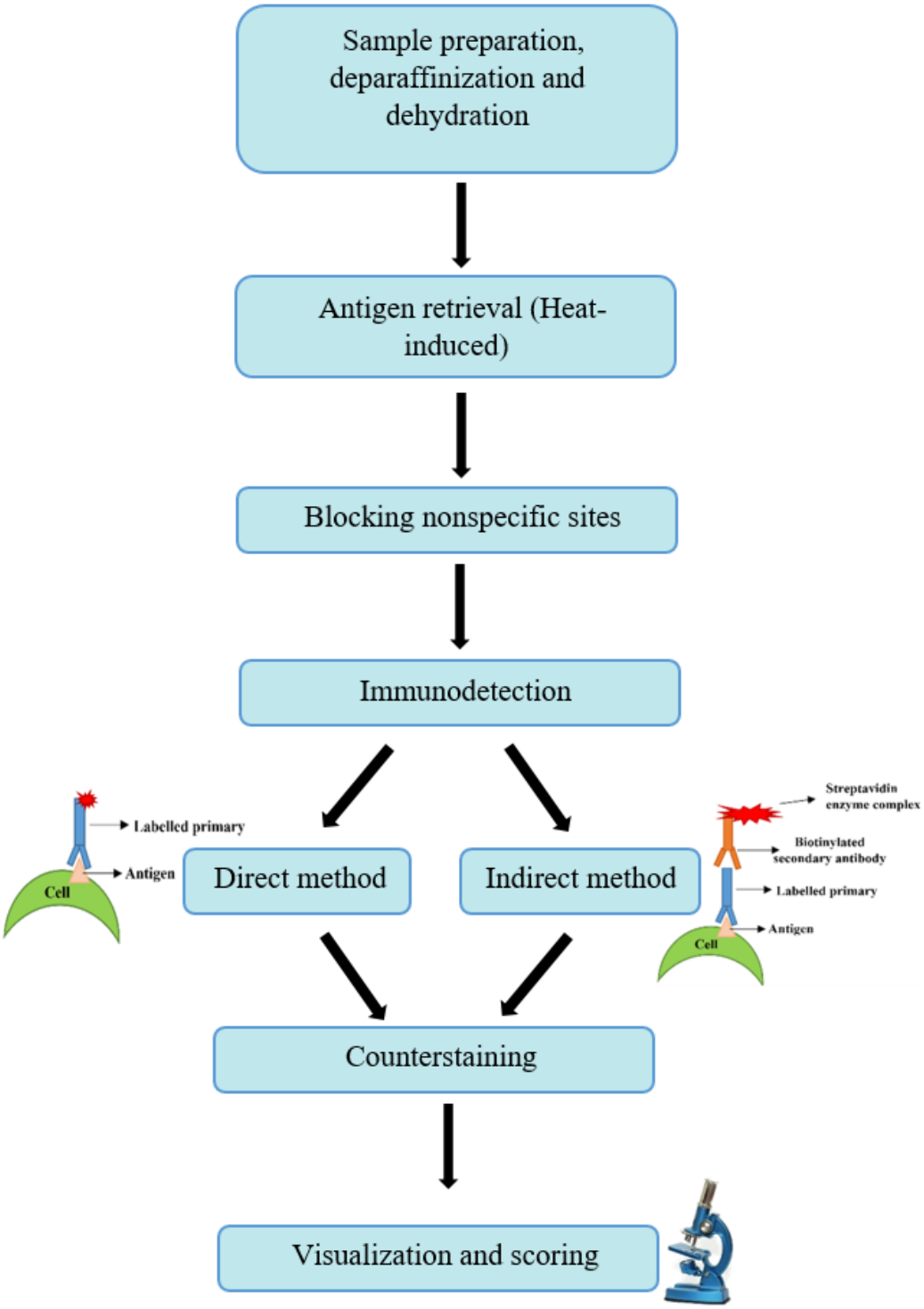


Figure 3.5 A chart flow of the steps involved in immunohistochemistry. The diagram shows schematic representation of the direct and indirect immunohistochemical methods.

3.3.2. Western blot

The separation of proteins by electrophoresis using polyacrylamide-urea gels and the transfer of proteins onto a nitrocellulose membrane was first introduced by Towbin and his colleagues in 1979 (Towbin et al., 1979). In 1981, Burnette developed the more widely used sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and the method was eventually termed western blotting (Burnette, 1981). This method has since been widely used in research laboratories around the world for the immunodetection and quantification of specific proteins within a complex cell homogenate (Figure 3.6). The complex mixture of proteins is first denatured by heating before being separated by gel electrophoresis based on their molecular weight and subsequently transferred onto a membrane. When the proteins are loaded onto the gel, they have a negative charge by binding to SDS and travel toward the positive electrode when a voltage is applied. It is important to run at low voltage as high voltage can overheat and distort bands. After separation, the protein mixture is transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane via an electric current which pulls proteins from the gel onto the membrane. Membranes have a high affinity for protein binding therefore the free protein binding sites need to be blocked using 5% bovine serum albumin or non-fat dried milk diluted in TBST (tris-buffered saline tween-20) to prevent nonspecific binding. An antibody specific to the protein of interest is then added to the membrane which then binds to the target protein if it is present. Washing is an important step between antibodies as it reduces background noise and removes unbound antibody. A secondary antibody tagged with an enzyme (usually horseradish peroxidase) is used to bind and label the primary antibody which can then be detected using chemiluminescence or fluorescence techniques. The reaction product produces luminescence, which is related to the amount of protein, and this can be detected using X-ray photographic film (Mahmood & Yang, 2012). Housekeeping proteins such as beta actin, alpha tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can be used as a loading control to ensure equal loading of proteins in the same membrane. Western blotting can be used as a semi-quantitative method to compare the expression of specific proteins in different lysates. The expression levels of proteins can be quantified by using a densitometer and there are available software programs for image analysis of bands on film (Jensen, 2012).

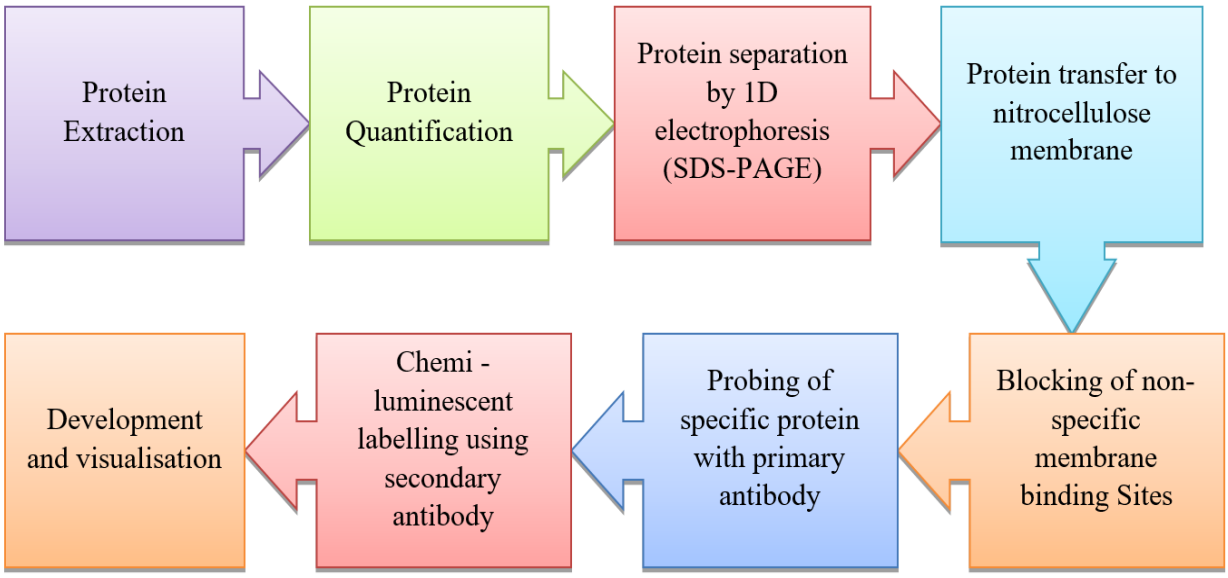


Figure 3.6 A typical workflow of the western blot technique for the separation and visualization of proteins.

3.4. Aims

Malignant pleural mesothelioma remains an aggressive disease associated with poor prognosis. First line treatment with a regimen of Cisplatin/Pemetrexed has a survival time of 12 months in about 42% of patients. There is currently no approved salvage regimen following failure of the first line treatment therefore, there is a need to develop novel therapeutic approaches. Severe effects of chemotherapy agents and drug resistance to these agents provides a rationale for the development of new modalities such as targeted-based cancer therapy. Over the past decade, targeted based therapeutic agents have been a major focus for cancer therapy and currently the US Food and Drug Administration (FDA), European Medical Agency (EMA), NICE (UK) and other related bodies have been evaluating and approving several agents including small molecule inhibitors and monoclonal antibodies. These agents target specific proteins and inhibit biologic transduction pathways that are hyperactive or overexpressed by tumour cells. There is some evidence that the Epidermal Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor receptor (VEGFR), Hepatocyte growth factor receptor (c-MET), Mammalian target of Rapamycin (MTOR) and Cyclooxygenase-2 (COX-2) pathways may be important in MPM. The potential role of Lipoxygenases (LOXs) remains unclear. Molecular interaction between the ligands (EGF,

VEFG, HGF), their receptors (EGFR, VEGFR, c-MET), MTOR, COX-2 and LOX are detailed in Figure 3.7.

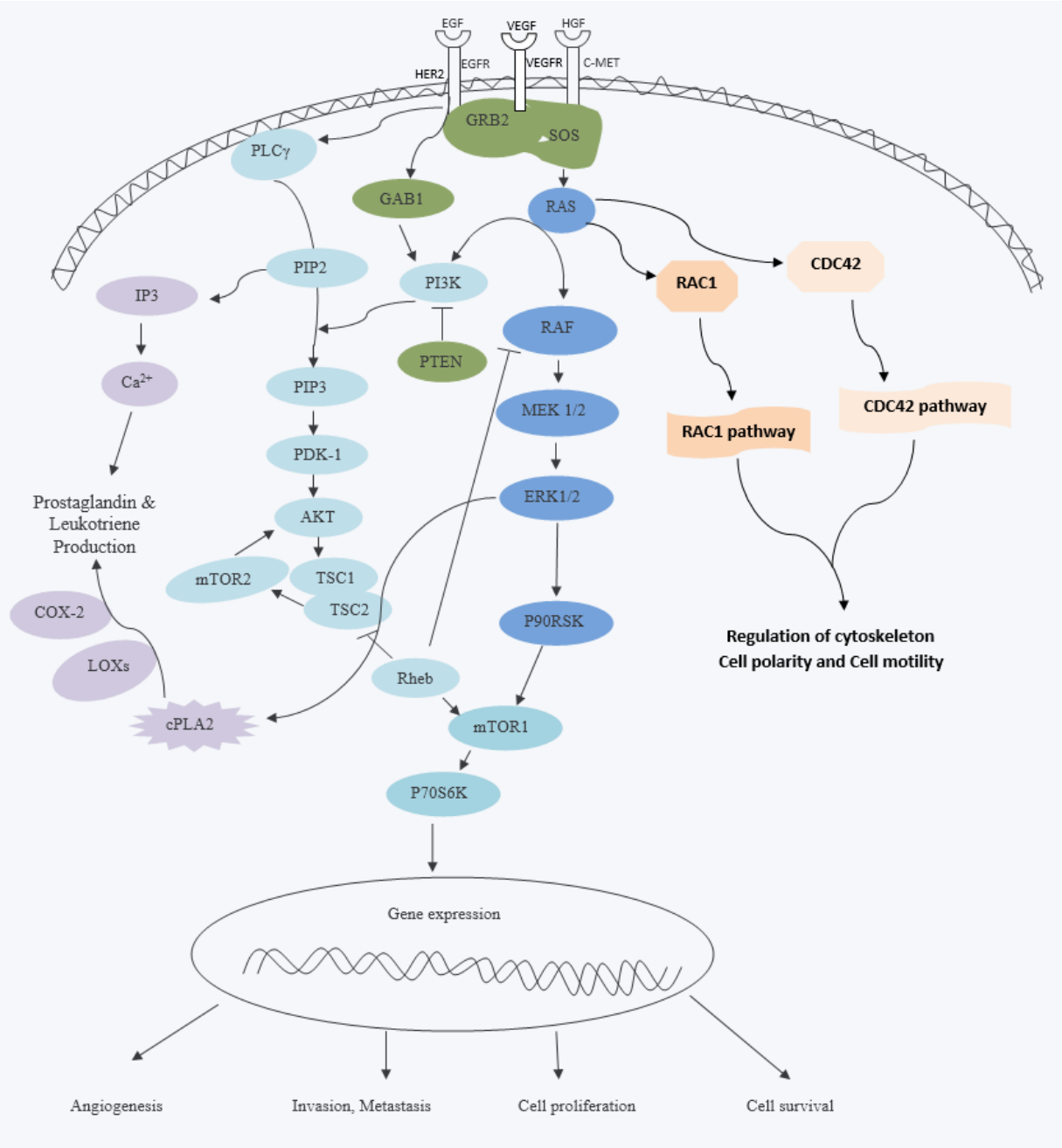


Figure 3.7 Interactions between signal transduction pathways. This figure demonstrates the between receptor kinases (when stimulated by their respective ligands) and activated intracellular signalling cascades. Activation of EGFR and VEGFR by EGF and VEGF ligands respectively results in the activation of the PI3K/AKT/MTOR pathway and the MAPK pathway. In addition to these two intracellular pathways, c-MET also stimulates the RAC1 pathway and CDC42 pathway responsible for the regulation of cytoskeleton, cell polarity and motility. MAPK (ERK) results in the increased transcription of COX-2 and LOX by stimulating the release of the cytosolic phospholipase A2 resulting in the release of arachidonic acid to for prostaglandins and leukotrienes.

A better understanding of the molecular pathogenesis in MPM is required for developing new diagnostic tools and new targeted therapies. MPM is molecularly characterised by the loss of tumour suppressor genes that result in the aberrant activation of tumour promoting molecular pathways. The identification of targeted agents that inhibits these molecular pathways as single agents or in combination might increase anti-proliferative effects *in vitro* and could inform future preclinical studies and clinical trials in MPM. Our study would evaluate the cytotoxic effect of inhibitors that induce cell death by inhibiting intracellular signalling but may not induce alteration in cell permeability. Therefore, cytotoxic assays based on alteration in cell permeability was not used in this study. For the purpose of this study, MTS assay was preferred to measure the antiproliferative and cytotoxic effect of inhibitors because of its ease of use and lower inconsistency in the assay compared to the MTT assays. We aimed to examine the expression of known oncogenic proteins in our cohort of MPM tissue samples cell lines and also investigate the effect of their respective inhibitors *in vitro* in Chapters 6-10.

The specific aims for this project are:

- To demonstrate the expression and prognostic relevance of the 5-LOX and 12-LOX proteins in MPM using immunohistochemistry and to evaluate the effect of Zileuton, Celecoxib, Baicalein and MK886 in MPM cell lines. Zileuton and Baicalein have not been evaluated in MPM. Expression of 5-LOX and 12-LOX in MPM tissue samples has not yet been reported.
- To demonstrate the expression of VEGFR-2 protein in MPM tissue samples and cell lines using immunohistochemistry and western blotting respectively. Evaluate the effect of a novel VEGFR-2 inhibitor MGDC 265 on MPM cell lines. The effect of MGDC265 has not been evaluated previously in MPM.
- To demonstrate the expression of HER2 protein in MPM tissue samples using immunohistochemistry and evaluate the effect of EGFR inhibition using Afatinib, Gefitinib and Selumetinib in MPM cell lines. The effect of Afatinib in MPM has not been previously reported.

- To demonstrate the expression of AKT and MTOR in MPM cell line using western blotting. Evaluate the effect of PI3K and MTOR inhibition using KU006974 and XL388 (MTOR1 and MTOR2 inhibitor) and NVPBEZ253 and VS5584 (PI3K/MTOR inhibitors). The effect of dual mTOR inhibitors in MPM cell lines has not been previously reported.
- To demonstrate the expression of the c-METprotein in MPM tissue samples and cell lines using immunohistochemistry and western blotting respectively. To evaluate the effect of Crizotinib, SU11274, Tivantinib and MetMAB in MPM cell lines. The effect of MetMAB in MPM has not been evaluated previously.
- To identify effective multi-targeting regimens that inhibits the proliferation of MPM cell lines. To evaluate the effect of multi-targeting on cell migration and downstream pathways.

CHAPTER FOUR

MATERIALS AND METHODS

Chapter 4 Materials and Methods

4.1 Archival tissue samples

A series of archival MPM tissues samples previously described and characterised by O'Kane et al 2005 was used within this project (O'Kane et al., 2005). Histopathology records and clinicopathological data of patients diagnosed with MPM within the Hull and East Yorkshire NHS trust from 1995 to 2000 were obtained from Hull Royal Infirmary, following ethical approval from the Hull and East Riding Local Research Ethics Committee (ref: 11/00/212). The samples were preserved in formalin-fixed paraffin embedded tissue blocks and consisted of 48 epithelioid cases, 27 mixed/biphasic cases and 18 sarcomatoid cases. Eight benign pleura samples were also included from males with primary pneumothorax.

4.1.1 Characteristics of patient cohort

Our cohort of mesothelioma samples initially consisted of 93 patients but over the years some of the blocks have been exhausted and in some instances some of the tissue specimen were unscoreable due to folding and other technical issues. In those circumstances, tissues that were not scoreable were not included. This explains the variation in the numbers mentioned in the result chapters for immunohistochemical analyses.

4.2 Cell lines

Cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (31870-074), supplemented with appropriate antibiotics and growth supplements (Appendix B). The cell lines used are listed below.

4.2.1 NCI-H2452 (ATCC, #CRL-5946)

An adherent cell line with epithelial origin obtained from a mesothelioma male patient (non-smoker). It was established in November 1990 and purchased from the American Type Culture Collection.

4.2.2 NCI-H2052 (ATCC, #CRL-5915)

This adherent cell line was derived from the metastatic site (pleura effusion) in a 65 years old male Caucasian with stage 4 mesothelioma. It was established in September 1998 and the patient was a smoker. This cell line has previously been described as a sarcomatoid subtype (Fiorelli et al., 2014). It was purchased from the American Type Culture Collection.

4.2.3 MSTO-211H (ATCC, #CRL-2081)

An adherent cell line derived from the metastatic site (pleural effusion) in a 62 years old Caucasian male with no prior chemotherapy or radiation diagnosed with biphasic (mixed) mesothelioma of the lung. It was established in 1985 and purchased from the American Type Culture Collection.

4.2.4 A549 (ECACC, #86012804)

An adherent non-small cell lung carcinoma cell line derived from a 58 year old Caucasian male. This cell line was used as a positive control and purchased from the European Collection of cell cultures (ECACC).

4.3 Inhibitors

Dimethyl Sulfoxide (DMSO; #D2650, Sigma Aldrich) was used as a drug vehicle at final concentrations less than 0.1% and was well tolerated by the cells. PBS or Sterile water was also used as a drug vehicle as recommended by some manufacturers. Control cells were exposed to the drug carrier in every experiment to eliminate the effect of DMSO on the cells. Table 4.1 is a list of all the inhibitors used, appropriate solvent and stock concentrations.

Table 4.1 List of inhibitors and chemotherapeutic agents used within the project and their stock concentrations. Pemetrexed, MetMAB and Licofelone were supplied on MTA as not commercially available at the time.

Drug	Pathway	Solvent	Supplier	Stock concentration
Afatinib	EGFR/HER2	DMSO	Selleckchem	50 mM
Baicalein	12 LOX	DMSO	Tocris	50 mM
Cediranib	VEGFR2	DMSO	Selleckchem	50 mM
Celecoxib	COX-2	DMSO	Sigma	25 mM
Cisplatin	DNA,	Sterile distilled water	Sigma	1 mM
Crizotinib	c-MET/ALK	DMSO	Tocris	50 mM
Dactolisib (NVPBEZ235)	PI3K/mTOR	DMSO	Selleckchem	2 mM
Gefitinib	EGFR	DMSO	Tocris	50 mM
Ku0063794	mTOR1&2	DMSO	Tocris	50 mM
Licofelone	COX/LOX5	DMSO	Prof Stephan Laufer	25 mM
MetMAB	c-MET	PBS	Genentech	600 µM
MGCD 265	VEGFR1-3/c-MET/RON/TIE	DMSO	Selleckchem	50 mM
MK886	FLAP	DMSO	Tocris	50 mM
Pemetrexed	TS	Sterile DDH_20	EliLilly	5 mM
Raltitrexed	TS	DMSO?	Selleckchem	50mM
Selumetinib	MEK	DMSO	Selleckchem	50 mM
SU11274	c-MET	DMSO	Tocris	50 mM
Tivantinib (ARQ197)	c-MET	DMSO	Selleckchem	50 mM
VS-5584	PI3K/mTOR	DMSO	Selleckchem	50 mM
XL388	mTOR1&2	DMSO	Selleckchem	50 mM
Zileuton	5 LOX	DMSO	Selleckchem	25 mM

4.3.1 Selective LOX and COX inhibitors

4.3.1.1 Celecoxib

Celecoxib was purchased from Sigma-Aldrich (#PZ008). It has a molecular mass of 381.37 g/mol and its chemical formula is $C_{17}H_{14}F_3N_3O_2S$ (Figure 4.1). Celecoxib is marketed by Pfizer under the trade name Celebrex. It belongs to the class of non-steroidal anti-inflammatory drugs. Celecoxib exerts its anti-inflammatory and analgesic properties by

blocking the synthesis of prostaglandins (Davies et al., 2000; Ricciotti & FitzGerald, 2011). The chemical structure consists of the *p*-sulfamoylphenyl group in its side chain which binds to the hydrophobic pocket of the COX-2 enzyme for selective inhibition (Grosser, 2006). Besides COX-2 inhibition, Celecoxib has been shown to exert antitumor activity by binding and inhibiting targets involved in antitumour response via both COX-dependent and COX-independent mechanisms as seen in other cancer types (Hsu, 2000; Han et al., 2004; Grösch et al., 2006; Kern et al., 2006; Schönthal, 2007).

Celecoxib is administered orally and rapidly absorbed, reaching peak plasma concentration between 2-4 hours. It is extensively metabolized in the liver by the cytochrome P_{450} (CYP) 2C9 isoenzyme and excreted in urine and faeces (Davies et al., 2000; Paulson et al., 2000). Celecoxib is currently used for the treatment of osteoarthritis, rheumatoid arthritis, acute pain including dysmenorrhoea at doses of 100-400 mg. The peak plasma drug concentration (C_{max}) of 400 mg single dose of celecoxib ranges between 602.7 µg/L and 810.9 µg/L (Davies et al., 2000). Epidemiological studies have shown that continuous uptake of Celecoxib and related compounds lowers the incidence of colonic polyps in patients with hereditary familial adenomatous polyposis (FAP) syndrome and reduces the risk of colon, breast, oesophageal cancers. It has been approved for the prevention of colon cancer in patients with FAP and several clinical studies are underway for its potential use in the treatment of advanced cancers.

4.3.1.2 Licofelone (ML3000)

Licofelone was obtained via Material Transfer Agreement from c-a-i-r biosciences GmbH (see appendix D). ML3000 was synthesized by Merckle GmbH and is being developed under the trade name Licofelone. It has a molecular mass of 379.9 g/mol and its chemical formula is $C_{23}H_{22}CINO_2$ (Figure 4.1). It is a dual COX/5-LOX inhibitor with analgesic, anti-inflammatory, antipyretic, antiplatelet aggregation properties (Tries & Laufer, 2001). Licofelone inhibits both cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase reducing the production of prostaglandins and leukotrienes. Unlike other single COX inhibitors, Licofelone further eliminates the shunting of arachidonic acid to the 5-LOX pathway when COX is being inhibited (Tries et al., 2002). *In vitro* studies have also shown that Licofelone might be a potential chemotherapeutic or preventive agent in prostate and colon cancers (Narayanan et al., 2007; Tavolari et al., 2008).

Plasma concentration of Licofelone peaked at 0.74-4 hours after oral administration, the plasma steady state concentration ranged from 1.13 µg/ml – 2.36 µg/ml (Ding & Cicuttini,

2003). Studies with murine models showed high concentration of Licofelone in the lung, liver, kidney, heart and intestine and elimination was through urine and faeces (Ding & Cicuttini, 2003). Licofelone is the first dual COX/5LOX inhibitor with significant therapeutic potentials for the treatment of osteoarthritis (OA) and it has a well-established gastrointestinal safety profile when compared with other NSAIDs (Alvaro-Gracia, 2004). It recently passed a phase III clinical trial for the treatment of OA.

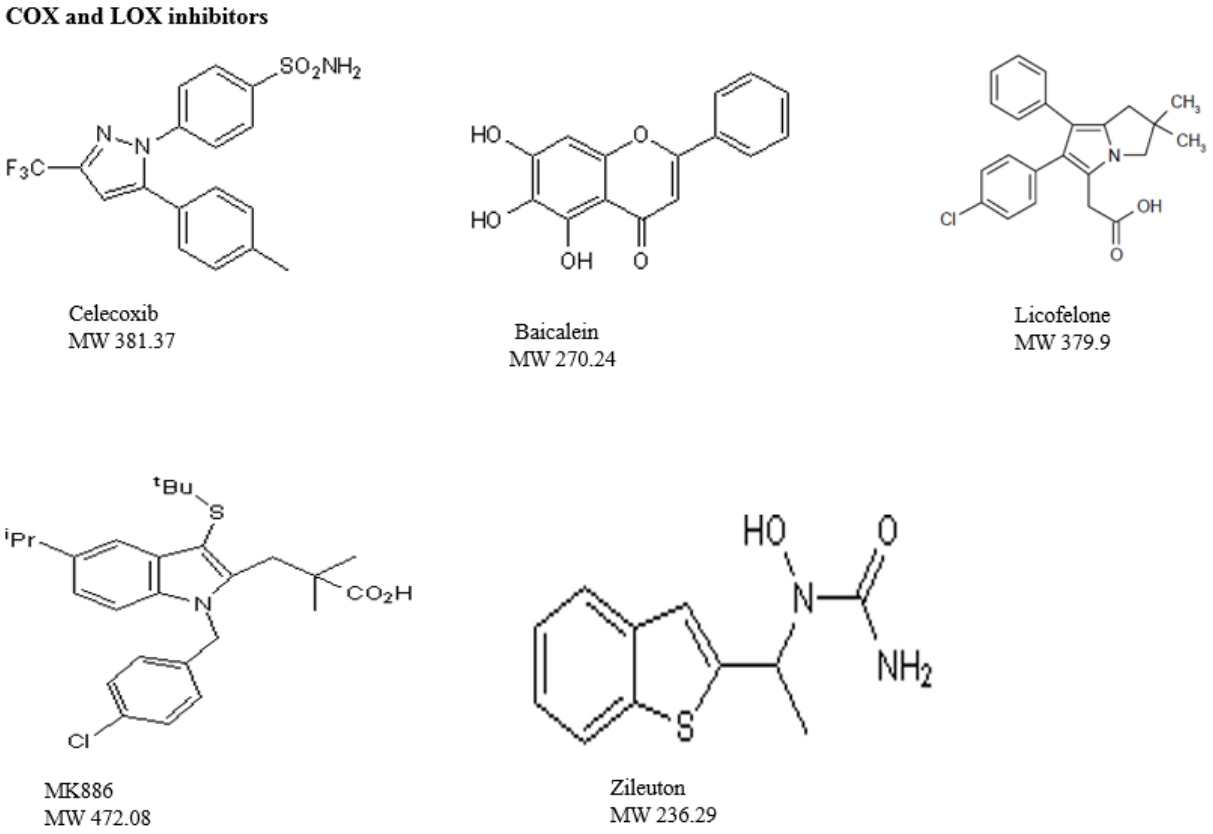


Figure 4.1 COX and LOX inhibitors and their relative molecular mass in $gmol^{-1}$. (Images from www.selleckchem.com)

4.3.1.3 MK886

MK886 was purchased from Tocris Bioscience (#1311). It has a molecular mass of 472.08 g/mol and its chemical formula is $C_{27}H_{35}ClNO_2S$ (Figure 4.1). MK886, synthesized by Merck Frosst is a highly potent and specific leukotriene biosynthesis inhibitor. It inhibits leukotriene synthesis by binding to the 5-LOX activating protein (FLAP) which further prevents 5-lipoxygenase membrane translocation and activation in a concentration-dependent

manner (Rouzer et al., 1990). FLAP, a membrane-bound protein transports arachidonic acid to 5-LOX for the efficient production of oxidized lipid products (LTs). Studies have also shown that MK886 induces apoptosis independently of FLAP in haematopoietic cell lines (Datta, 1998; Datta et al., 1999).

Studies conducted by Depre *et al*, showed that MK886 exerts maximum effect within 1-2 hours. The C_{max} values of 3.9 ± 1.6 µg/ml and 4.0 ± 1.9 µg/ml were obtained for 500 mg and 750 mg doses respectively (Depre et al., 1993). Preclinical data has shown several antiproliferative effects of MK886 in various malignancies but is not yet approved for further development.

4.3.1.4 Baicalein

Baicalein was purchased from Tocris Bioscience (#1761). It has a molecular mass of 270.24 g/mol and its chemical formula is $C_{15}H_{10}O_5$ (Figure 4.1). Baicalein is a flavonoid extracted from the root of *Scutellaria baicalensis*, a member of the mint family used in Chinese and Japanese medicine for a numerous ailments especially chronic hepatitis. The mechanism of action of this naturally occurring compound is yet to be thoroughly understood however, it has been described as a 12-LOX and 15-LOX inhibitor with higher affinity for 12-LOX. Baicalein has anti-inflammatory, antithrombotic and antioxidant effects and also induces cell cycle arrest in cancer cells. Antitumour effect of baicalein has been extensively studied *in vitro* and it has been reported to modulate the PI3K/Akt and ERK pathways, cyclin dependent kinases, p53, apoptotic proteins, p38 and induce autophagy and which subsequently suppresses the proliferation of tumour cells (Deschamps et al., 2006; Chao et al., 2007; Leung et al., 2007; Kong et al., 2010; Guo et al., 2011; Huang et al., 2012; Czapski et al., 2012; Zhang et al., 2013; Chen et al., 2013; Aryal et al., 2014; Song et al., 2014). A recent first in human phase I dose escalating trial showed that baicalein is well tolerated in humans at doses ranging from 100 mg to 2800 mg. Therefore, the maximum tolerated dose was not achieved (Li et al., 2014)

4.3.1.5 Zileuton

Zileuton was purchased from Tocris Bioscience (#3308). It has a molecular mass of 236.29 g/mol and its chemical formula is $C_{11}H_{12}N_2O_2S$ (Figure 4.1). It was manufactured by Abbott laboratories and is now marketed by Cornerstone Therapeutics Inc under the trade names ZYFLO and ZYFLO CR. Zileuton selectively inhibits the 5-lipoxygenase (5-LOX) enzyme

which catalyses the formation of leukotrienes from arachidonic acid. Leukotrienes are molecules that contribute to chronic inflammation which could result to cancer moreover, 5-LOX levels are often elevated in cancers. *In vitro* and *in vivo* preclinical studies have demonstrated the chemopreventive and chemotherapeutic characteristics of zileuton in several cancers (Hussey & Tisdale, 1996; Steele et al., 1999; Wenger et al., 2002; Chen et al., 2004; Barry et al., 2009; Meng et al., 2013; Gounaris et al., 2015). Unfortunately, the chemotherapeutic potential of zileuton was not observed in the Phase II clinical trial in NSCLC patients when investigated in combination with a COX-2 inhibitor (Edelman et al., 2008). It is approved for the treatment of chronic asthma in adults and children. Zileuton is administered as an oral dose of 600 mg four times per day and the plasma steady state concentration ranges from 9.28 to 4.66 mg/L (Awni et al., 1995).

4.3.2 EGFR inhibitors

4.3.2.1 Afatinib (BIBW2992)

Afatinib (tradename: Gilotrif®) was purchased from Selleckchem (#S11011). It has a molecular mass of 485.94 g/mol and its chemical formula is $C_{24}H_{25}ClFN_5O_3$ (Figure 4.2). It is a dual irreversible tyrosine kinase inhibitor of the ErbB family currently approved as a first-line treatment for patients with metastatic non-small cell lung cancer (NSCLC) whose tumours have EGFR exon 19 deletions or exon 21 (L858R) substitutions mutations. Afatinib covalently binds to specific cysteine residues within the catalytic domain of the receptors to prevent the binding of ATP resulting in the down regulation of EGFR signalling. The domains involved are Cys^{797} in EGFR, Cys^{805} and Cys^{803} in HER2 and HER4 respectively. Its irreversible binding to the catalytic domain of the receptors prevents the formation of dimers that promotes receptor tyrosine kinase activity and is intended to improve the efficacy of an inhibitor and prevent resistance (Li et al., 2008; Solca et al., 2012). Preclinical studies using a variety of enzymological, cell-based and in vivo assays have shown that Afatinib can specifically inhibit *in vitro* and *in vivo* enzymatic activity of wild-type EGFR and HER2, as well as erlotinib-sensitive EGFR L858R mutant and the erlotinib-insensitive L858R/T790M double mutant. Although the potency of Afatinib on the double mutant EGFR is reduced (Kwak et al., 2005; Li et al., 2008; Sos et al., 2010; Cha et al., 2012; Ou, 2012). Apart from targeting EGF receptors, Afatinib also inhibits downstream signalling pathways such as Ras/Erk, PI3K/Akt and STAT pathways in cell lines derived from cancers of the lung, breast

and pancreas which then results in significantly reduced cell growth (Ioannou et al., 2011; Canonici et al., 2013; Lee et al., 2013; Mack et al., 2013; Suzawa et al., 2016). The recommended dose is 40 mg orally, once a day and its maximum mean plasma concentration at steady state is 29 ng/mL to 63.4 ng/mL (Gordon et al., 2013; Mancheril et al., 2014).

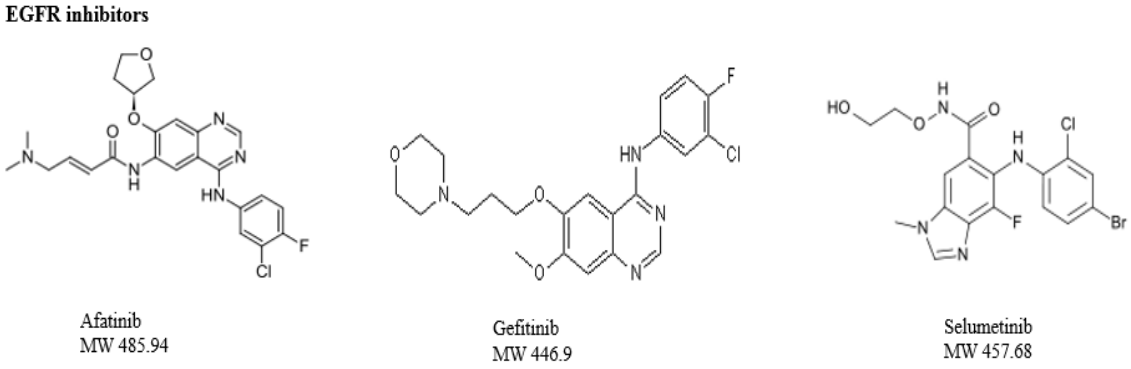


Figure 4.2 Inhibitors of the EGFR pathway. Chemical structures and relative molecular mass in $gmol^{-1}$. (Images from www.selleckchem.com)

4.3.2.2 Gefitinib (Iressa)

Gefitinib was purchased from Tocris Bioscience (#3000). It has a molecular mass of 446.9 g/mol and its chemical formula is $C_{22}H_{24}ClFN_4O_3$ (Figure 4.2). Gefitinib is an orally active selective EGFR-TKI that belongs to the first generation of EGFR tyrosine kinase inhibitors. As a reversible inhibitor, it binds to the ATP binding site of the kinase to inhibit EGFR autophosphorylation and its downstream signalling. It was first approved by the FDA in 2003 as a third-line therapy for the treatment of locally advanced or metastatic NSCLC but subsequently failed to show an overall survival benefit in a confirmatory phase III trial (ISEL). In 2004, three independent studies demonstrated a dramatic response to gefitinib in tumours that harbour somatic EGFR gene mutations in ATP binding cleft the kinase domain (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004; Thatcher et al., 2005). Recently, it was approved as first-line therapy for the treatment of NSCLC patients with activating mutations in the EGFR-TK domain (exon 19 deletions or exon 21 (L858R) substitution). The recommended dose is 250 mg orally once a day and its maximum mean plasma concentration at this dose was 159 ng/mL (Swaisland et al., 2005).

4.3.2.3 Selumetinib (AZD6244)

Selumetinib was purchased from Selleckchem. It has a molecular mass of 457.68 g/mol and its chemical formula is $C_{17}H_{15}BrClFN_4O_3$ (Figure 4.2). It is a potent, selective, orally available and non ATP-competitive small molecule inhibitor of the mitogen-activated protein kinase (MAPK) kinase MEK1/2 immediately downstream of RAF (Figure 2.3). Selumetinib inhibits both MEK1 and 2 and prevents the activation of ERK1/2 and transcription factors which results in the inhibition of cell proliferation. Preclinical studies conducted *in vitro* or *in vivo* with human xenograft tumour models have shown that selumetinib is able to reduce tumour growth, induce apoptosis and is very effective in cell lines with *BRAF*, *KRAS* or *NRAS* mutations as a single agent or in combination with chemotherapy or other targeted therapies (Davies et al., 2007; Yeh et al., 2007; Metro et al., 2013). A randomised controlled phase II trial in previously treated advanced NSCLC patients with K-Ras mutation showed that median progression free survival and overall response rate favoured the selumetinib arm (Jänne et al., 2013). It is currently in different phases of approximately 30 clinical trials in several cancer types. The maximum tolerated dose was achieved at its recommended dose of 100 mg twice daily and the mean plasma concentration was 1600 ng/mL (O'Neil et al., 2011).

4.3.3 c-MET inhibitors

4.3.3.1 Crizotinib (Xalkori®)

Crizotinib was purchased from Tocris Biosciences (#4368). It has a molecular mass of 450.34 g/mol and its chemical formula is $C_{21}H_{22}Cl_2FN_5O$ (Figure 4.3). It was synthesized primarily as a c-MET inhibitor via the modification of PHA-665752, a first generation c-MET inhibitor with poor pharmacological properties. Crizotinib is an oral, potent ATP-competitive inhibitor of c-MET, ROS1 and ALK (anaplastic lymphoma kinase) kinases and their oncogenic variants. The binding of crizotinib to the ATP-site of c-MET kinase domain interferes with ATP and substrate binding therefore inhibiting autophosphorylation of the kinase both *in vitro* and *in vivo* (Christensen et al., 2007; Zou et al., 2007; Cui et al., 2011; Curran, 2012). It was approved for the treatment of ALK-positive NSCLC patients in 2013 and in 2016 for the treatment of ROS1 positive NSCLC patients based on successful clinical trials (Hirsh et al., 2013; Shaw et al., 2013, 2014; Mazières et al., 2015). Crizotinib has also been found to be effective as a monotherapy in some lung cancer patients whose tumours were void of ALK and ROS rearrangements but had de novo c-MET amplification (Ou et al., 2011; Schwab et

al., 2014). The recommended dose is 250 mg orally twice a day and the mean plasma concentration at this dose ranges from 135 to 256 ng/mL (Curran, 2012; Xu et al., 2015).

4.3.3.2 SU11274

SU11274 was purchased from Tocris Bioscience (#4101). It has a molecular mass of 568.09 g/mol and its chemical formula is $C_{28}H_{30}ClN_5O_4S$ (Figure 4.3). It is a selective and ATP-competitive inhibitor of c-MET. SU11274 targets the ATP-binding site of c-MET and blocks HGF-dependent c-MET activation as well as the oncogenic variant Tpr-MET. In enzymatic assays, SU11274 exhibited greater than 50-fold selectivity for c-MET than other kinases. It has also been shown to inhibit the phosphorylation of c-MET downstream kinases and induce cell cycle arrest and apoptosis in cancer cells including mesothelioma (Sattler et al., 2003; Wang et al., 2003; Berthou et al., 2004; Jagadeeswaran et al., 2006). SU11274 was recently reported to sensitize prostate cancer cells to ionizing radiation (Yu et al., 2012). SU11274 is yet to undergo clinical trial.

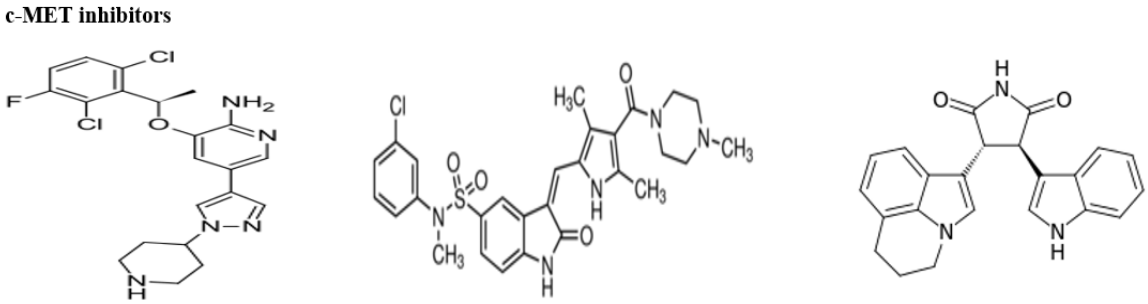


Figure 4.3 c-MET inhibitors. Chemical structure and relative molecular mass in $gmol^{-1}$. (Images from www.selleckchem.com)

4.3.3.3 Tivantinib (ARQ 197)

Tivantinib was purchased from Selleckchem (#S2753). It has a molecular mass of 369.42 g/mol and its chemical formula is $C_{23}H_{19}N_3O_2$ (Figure 4.3). It is the first selective non-ATP competitive c-MET inhibitor that acts by locking the kinase in a closed and inactive form when bound. Tivantinib was found to selectively inhibit c-MET in a panel of 230 human

protein kinases without affecting the concentration of ATP. In cancer cell lines and xenografts, Tivantinib was found to inhibit HGF-stimulated and constitutive c-MET phosphorylation, and also downstream proteins such as Akt, MAPK and STAT-3 with IC_{50} of 100 -300 nM. Exposure to tivantinib resulted in the inhibition of metastasis, invasion, proliferation, and induction of caspase dependent apoptosis in cell lines with constitutive MET activity (Jeay et al., 2007; Anderson et al., 2007; Li et al., 2007; Gu et al., 2009; Munshi et al., 2010). Two phase I clinical trials in various solid tumours showed that tivantinib was well tolerated and also demonstrated the safety and antitumour activity of the inhibitor (Rosen et al., 2011; Yap et al., 2011). A Phase II trial of tivantinib in combination with erlotinib significantly improved progression free survival and overall survival (Sequist et al., 2011). Tivantinib is in several phase of clinical trials for different malignancies, as a single agent and in combination with other cytotoxic agents. Maximum tolerated dose was achieved at 360 mg twice daily and the mean plasma concentration at that dose is 1655 ng/mL (Goldman et al., 2012).

4.3.4 PI3K/AKT/mTOR inhibitors

4.3.4.1 NVPBEZ235 (Dactolisib)

NVPBEZ235 was purchased from Selleckchem (#S1009). It has a molecular mass of 469.55 g/mol and its chemical formula is $C_{30}H_{23}N_5O$ (Figure 4.4). NVPBEZ235 is an orally available PI3K and mTOR1/mTOR2 inhibitor that potently inhibits class 1 PI3K activity, mTOR1 and mTOR2 catalytic activity in a reversible manner by binding to their ATP-binding sites (Maira et al., 2008). Preclinical data has shown that NVPBEZ235 has strong anti-proliferative effects and induces cell cycle arrest at low IC_{50} concentrations in several tumour xenografts regardless of genetic alterations (Serra et al., 2008; Baumann et al., 2009; Cao et al., 2009; Li et al., 2015). It was the first PI3K inhibitor to enter clinical trials about a decade ago. There has been several phase I/II studies testing NVPBEZ235 in several tumour types especially breast cancer but only one phase I result has been published so far. A phase I study reported by Bendell et al (2015) recommended a maximum tolerated dose of 300 mg twice a day. Prolonged stable disease was observed in 30.3% of the patients irrespective of the tumour type. There was also no biomarker used for selection in this study. The mean plasma concentration at the recommended dose was 655.6 ng/mL (Bendell et al., 2015).

4.3.4.2 KU0063794

KU0063794 was purchased from Tocris Bioscience (#3725). It has a molecular mass of 465.54 g/mol and its chemical formula is $C_{25}H_{31}N_5O_4$ (Figure 4.4). It was developed by AstraZeneca using PI-103, a dual PI3K/mTOR inhibitor as lead compound. It is an ATP-competitive inhibitor of both mTOR 1 and mTOR 2 with an IC_{50} of 10nM but does exhibit inhibitory activity against class I PI3Ks. KU0063794 inhibits cell proliferation in NSCLC cells that are sensitive and resistant to EGFR TKIs by interrupting the AKT/FOXO1 signalling pathway in resistant cells (García-Martínez et al., 2009; Fei et al., 2013). In renal carcinoma cells, KU0063794 was able to inhibit the phosphorylation of p70S6K, 4E-BP1 and Akt which are downstream of the mTOR complexes when compared with rapamycin analogs (Malagu et al., 2009; Zhang et al., 2013). KU0063794 is still in preclinical development.

4.3.4.3 VS-5584

VS-5584 was purchased from Selleckchem (#S7016). It has a molecular mass of 354.41 g/mol and its chemical formula is $C_{17}H_{22}N_8O$ (Figure 4.4). It is a novel, potent and selective inhibitor of all class I PI3K isoforms and mTOR1/2. Apart from blocking the phosphorylation of cellular substrates in the PI3K/Akt/mTOR pathway in different tumour models, VS-5584 also preferentially targets cancer stem cells (Hart et al., 2013; Trombino et al., 2014; Kolev et al., 2015; Shao et al., 2015). In February 2015, it received approval as an orphan drug for the treatment of mesothelioma. VS-5584 is currently in phase I/II clinical trial for advanced non-hematologic malignancies or lymphoma and in combination with Defactinib in mesothelioma patients (NCT01991938; NCT02372227).

4.3.4.4 XL-388

XL-388 was purchased from Tocris Bioscience (#4893). It has a molecular mass of 455.5 g/mol and its chemical formula is $C_{23}H_{22}FN_3O_4S$ (Figure 4.4). It is a novel, selective ATP-competitive inhibitor of both mTOR1 and mTOR2 developed by Exelixis. It inhibits mTOR1 and mTOR2 at IC50 values of 8 nM and 166 nM respectively. In prostate and breast cancer xenograft models, XL388 displayed strong pharmacodynamics and pharmacokinetic effects and significantly reduced tumour growth at nanomolar range. In MCF-7 cell lines, XL388 inhibits mTOR1 phosphorylation of p70S6K (Thr389) and mTOR2 phosphorylation of Akt (Ser473) at IC_{50} values of 94 nM and 350 nM respectively. The combination of XL388 with chemotherapeutic agents also produced a synergistic effect in both cell lines and xenografts

(Miller, 2009; Liu et al., 2009; Schenone et al., 2011; Takeuchi et al., 2013). XL388 is still in early preclinical development.

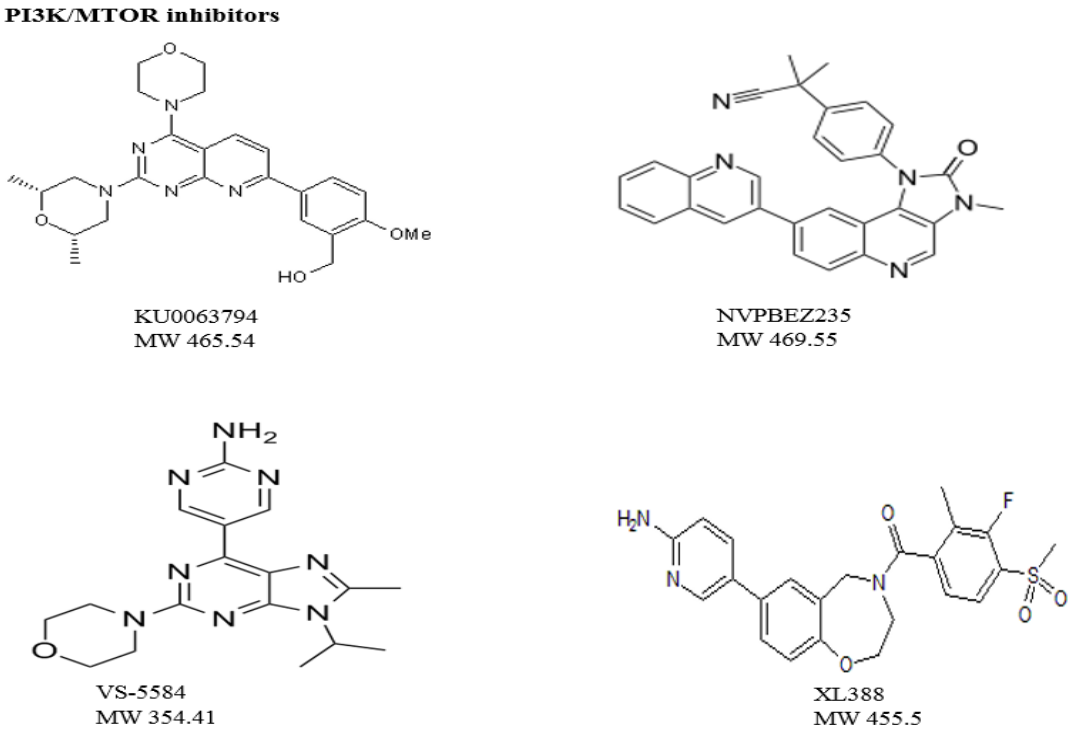


Figure 4.4 PI3K/Akt/mTOR pathway inhibitors. Chemical structure and relative molecular mass in $gmol^{-1}$. (Images from www.selleckchem.com)

4.3.5 VEGFR inhibitors

4.3.5.1 Cediranib (AZD2171)

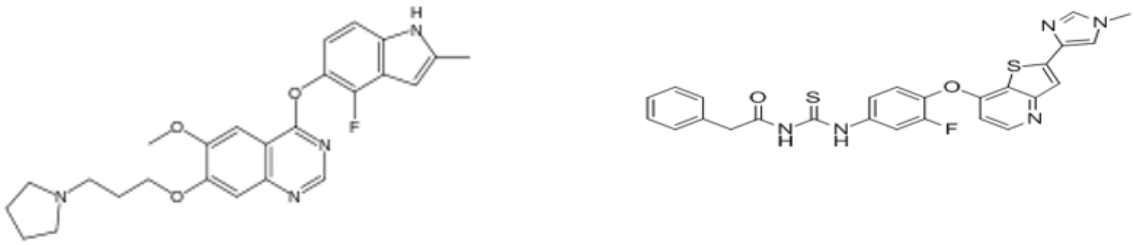
Cediranib was purchased from Selleckchem (#S1017). It has a molecular mass of 450.51 g/mol and its chemical formula is $C_{25}H_{27}FN_4O_3$ (Figure 4.5). Cediranib is a highly potent, and selective oral inhibitor of VEGF receptors 1-3 with additional inhibitory activity against PDGF and c-KIT. Preclinical data showed cediranib to be effective in several cancer types including colon, breast, ovarian, lung and prostate (Wedge et al., 2005; Goodlad et al., 2006; Miller et al., 2006; Gomez-Rivera et al., 2007; Takeda et al., 2007; Smith et al., 2007). Initial dose-escalating phase I trials showed good tolerability and pharmacokinetic properties (Drevs et al., 2007; Ryan et al., 2007; Laurie et al., 2008). Promising preclinical studies led to several Phase II clinical trials in NSCLC, colorectal and other solid tumours but limited success has been demonstrated in subsequent phase II/III trials in these tumour types (Ramalingam et al., 2010; Kato et al., 2012; Alberts et al., 2012; Hyams et al., 2013; Laurie

et al., 2014) . In MPM patients, preliminary data from a Phase I trial reported a preliminary response rate of 53%, promising median progression free survival and overall survival of 10 months and 16 months respectively with a recommended dose of 20 mg daily (Tsao et al., 2013). It was also tested in a Phase II trial as monotherapy in second line at doses of 45 mg daily but a disease control rate of 42%. In another Phase II trial, cediranib did not meet its primary endpoint and only higher doses were associated with improved disease control rate but with increased toxicity (Garland et al., 2011; Campbell et al., 2012). It is currently being tested in combination with pemetrexed/cisplatin in a phase I/II trial (NCT01064648). Recently, it was reported to significantly improve overall survival and progression free survival in gynaecological cancers as a single agent and when combined with other inhibitors (Liu et al., 2014; Bender et al., 2015; Symonds et al., 2015; Ledermann et al., 2016). There are 28 ongoing clinical trials including Phase III trials investigating cediranib as a single agent and in combination with standard chemotherapeutic agents in several tumour types (www.clinicaltrials.org). The recommended therapeutic dose of cediranib is 30 mg once daily and the mean plasma concentration at this dose is 70 ng/mL (Drevs et al., 2007; Zhu et al., 2013)

4.3.5.2 MGCD-265 (Glesatinib)

MGCD-265 was purchased from Selleckchem (#S13161). It has a molecular mass of 517.60 g/mol and its chemical formula is $C_{26}H_{20}FN_5O_2S_2$ (Figure 4.5). MGCD-265 is a novel orally active, potent, multi-targeted ATP-competitive inhibitor of VEGFR-1/2/3 and c-MET with additional inhibitory activity against and Ron and Tie2 (Saavedra et al., 2009). Results from the phase I trials of MGCD-265 in advanced solid tumours are yet to be published but pre-publication results from conference proceedings have been reviewed by Padda et al (2012) (Padda et al., 2012). MGCD-265 is still in early clinical development with two phase 2 clinical trials investigating its efficacy in patients with non-small cell lung cancer and squamous cell carcinoma of the head and neck (NCT00697632; NCT02544633).

VEGFR inhibitors



Cediranib
MW 450.51

MGCD265
MW 517.60

Figure 4.5 VEGFR inhibitors. Chemical structure and relative molecular mass in $gmol^{-1}$. (Images from www.selleckchem.com)

4.4 Methods

4.4.1 Cell culture

All cell lines were cultured under sterile conditions in a Class II tissue culture hood. Equipment such as water bath, cell culture hood, incubator, pipettes etc. were cleaned thoroughly with Virkon disinfectant and sprayed with 70% alcohol before every experiment to prevent contamination. Mycoplama tests were also carried out monthly.

4.4.2 Thawing cells

Before thawing cells, the cell culture medium was pre warmed in a 37^0C water bath for approximately 30 mins. Preserved cells were retrieved from -80^0C or a liquid nitrogen vessel, placed in a sealed plastic bag and suspended in the heated water bath to thaw quickly. Once thawed, the cells were transferred to a sterile 30 mls universal tube and 9 ml pre warmed cell culture medium was added drop-by-drop to achieve a 1:10 dilution and enable the cells to slowly adapt. The cell suspension was then centrifuged (Sigma-Aldrich 2-5; SciQuip centrifuge) at 1600 rpm for 3 mins to pellet the cell and remove the freezing medium (Appendix B). The supernatant was discarded and the pellet re-suspended in a suitable volume of fresh prewarmed culture medium. The cell suspension was transferred into either a T25 ($25cm^3$) or a T75 ($75cm^3$) tissue culture flask depending on the size of the cell pellet. The flask of cells was then placed in a humidified incubator at 37^0C with 5% CO_2.

4.4.3 Passaging cells

Cells were cultured in RPMI cell culture medium and passaged three times a week with a maximum passage of 20. Before passaging and changing flask, cell culture medium was warmed to 37^0C in the water bath for 30 mins to maintain a constant temperature and reduce cellular alterations. Flasks were assessed under an inverted light microscope (Olympus) for confluence and contaminants. Cells were passaged or cryopreserved if the flask is >80% confluent. Flasks were placed in the cell culture hood and depleted media discarded. Three millilitres of warm PBS was used to rinse off remaining media in order for the media not to inactivate the trypsin. Three millilitres of pre-warmed TrypLE Express (#12604-013, Invitrogen), a microbially produced recombinant enzyme used for the dissociation of adherent mammalian cells, was added to the flask and incubated for 3 mins. After incubation, the flask was tapped gently to dislodge cells from the flask's surface and 7mls of medium was added to hinder the action of TrypLe. The cell suspension was transferred into a 30 ml sterile universal tube and centrifuged at 1600 rpm for 3 mins. The supernatant was discarded and the cell pellet was re-suspended in an appropriate volume of medium before splitting between flasks depending on confluency.

4.4.4 Cryopreservation of cells

Freezing medium consisting of 10% Dimethyl sulphide (DMSO) (#D2650, Sigma Aldrich) in RPMI was pre-warmed in a water bath at 37^0C for 30 mins. Cells were dissociated from flask as described in section 4.4.3 and 1 ml of freezing media was added slowly. The cell suspension was transferred into a 1 ml cryovial and stored at -80^0C before transferring to a liquid nitrogen vessel for long term storage.

4.5 Cell count

A Neubauer haemocytometer (a thick glass, microscopic slide) (Figure 4.6) was used to determine cell concentration. Adhered cells were dislodge from the flask as described in section 5.4.3, transferred into a 30 ml sterile universal tube and centrifuged at 1500 rpm for 3mins. Cells were re-suspended in 5-10 mls of media depending on the pellet size and mixed thoroughly. Twenty five micro litres of the homogenous cell suspension was taken and added to 25 µl of sterile filtered 0.4% Trypan blue (Sigma-Aldrich) in a 0.5 ml eppendorf tube. Trypan blue is used to assess cell viability and aids visualization of cells; viable cells have undamaged cell membrane therefore do not absorb the dye while non-viable cells do. The mixed suspension was left for few minutes for cells to absorb the dye.

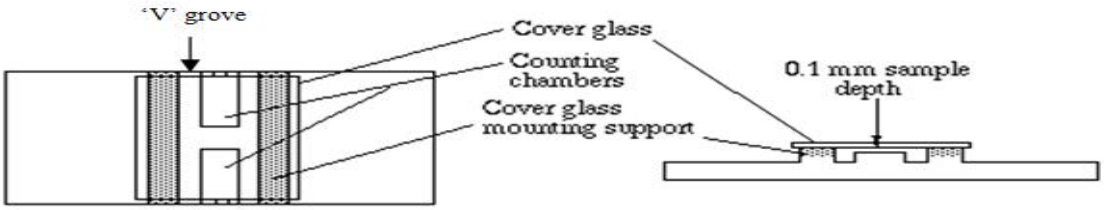


Figure 4.6 A diagrammatic representation of a haemocytometer. The cell sample mixed with trypan blue is pipetted into the V grove and transferred onto the surface of the counting chamber (Figure 4.7) by a capillary action.

Twenty five micro litres of the mixed suspension with trypan blue and cells was carefully pipetted on to the haemocytometer 'V' grove underneath a glass coverslip. The slide was placed under an inverted light microscope and counted with a hand-held cell counter. A specific counting pattern was established to avoid bias, cells were counted in if they overlap the top and right ruling of a 1/25 mm^2 (Figure 4.7).

The surface of the haemocytometer is divided into nine large 1 mm x 1 mm squares and the depth of the chamber is 0.1 mm. Five large squares were counted as numbered in Figure 4.7. Since each large square has a surface area of 1 mm^2 and a depth of 0.1 mm the volume will be 0.l mm^3. The dilution factor of the trypan blue was taken into account and it was important that each large square had 20-50 cells. For example if the total number of cells in the five squares was 120 cells per 0.5 mm^3, then the number of cells will be equal to (120 x 2) ÷ 0.5 = 480 cells/mm^3 equivalent to 480 cells/µl.

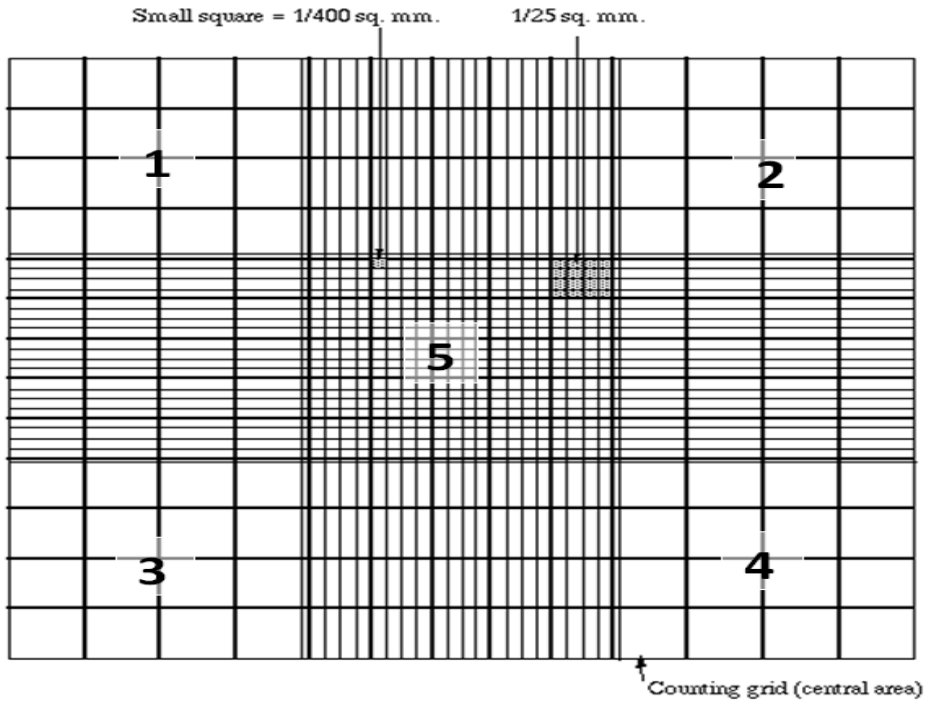


Figure 4.7 An illustration of the counting chamber of a haemocytometer. Cells at the top and right hand rulings of each 1/25 sq.mm were counted as 'in' and those on the bottom and left hand rulings were counted as 'out' to avoid bias.

4.6 Seeding cells onto 96 well plates

Cells were first counted as described in Section 4.5 to determine number of cells per µl of cell suspension. The factors considered when seeding 96 wells plates are: the number of cells needed per wells, the final volume of media in each well and the number of wells to be seeded.

For example: If there was a total of 900 cells/µl and 5000 cells are needed per well in 6 wells, the amount of cell suspension containing 5000 cells was calculated as 5000 ÷ 900 = 5.6 µl. To seed cells in 6 wells, a total of 8 wells are calculated to account for pipetting errors. Therefore, for 8 wells; 5.6 µl x 8 = 44.8 µl of cell suspension was required. The final volume of media loaded in each well was 100 µl hence, a total of 800 µl of media is required for 8 wells. To attain the final volume of media required, the volume of cell suspension is subtracted from 800 (total number of volume for 8 wells) i.e. (800 – 44.8 µl) = 755.2µl. Finally, 44.8 µl of cell suspension was added to 755.2 µl of media and mixed thoroughly to give a homogenous solution. The solution was poured in a reservoir and swirled for additional mixing before transferring into the 96 well plate with a multi-channel pipette.

4.7 Chemosensitivity Assays

Over the years chemosensitivity assays have been used to conduct initial screening of therapeutic agents, tailoring chemotherapeutic agents to individual patients and correlation of *in vitro*, preclinical *in vivo* and clinical response to novel therapeutic agents (Blumenthal et al., 2005). These assays include several clonogenic and non-clonogenic assays. Each of these assays has its advantages and disadvantages therefore since the interest of this project was to measure cell viability in response to several inhibitors a less rigorous non-clonogenic method was employed.

4.7.1 CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay)

This is a colorimetric method to determine the number of viable cells in proliferation or cytotoxic assays based on the reduction of the MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) to a coloured formazan product that is soluble in cell culture medium. The assay is based on the cellular conversion of the tetrazolium salt by NADH produced by dehydrogenase enzymes in metabolically active cells into a formazan product. Formazan absorbs at a wavelength of 492 nm which can be read on a standard spectrophotometric ELISA plate reader. The quantity of formazan product as determined by its absorbance is directly proportional to the number of living cells in culture (Cory et al., 1991). Hence, adjusted absorbance can be used as an indicator of cell number, and correspondingly, changes in absorbance can indicate cell growth, stagnation or death.

4.7.1.1 MTS Assay protocol

Each MTS assay for was performed in a 96 well flat-bottom plate (Figure 4.8) over 5 consecutive days.

Day 1 (Cell plating): Cells were seeded as outlined in Section 4.6 at 1000 cells per well for all cell lines in at least six replicates for each drug concentration. The number of cell per well was decided following cell growth optimizations (appendix C). The cells were suspended in an appropriate volume of media and 100 µl was dispensed on to the labelled central wells of the plates using a multichannel pipette. Hundred micro litres of media without cells was also added to each well along the border of the plate to prevent excessive evaporation from the central wells of the 96 well plates. One hundred micro litres of ddH_20 was added to the first

well on the top left hand corner of the plate labelled A1. It was taken as the blank and subtracted from the absorbance of all other wells (Figure 4.8).

Two plates were labelled; Plate A for cell growth and baseline reading on Day 2 and Plate B for treated cells and day 5 control cell growth reading. Both plates were incubated for 24 hours in a 37^0C incubator with 5% CO_2 to allow the cells adhere to the bottom of the wells.

Day 2-4: Twenty micro litres of MTS reagent (G3582; Promega) was added to wells containing cells and three media-only wells (for background reading) in Plate A. The plate (A) was incubated for three hours and the absorbance read using a microtitre plate reader at 492 nm wavelength (Labsystems Multiskan MS. Thermo Electron Corporation).

Stock solution for the drugs were prepared as detailed in Table 4.1 and further freshly diluted daily to concentrations ranging from 3 nM to 300 µM or 1 mM depending on the inhibitory effect of the drug. Stock solutions were stored at -20^0C or -80^0C depending on manufacturer's recommendation. The media in Plate B was discarded by gently inverting the plate over a virkon tub and gently tapping it on sterile absorbent paper. Corresponding drug concentrations was applied to each six replicate daily for 72 hours. DMSO was used as drug carrier for most drugs at different concentrations but not exceeding 0.1% diluted in media. Media and distilled H_20 were also changed in corresponding wells daily.

For combination experiments a fixed concentration of Inhibitor A was added to varying concentrations of Inhibitors B and vice versa in a reservoir. Both drugs were thoroughly mixed before adding to the wells with a multichannel pipette. In some combination experiments, IC25s were combined and the effect of the combination was compared to the IC25 of the individual single agents.

Day 5: Media containing drug was discarded and fresh media added into all wells in Plate B. This was to eliminate the effect of pigments present in some of the inhibitors. Twenty micro litres of MTS reagent was added to the wells containing cells and three media-only wells. The plate was then incubated for 3 hours in 37^0C with 5% $CO_{2.}$ After the incubation, absorbance reading was taken using a microplate reader at 492 nm wavelength. Each MTS assay experiment was carried out independently three times with six replicates in each experiment.

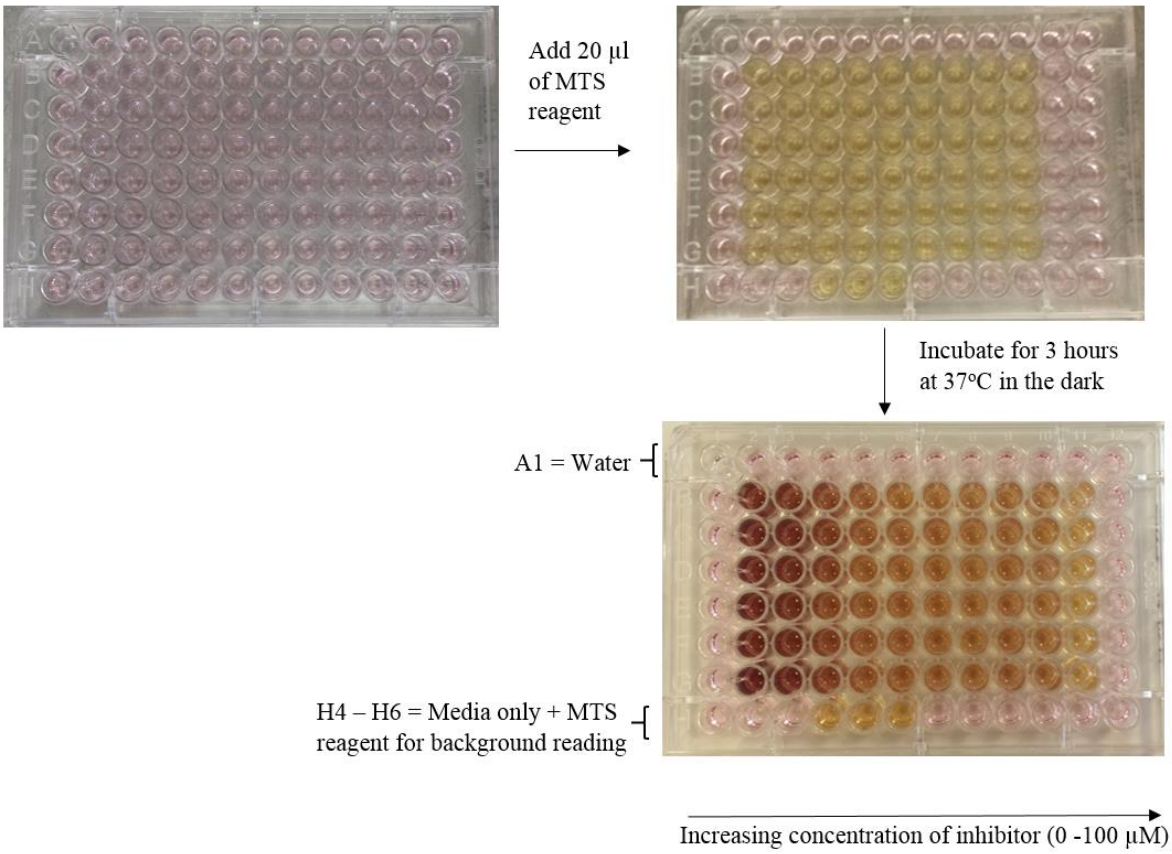


Figure 4.8 A pictorial representation of an MTS assay. Twenty microliters of MTS reagent was added to the wells containing cells and three media-only wells then incubated for 3 hours before reading the absorbance with a spectrophotometer.

4.7.1.2 Calculation of cell proliferation based on absorbance values

The average value from the six replicate wells with cells was obtained as well as the average value of the triplicate blank wells. The average value of the blank wells was subtracted from the average value of treated cells to account for background reading and referred to as adjusted absorbance. Cell growth percentage was calculated as:

$$\left\{ \frac{\text{Adjusted absorbance of day 5 control cells – Adjusted absorbance of day 2 control cell}}{\text{Adjusted absorbance of day 2 control cells}} \right\} X\,100$$

Percentage of viable cells for each drug concentration was calculated as:

$$\left\{ \frac{\text{Adjusted absorbance of day 5 treated cells}}{\text{Adjusted absorbance of day 5 control cells}} \right\} X\,100$$

Control cells are the untreated cells.

Combination index was calculated based on the (Chou & Talalay, 1984) method as:

$$D1/D1_a + D2/D2_a + (D1 \times D2)/ (D1_a \times D2_a)$$

Where D1 = Dose of drug A required to produce 50% cell viability when combined with fixed concentration of drug B

D1a = Dose of drug A alone required to produce 50% cell viability

D2 = Dose of drug B required to produce 50% cell viability when combined with fixed concentration of drug A

D2a = Dose of drug B alone required to produce 50 % cell viability

The result of the combination index was further interpreted as:

< 0.1 very strong synergism
0.1– 0.3 strong synergism
0.3 - 0.7 synergism
0.7 – 0.9 moderate to slight synergism
0.9 – 1.1 nearly additive
1.1 – 1.45 slight to moderate antagonism
1.45 – 3.3 antagonism
> 3.3 strong to very strong antagonism

4.7.1.3 Statistical analysis

Three independent experiments were conducted for each single drug or combination experiments. The relative cell growth for each drug concentration was calculated and uploaded onto Graphpad Prism 6 software where the mean +/- standard deviation values were generated. The X-axis was labelled in molar log scale and the Y-axis represented the percent of viable cells. The IC50 values were generated using a non-linear sigmoidal dose response curve analysis. The data for combination experiment in each cell line was analysed using a paired two-sample t-test to compare single treatment with combinations. A confidence level of $p < 0.05$ was considered statistically significant.

4.8 Immunohistochemistry

This is a process of detecting proteins in cells of a tissue section by exploiting antibodies that binds specifically to the antigen in the biological tissue (Section 3.3.1). The tissue is further subjected to immunoperoxidase staining before visualizing under a microscope.

4.8.1 De-waxing and Rehydration

Formalin fixed paraffin-embedded tissue sections from archival tissue samples described in section 4.1 were cut into 4 µm sections onto Super Frost Plus slides (#00594, Menzel-Glaser, Germany) using a Microtome (Leica RM 2135). The slides were placed in a 37^0C incubator to dry overnight to increase the adhesion of the tissue sections to the surface of the glass slide. The sections were de-waxed by placing the slides in metal racks and incubating in pre warmed Histoclear II (#HS-200, National Diagnostics, Hull) for 10 mins. The slides were further dipped in 2 separate pots of fresh Histoclear II for 10 secs each at room temperature. The slides were rehydrated by passing them in 3 separate pots containing 100% ethanol for 10 seconds in each to ensure all paraffin wax had been removed. Endogenous peroxidase activity was blocked by incubating the slides in 400 ml methanol containing 8 ml fresh hydrogen peroxide (H_20_2) for 20 minutes. The slides were rinsed off with tap water and ready for antigen retrieval.

4.8.2 Epitope retrieval

Formalin-fixed paraffin-embedded (FFPE) tissue sections may require the restoration of immuno- reactivity of antigens which is lost as a result of formalin fixation. The antigen retrieval principle is based on the application of heat to FFPE tissue sections in an aqueous medium. After deparaffinising and rehydrating the tissue sections, the slides were placed in a pressure cooker containing 1500 ml dH20 and 15 mls Antigen Unmasking Solution (H-3300, Vector Laboratories Inc., CA, USA) for 3 minutes at 15 psi. The unmasking solution is a citrate-based solution with pH 6.0. The pressure cooker was cooled under running tap water and the slides were transferred to a pot containing 400 mls of TBS.

4.8.3 Blocking non-specific binding sites

The slides were assembled onto a Sequenza system (Shandon, Basingstoke, UK) using clean cover plates. TBS was applied to the assembled slides to ensure there were no air bubbles and the slides were properly fitted. Non-specific binding sites were blocked using 100 µl of 2.5% normal horse serum from the Vectastain Ready-to-use Quick Kit (#PK-7800, Vector Laboratories Inc, CA, USA); a prediluted blocking serum. The slides were incubated for 10 mins at room temperature and subsequently rinsed with TBS for 5 mins each time.

4.8.4 Blocking endogenous avidin and biotin

Endogenous biotin, biotin receptors and avidin binding sites were blocked to reduce background signals and other non-specific binding. The Avidin-Biotin blocking kit (SP-2001,

Vector Labs) containing an avidin solution and a biotin solution was used. One hundred µl of Avidin D solution was added on to the slides for 15 mins at room temperature and rinsed with TBS for 5 mins. This was followed by the addition of 100 µl of biotin solution to the slides and incubated for 15 minutes and a 5 minutes rinse with TBS.

4.8.5 Incubation with primary and secondary antibody and antibody detection

A 1.5% blocking solution was prepared using normal horse serum (from the R.T.U. Vectastain quick kit) and TBS. The antibody was then diluted in the blocking solution to achieve an optimum antibody concentration. One hundred µl of desired antibody concentration was added to each slide and incubated for 2 hours at room temperature and a humidified environment by putting covers on the sequenza racks (see Table 4.2 for antibodies). For every immunohistochemical experiment a negative control was included with every batch of antibody and incubated with 100 µl of 1.5% blocking solution. Slides were then rinsed twice for 5 mins with TBS.

Slides were incubated with 100 µl of pre-diluted biotinylated universal pan-specific (anti-mouse/rabbit/goat IgG) secondary antibody (included in the Vectastain R.T.U Quick kit; #PK-7800, Vector Laboratories Inc. CA, USA) for 20 minutes at room temperature in a humidified environment. The slides were then washed with TBS for 5 mins and incubated with 100 µl preformed streptavidin/peroxidase complex reagent (included in the Vectastain R.T.U Quick kit; #PK-7800, Vector Laboratories Inc. CA, USA) for 10 mins. The slides were finally rinsed with TBS for 5 mins and transferred into metal slide racks and placed in a pot containing 400 ml TBS.

4.8.6 Antibody visualisation

A 0.02% DAB (3,3-diaminobenzidine tetrahydrochloride, a commonly used chromogen), was prepared (see appendix B) and added to 400 ml TBS containing 0.125% Hydrogen peroxide (H_20_2). The slides were incubated in the solution and closely monitored for the development of brown staining of the tissue sections using a light microscope. The slides were not incubated for more than 30 mins to avoid DAB precipitates. An enzymatic reaction occurs when DAB is added to H_20_2 in the presence of horseradish peroxidase to produce an insoluble brown DAB precipitate and water in the location of the tissue where the antibody has bound to the antigen.

4.8.7 Enhance, counterstain and differentiate

The staining was enhanced by incubating the slides in a copper sulphate solution containing 0.5% copper sulphate in 0.9% sodium chloride solution for 5 minutes. The tissue sections were counterstained in filtered Harris Haematoxylin (#HHS32, Sigma Aldrich), by incubating for 20 seconds and the slides washed under running water to remove excess haematoxylin. Slides were incubated by gently agitating in acid alcohol (70% alcohol and 1% conc. HCL) for 10 seconds to remove excess stain and define nuclei, by differentiating the counterstain. The slides were then rinsed thoroughly in running tap water.

4.8.8 Rehydration, clearing and mounting

The tissue sections were rehydrated by passing the slides through 3 sequential 100% ethanol solutions by gentle agitating for 10 seconds in each. The sections were cleared in Histoclear II (#HS-200, National Diagnostics) by passing them through 3 separate Histoclear solutions and gently agitating for 10 seconds in each. Coverslips were than applied to the slides using histomount (National; Diagnostics, Hull, UK) and allowed to air-dry overnight.

4.8.9 Histological scoring

Dried immunostained slides were assessed using a light microscope and a scoring system was developed. The slides were scored by two independent scorers including a consultant histopathologist (Dr Anne Campbell) specialized in MPM. Each scorer had been trained prior to scoring by the consultant histopathologist. A two-tier system was employed such that protein expression in >25% of the tumour cells were scored as positive (1) while protein expression in <25% of the tumour cells were scored as negative (0). In case of a controversial conclusion on the status of a slide, it was resolved by the conclusion of the consultant histopathologist.

4.8.10 Statistical analysis

Statistical analysis was performed using the SPSS software version 21 and 22 (SPSS, Chicago, USA). Univariate analysis for protein expression and histological subtype was performed using Kaplan Meier curves with log rank analysis. Multivariate analysis was calculated using Cox regression to assess the effect of protein expression on survival, independent of histological subtype, which has been shown to be an independent prognostic variable in MPM.

Table 4.2 List of primary antibodies used within the project

Antibody (company)	Catalogue number	Molecular weight (kDa)	Host species	Blocking agent	Dilution	Application
Anti-alpha tubulin (Abcam)	ab7291	50	Mouse	5% non-fat milk	1:1000	WB (loading control)
Anti-Beta actin (Abcam)	ab8227	40	Rabbit	5% non-fat milk	1:1250	WB (loading control)
5LOX (Abcam)	ab169755	78	Rabbit	Normal Horse serum	1:100	IHC
	ab39347		Rabbit	5% non-fat milk	1:250	WB
12LOX (Abcam)	ab23678	75	Rabbit	Normal Horse serum	1:100	IHC
				5% non-fat milk	1:500	WB
c-MET (C-12) (SantaCruz Biotechnology)	sc-10	145	Rabbit	Normal Horse serum	1:100	IHC
	sc-10		Rabbit	5% non-fat milk	1:200	WB
HER2 (BD Biosciences)	15811A	Clone 3B5	Mouse	Normal Horse serum	1:35	IHC
EGFR (Abcam)	ab2340	185	Rabbit	5% non-fat milk	1:250	WB
VEGFR2 (5B11) (Cell signalling)	2479	230	Rabbit	Normal Horse serum	1:75	IHC
			Rabbit	5% non-fat milk	1:250	WB
p-AKT (R&D)	AF887	60	Rabbit	5% non-fat milk	Not optimised	WB
PTEN (R&D)	MAB847	50	Mouse	5% non-fat milk	Not optimised	WB
ALDHA1 (R&D)	MAB5869	56	Mouse	5% non-fat milk	1:1000	WB
ERK 2 (SantaCruz Biotechnology)	sc 154	44	Mouse	5% non-fat milk	1:500	WB
p-ERK1/2 (SantaCruz Biotechnology)	sc 7383	42/44	Mouse	5% non-fat milk	1:500	WB

Caspase 3 (clone 4-1-18) (Biolegend)	622701	32 cleave d -17	Mouse	5% non-fat milk	Not optimized	WB

4.9 Western Blot Analysis

Western blotting was used to determine the expression of proteins in cell lines.

4.9.1 Protein extraction

Cultured cells (section 4.2) were pelleted and resuspended in 5 mls of sterile cold phosphate buffer saline (PBS) (see Appendix B) three times to ensure the removal of media. The cells were resuspended in 1 ml of PBS and centrifuged at 3000 rpm for 3 mins and the supernatant discarded. Depending on pellet size, cells were resuspended in 500-1000 µl of western blot lysis buffer (see Appendix B) and vortexed for 5 mins before placing on an end-over-end rotator at 4^0C overnight. After 16 hours, samples were centrifuged at 16,000 xg for 15 mins at 4^0C, carefully transferred into new pre-chilled eppendorf polypropylene tubes and cell debris discarded. The samples were then stored at -80^0C.

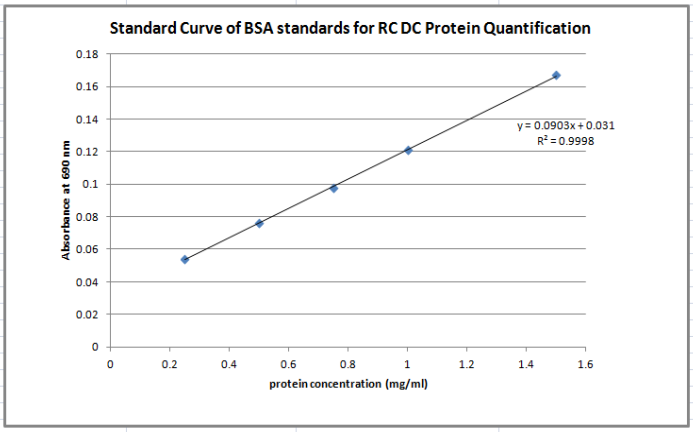
4.9.2 Protein Quantification

The Biorad RC DC protein assay kit (#500-0122, Bio-Rad) was used in the quantification of protein samples. It is a colorimetric assay that determines protein concentration in the presence of detergents such as SDS and reducing agents such as β-mercaptoethanol based on the modification of the Lowry protocol. Proteins react with copper ions under alkaline conditions to form complexes that react with folin phenol reagent (specifically phosphor-molybdic-phosphotungstic reagent in folin phenol) (Lowry et al 1951). The reagent is slowly reduced to molybdenum/tungsten blue and changes colour from yellow to blue. The samples to be quantified are estimated against the standard curve of a selected protein standard solution with known concentrations. The higher the concentration of the protein, the more molybdenum/tungsten blue is produced and the darker the solution which is further estimated by reading the absorbance.

Standard Bovine serum Albumin (BSA) (#A2153-100G) was used as protein standard and five linear range dilutions were made (1.5, 1.0, 0.75, 0.5, and 0.25 mg/ml) from a stock solution of 2 mg/ml diluted in dH_20. Protein lysates were retrieved from the -80^0C freezer and placed on ice to thaw. When thawed the samples were vortexed and diluted with DH_20 in 1:5, 1:10 and 1:20 dilutions with replicates.

Twenty-five microlitres of BSA standards and protein lysates dilutions were placed in 1.5 ml eppendorf tubes and 125 µl of RC Reagent I (#500-0117, Biorad) was added. Samples were vortexed and incubated at room temperature for 1 min. One hundred and twenty-five microlitres of RC Reagent II (#500-0118, Biorad) was added and the samples vortexed for 10 secs. The samples were centrifuged at 14,800 rpm for 5 mins and supernatant discarded by inverting the tubes on absorbent paper to ensure the liquid is completely drained leaving the pellets at the bottom of the tubes. Reagent 'A' was prepared according to manufacturer's instruction by adding 5 µl of DC reagent 'S' to every 250 µl of DC reagent 'A'. One hundred and twenty-seven microlitres of Reagent 'A' was added to each eppendorf tube and vortexed for 5 mins at room temperature to dissolve pellet. One millilitre of DC Reagent B was added to each tube; the samples were inverted to mix and incubated for 15 mins at room temperature. Two hundred microlitres of each protein sample and standard were transferred in replicates on to a 96 well plate by inverse pipetting. Two hundred microlitres of DH_20 was placed in A1 well as blank and the absorbance read using a Multiskan MS plate reader (Thermoelectron, UK) at a wavelength of 690 nm. The variation in the colour of each standard is reflected in the absorbance values increasing with an increase in protein concentration. A protein standard curve of absorbance against standard (BSA) protein quantity was plotted using the values from the automated plate reader. The minimum accepted value for the line of best fit was 0.90. Regression analysis in Microsoft Excel 2007 was used to calculate the concentration of each unknown protein sample and the amount required to load onto a gel (Figure 4.9).

Concentration of standard (mg/ml)	Absorbance of standard (1)	Absorbance of standard (2)	Average Absorbance
0.25	0.054	0.054	0.054
0.5	0.076	0.077	0.0765
0.75	0.098	0.098	0.098
1	0.122	0.12	0.121
1.5	0.167	0.167	0.167



m	0.0903 y = mx + c
c	0.031 x = (y-c)/m

Sample	Absorbance of unknown	Concentration of unknown (x) mg/ml	Final Concentration Corrected for dilution (mg/ml)	Average Concentration (mg/ml)
H2052 lysate A (1:5)	0.1035	0.802879291	4.014396456	
H2052 lysate A (1:10)	0.072	0.454042082	4.540420819	
H2052 lysate A (1:20)	0.0655	0.382059801	7.641196013	5.398671096

Figure 4.9 Standard curve of BSA standards. Showing a representative example of the protein standard curve of absorbance against protein concentration. The concentration of each unknown protein sample is read off this graph using Microsoft excel 2010**.**

4.9.3 One Dimension Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten microlitres of β-mercaptoethanol (#M-7522, Sigma Aldrich) was added to every 190 µl of western blot sample buffer (without phosphatase and protease inhibitors). The β-mercaptoethanol disrupts the structure of proteins by cleaving to its disulphide bonds. Twenty to fifty micrograms of protein samples were diluted with the prepared sample buffer to a volume of 25 µl in 0.5 ml microfuge tubes. The samples were vortexed, placed in a thermocycler (Techne) and heated at 95^0C for 5 mins to denature the proteins. After the heating cycle, the samples were immediately placed to ice to prevent a reverse reaction. The samples were vortexed and centrifuged at 15,000 xg for 30 seconds. A 12 well Precise 12% Tris-HEPES homogenous gel (#25222, Thermo scientific) or a 12 well Precise 4-20% Tris-HEPES gradient gel (#255224, Thermo scientific) was placed vertically in an electrophoresis unit in addition to a blank plastic block. The advantage of using gradient gel is that it allows the separation of a broader range of proteins than those with single acrylamide percentage also known as linear gels. Western blot running buffer (appendix B) was added to the lower and upper compartment of the unit and the bolts were fastened to prevent transfer of the running buffer between the compartments. Ten microliters of molecular weight marker

Precision Plus Protein Western C standards (#161-0376, Bio-Rad) was loaded onto the first well of the gel to determine the weight of probed proteins. Twenty-five microliters containing 20-50 µg of the protein samples were also loaded onto separate wells and 140V of electric current was applied at room temperature for 30-90 minutes depending on the weight of the protein being probed.

4.9.4 Protein transfer to Nitrocellulose Membrane using the iBlot System (IB 1001, Invitrogen)

Nitrocellulose iBlot transfer stacks (#IB3010-01, Invitrogen) containing filter paper, a disposable anode and cathode stack were used. The foil sealing of the anode stack was removed and the anode stack placed on the blotting surface of the iBlot system (Figure 4.10). The gel was carefully placed on the nitrocellulose transfer membrane (0.2 µm pore size) attached to the anode stack and a pre-soaked iBlot filter paper (soaked in DH_20) was placed on the gel. Air bubbles were removed using the blotting roller provided. The cathode stack was placed over the pre-soaked filter paper with the electrode side facing up. A disposable sponge was placed with the electrode aligning with the metal contact on the iBlot system and the lid was closed. A continuous red light indicating a closed circuit was lit and the start/stop button pressed after ensuring the selection of the correct program and time (program 2 for 6 mins) as recommended by the manufacturer. At the end of the transfer, the membrane was immediately removed and the components discarded.

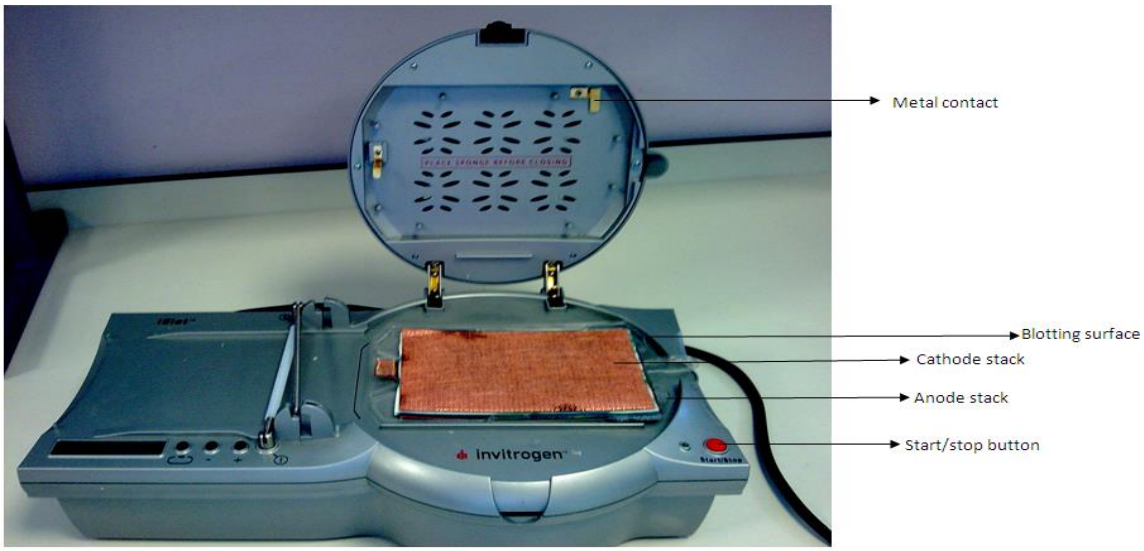


Figure 4.10 An annotated iBlot System. The iblot system is loaded with a commercially obtained transfer stack containing the 0.2 micron nitrocellulose membrane.

4.9.5 Blocking of binding sites on nitrocellulose membrane

Nitrocellulose membrane aids in the immobilization of proteins due to its non-specific ability to bind amino acids. The membrane was blocked to prevent non-specific binding between the membrane and the antibody used for detecting the target protein. Following the transfer of protein onto the nitrocellulose membrane, the membrane was placed in a Nalgene staining box and blocked with 20 mls of blocking solution (either 5% low-fat Marvel milk powder in TBS Tween-20 or 5% Bovine serum albumin) (see appendix B) overnight at 4^0C on an orbital rocker (Stuart Scientific).

4.9.6 Immunoblotting

The optimised volume of primary antibody (see Table 4.2) was added to 10 mls of 5% blocking solution and incubated for two hours at room temperature or overnight at 4^oC on an orbital shaker. The membrane was washed 3-6 times for 5 mins in 10 mls TBS/TWEEN-20 to remove unbound antibody. The required amount of corresponding secondary HRP conjugated antibody (see Table 4.2) was added to 10 mls of blocking solution and added to the membrane. The secondary antibody and membrane were incubated for 1 hr on an orbital rocker. Three microliters of Precision Streptactin-HRP conjugate (#161-380, Bio-Rad) was added to the solution containing the secondary antibody to bind the molecular weight marker for easy detection. The secondary antibody used were horseradish peroxidise (HRP) conjugated and reacts with the chemiluminescent substrate for easy detection with photographic materials.

4.9.7 Protein detection

The Supersignal West Pico Chemiluminescence substrate kit (#34080, Thermo scientific) containing hydrogen peroxide and luminal enhancer was used. The chemiluminescent substrate is a highly sensitive enhanced substrate for detecting horseradish peroxidase (HRP) present in the secondary antibodies and hereby emitting a chemiluminescent signal that can be x-rayed on photographic films. Five millilitres of each solution was added to the membrane in a Nalgene box and agitated gently for 5 mins in the dark. The membrane was placed between two plastic sheets in an X-ray cassette. A piece of CL-Xposure film (#34090, Thermo scientific) was placed on the plastic sheet and the x-ray cassette was closed for a required amount of time depending on the strength of the chemiluminescent signal. After exposure, the film was passed through 250 mls of 25% Kodak developer (#P7042, Sigma-

Aldrich) till bands became visible or approximately 60 seconds. The film was then passed through 250mls of 5% acetic acid and fixed in 250 mls of 2% Kodak fixer (#P7167, Sigma-Aldrich) for 30 seconds in each before washing under running tap water. The film was air dried and evaluated.

4.9.8 Loading controls

Loading controls were used on membrane to ensure equal protein loading in each well of a gel. The loading controls used were alpha-tubulin (~50 kDa) (ab7291, Abcam) or beta-actin (~42 kDa) (ab8227, Abcam). It was also necessary to assess relative protein expression between samples by normalizing the protein bands to the loading control bands.

4.9.9 Densitometry

This is a quantitative measurement of the optical density in photographic films as a result of exposure to light. Developed x-ray films were scanned with a GS-800 calibrated densitometer (Bio-Rad) and assessed with ImageJ software (National Institute of Health). Densitometry was performed by comparing the density of unsaturated bands of the protein of interest and normalizing them against respective loading control bands. The relative density of the bands were obtained and used to calculate adjusted relative density when compared to the positive control band.

4.10 Wound healing assay

This protocol was adapted from (Jonkman et al., 2014). Briefly, Cells were seeded in a T25 flask and incubated to attain 60% confluence. When confluent, a scratch was created in the cell monolayer using a 1 ml pipette tip. The flask was thoroughly rinsed twice with warmed fresh PBS to remove all detached and loosely attached cells. Media containing the appropriate drug either as a single agent or in combination was then added to the flask. Cell migration into the wound was monitored over time and 100x images were taken with an Olympus digital camera (c-5060) mounted onto an Olympus CK2 inverted microscope at 0 hours, 24 hours and 48 hours. Each experiment was carried out three times. The width of each wound was measured at the same position on all images, the average (of 3 points) was taken and the relative percentage of wound closure at 24 hours and 48 hours with respect to 0 hours was calculated.

CHAPTER FIVE

EXPRESSION OF LIPOXYGENASES (LOXs) AND THE EFFECT OF COX-2 and LOX INHIBITORS ON MESOTHELIOMA CELL LINES

Chapter 5 Expression of LOXs and the effect of COX-2 and LOX inhibitors on mesothelioma cell lines

5.1 Arachidonic acid metabolites in MPM

As previously mentioned in Section 2.4, arachidonic acid is metabolised by lipoxygenase (LOX) enzymes to form leukotrienes (LTs) and by cyclooxygenase (COX) enzymes to form prostanoids, including prostaglandin E_2 (PGE_2) which has been implicated in inflammation and carcinogenesis (Clària & Romano, 2005; Goossens et al., 2007; Greenhough et al., 2009; Schneider & Pozzi, 2011; Salvado et al., 2012) (Figure 5.1). COX exists in two forms, COX-1 and COX-2. COX-2 is overexpressed in a wide variety of tumours and this feature has been correlated with the malignant properties of cancers. Inhibition of COX-2 has been reported to reverse malignant behaviour such as antiapoptosis, angiogenesis and invasion (Fu et al., 2004; Cerella et al., 2011) and epidemiological evidence suggests that regular use of COX-2 inhibitors may reduce the risk of several cancers (Chan et al., 2005).

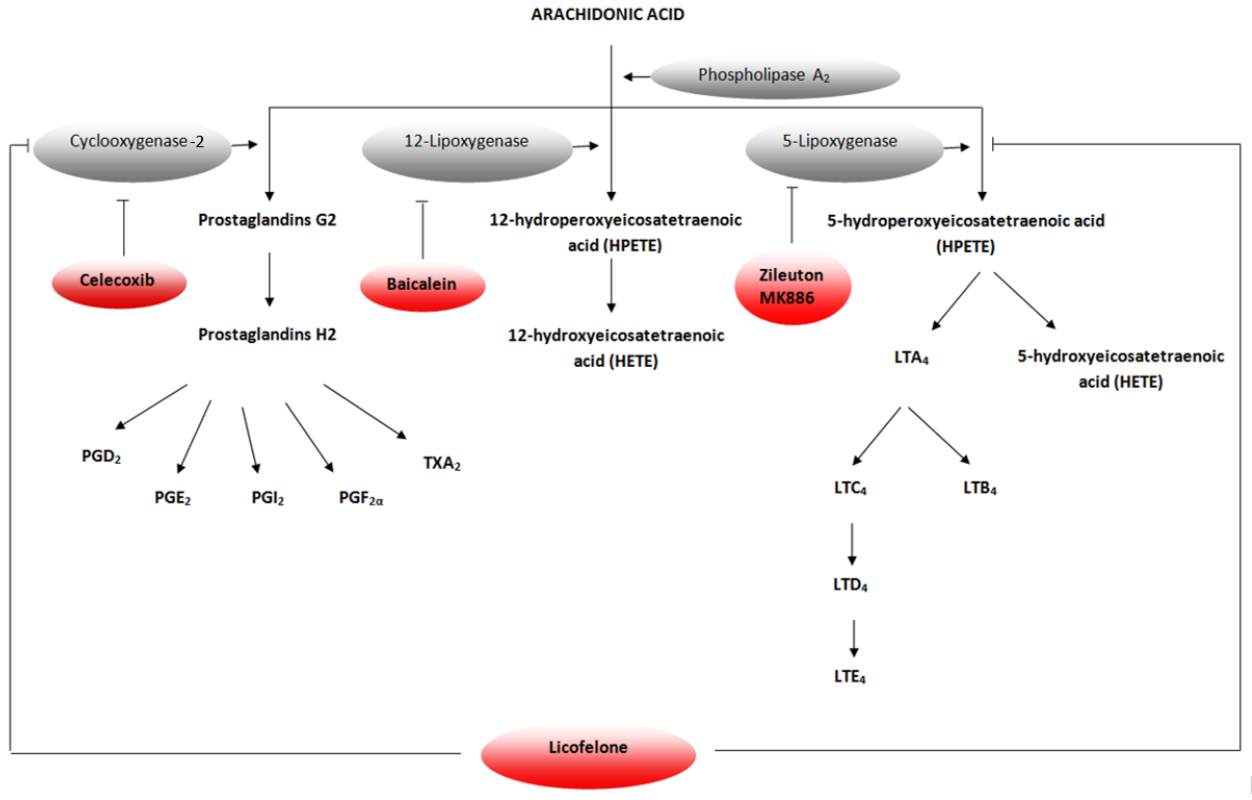


Figure 5.1 Schematic representation of the arachidonic acid pathways and inhibitors. Arachidonic acid in the cytosol is released by the phospholipase A2 enzyme and metabolised by cyclooxygenases and lipoxygenases to yield eicosanoids. The inhibitors used in this chapter for COX-2, 12-LOX and 5-LOX are shown in red. Adapted from (Wang & DuBois, 2010).

Immunohistochemical studies have shown that COX-2 protein is overexpressed in 59 to 100% of mesothelioma tumour samples (Table 5.1). Edwards et al (2002) reported COX-2 expression as a strong prognostic factor and that it contributes independently to other clinical and histopathologic factors to determine poor survival. Mineo et al (2010) demonstrated the combination of high COX-2, low p21 and low p27 expression as a negative prognostic indicator in MPM.

Table 5.1 COX-2 expression in MPM by immunohistochemistry

Author	No of cases	Histological subtype	Antibody used	COX-2 expression	Prognostic status
(Marrogi et al., 2000)	30	Epithelial – 23 Biphasic – 4 Sarcomatoid - 3	C22420; (Transduction Laboratories, Lexington, KY)	30/30 (100%)	Not reported
(Edwards et al., 2002)	18	Not reported	SC-1745 (Santa Cruz)	18/18 (100%)	Yes – poor survival p=0.0005
(Baldi, 2004)	29	Epithelial – 16 Biphasic – 7 Sarcomatoid - 6	SC-1745 (Santa Cruz)	19/29 (65.5%)	Yes – poor survival P=0.01
(O'Kane et al., 2005)	86	Epithelial – 42; Biphasic – 28; Sarcomatoid - 16	COX-2, Clone 33 (BD Biosciences, CA, USA)	51/86 (59%)	Yes – good survival p=0.002

NS398, a COX-2 inhibitor has been shown to have a time and dose dependent antiproliferative effect in the mesothelioma cell line VAMT-1 when compared to a non-malignant mesothelial isolate (CHTN18650) (Marrogi et al., 2000). Celecoxib was shown to significantly reduce the proliferation of primary mesothelioma cells *in vitro* (Catalano et al., 2004). The Cawkwell research group have also previously shown using immunohistochemistry (IHC) that COX-2 is overexpressed in MPM and that the specific COX-2 inhibitor DuP-697 can potentiate the *in vitro* cytotoxic effects of pemetrexed in MPM cell lines (O'Kane et al., 2005; O'Kane et al., 2010). In mice, oral administration of Rofecoxib (COX-2 inhibitor) significantly reduced the growth of tumours in mesothelioma bearing mouse when combined with an adenovirus expressing murine interferon-beta

(Ad.IFN-β) (DeLong et al., 2003). Combination of Rofecoxib with Gefitinib (an EGFR inhibitor) showed a synergistic effect in the mesothelioma cell line 1st-Mes-2 (Stoppoloni et al., 2010). Romano et al (2001) are the only authors that have demonstrated the expression of 5-LOX and 12-LOX in human mesothelial cells and that metabolically active 5-LOX is selectively overexpressed in mesothelioma cells. The RT-PCR analysis showed that 5-LOX mRNA was present in mesothelioma cells but not in the normal mesothelial cells. AA-861, a specific 5-LOX inhibitor and NDGA, a general LOX inhibitor significantly inhibited mesothelioma cell proliferation in a time dependent manner (Romano et al., 2001). So far, there is no published data demonstrating the expression of 5-LOX and 12-LOX in tumour samples. COX-2, 5-LOX and 12-LOX appears to have similar mechanisms in the regulation of cell viability but utilize different signalling pathways. It has been suggested that arachidonic acid might be shunted from one pathway to the other when a particular pathway is being inhibited therefore suppressing the inhibitory effect of a single agent (Ye et al., 2005; Ganesh et al., 2012; Park et al., 2012). Currently there are no published studies that demonstrates the anti-tumour effect of combining COX-2 and LOX inhibitors. There is also no study that has reported the expression of lipoxygenases in MPM and this needs further evaluation.

Aims:

- To assess the expression of 5-LOX protein and its prognostic relevance in MPM tissue samples using immunohistochemistry
- To evaluate the effect of the co-expression of 5-LOX, COX-2 and LOX-12 proteins on survival
- To assess the expression of 5-LOX and 12-LOX in MPM cell lines using western blotting
- To assess the antiproliferative effect of Celecoxib (COX-2), MK886 (FLAP), Zileuton (5-LOX), Baicalein (12-LOX) and Licofelone (COX/5-LOX) in MPM cell lines
- To assess the antiproliferative effect of combining Celecoxib and Baicalein in MPM cell lines

5.2 Materials and Methods

5.2.1 Archival tissue samples

Research Ethics Committee approval was granted for the study (ref 11/00/212). Archival MPM tissue samples were obtained from patients diagnosed between 1992 and 2000 at Hull Royal Infirmary, UK. The majority of patients were male (76/83; 92%). The original diagnostic histology slides were reviewed by a Consultant histopathologist specialising in MPM (AC) and clinicopathological details for all samples were available. The samples selected for use in this chapter were 44/83 (53%) epithelial, 25/83 (30%) biphasic (mixed) and 14/83 (17%) sarcomatoid histological subtypes. In order to investigate benign pleural tissue, 8 archival samples were obtained which were derived from male patients who had undergone thoracoscopic intervention to prevent recurrence of spontaneous pneumothorax. These 8 samples were classified histologically by the histopathologist into non-reactive (n=2), mildly reactive (n=2) and very reactive (n=4) based on the reactivity of the mesothelial cells.

5.2.1 Histological subtypes

Histological subtype is a known significant prognostic indicator in mesothelioma therefore our entire cohort of samples was assessed to ensure it was truly representative of mesothelioma (Section 4.1). Kaplan Meier survival analysis was used to assess the statistical significance differences between the subtypes (Figure 5.2).

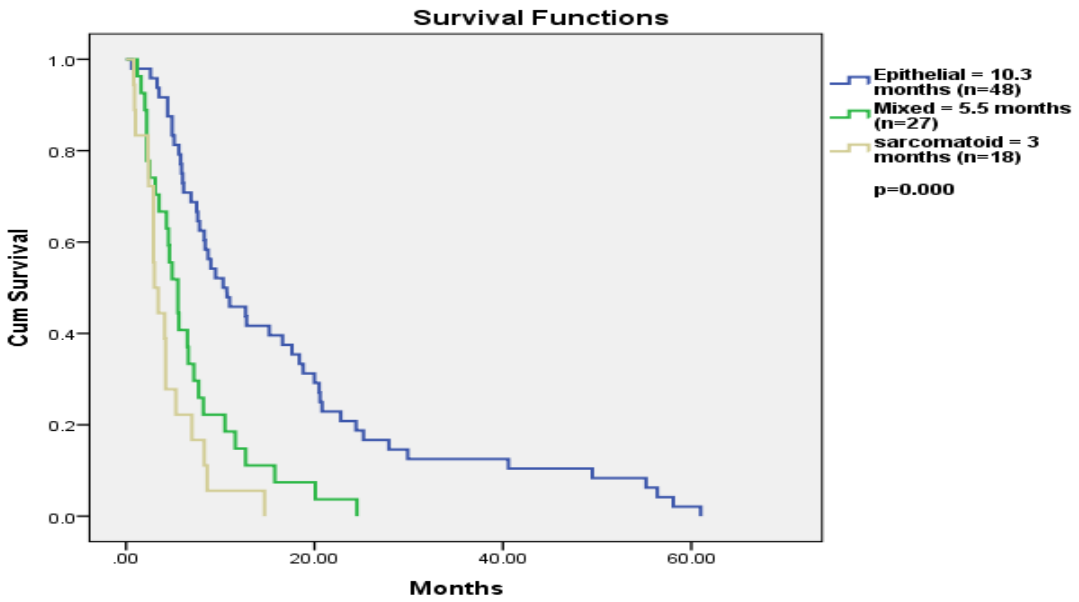


Figure 5.2 Kaplan Meier survival curves demonstrating improved survival in the epithelial subtypes ($p<0.01$). Median survival times were 10.3, 5.5 and 3 months in epithelial (blue line), biphasic/mixed (green line) and sarcomatoid (yellow line) subtypes respectively.

In some instances histological subtypes were stratified into only two categories (epithelial and non-epithelial) since there was no significant difference in the survival times between the biphasic/mixed and sarcomatoid subtypes as demonstrated by log rank analysis (Figure 5.3). The number of samples in the biphasic/mixed and sarcomatoid subtypes is relatively low hence combining them might improve statistical power. All analyses were carried out when the data is split into two histological categories and also three histological categories to ensure that there is no bias in the results obtained.

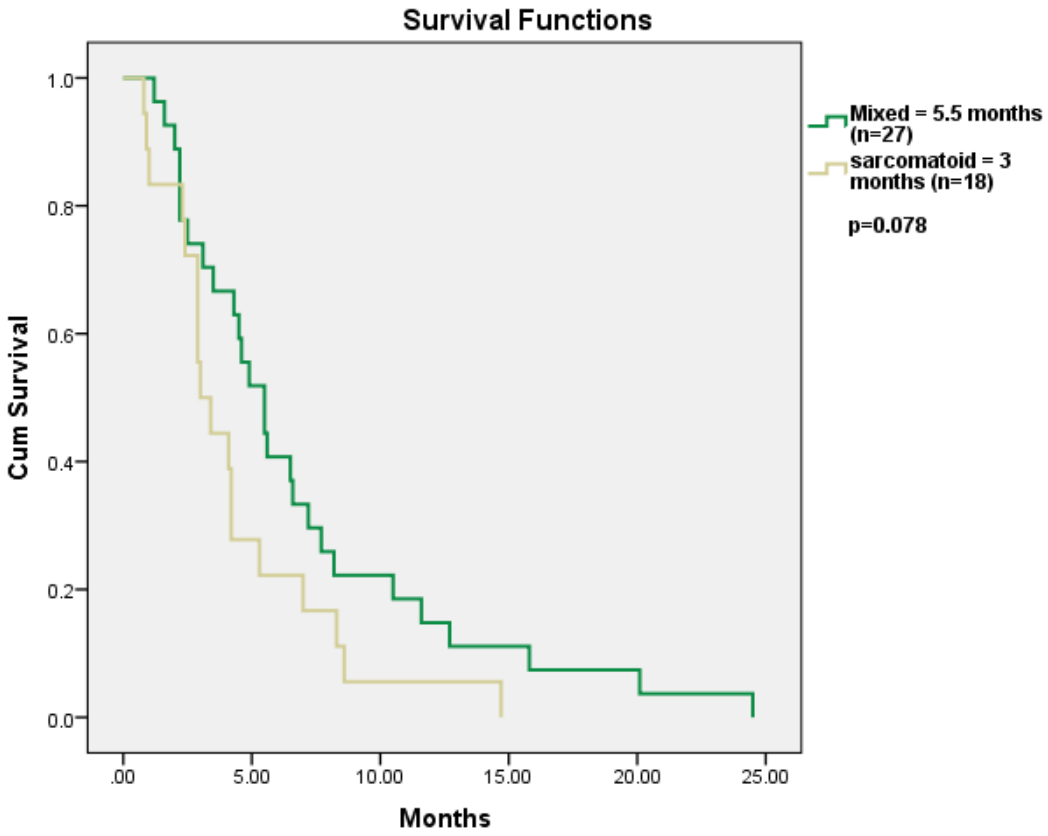


Figure 5.3 Kaplan Meier survival curves showing that there is no significant difference in survival times between the biphasic/mixed and sarcomatoid subtypes. Survival time for the mixed subtype was 5.5 months (green line). Survival time for the sarcomatoid subtype was 3 months (yellow line). The log rank test for comparison showed that there was no significant difference in the survival times of both subtypes p=0.078.

5.2.2 Immunohistochemistry

The 83 archival MPM tissue samples and 8 benign pleural tissue samples were analysed by IHC as previously described in section 5.8. In brief, endogenous peroxidase enzymes were blocked and antigens were heat-retrieved by boiling under pressure for 3 minutes at 15 psi in 1:100 Antigen Unmasking Solution (H-3300, Vector Laboratories Inc.) within a stainless steel pressure cooker. Non-specific staining was blocked using normal horse serum (#PK-7800, Vector Laboratories Inc.) and endogenous biotin and avidin binding sites were also blocked (#SP-2001, Vector Laboratories Inc.). Incubations with anti 5-LOX or anti 12-LOX antibodies were performed using a dilution of 1:100 for 2 hours (Table 5.2). The 12-LOX experiment was carried out in collaboration with Dr Agarwal. Negative (antibody-omitted) control and positive control slides, which consisted of archival colorectal cancer tissue, were

included in each batch. Antibody localisation was detected and visualised using a streptavidin/peroxidase method (#PK-7800, Vector Laboratories Inc.) with DAB as chromogen. Slides were counterstained with haematoxylin and independently reviewed by a 3 observers, including a Consultant histopathologist (AC) specialising in MPM. Discordant scores were reviewed in open discussion. For MPM samples, "positive" protein expression was recorded if there was moderately strong staining in more than 25% of the malignant cells and "negative" protein expression was recorded if no, or only weak, staining was seen or if staining was seen in less than 25% of the malignant cells.

Table 5.2 5-LOX and 12-LOX antibodies used in this chapter

Antibody (company)	**Catalogue number**	**Molecular weight (kDa)**	**Host species**	**Blocking agent**	**Dilution**	**Application**
Anti-alpha tubulin (Abcam)	ab7291	50	Mouse	5% non-fat milk	1:1000	WB (loading control)
5-LOX (Abcam)	ab169755	78	Rabbit	Normal Horse serum	1:100 (2 hours)	IHC
	ab39347	78	Rabbit	5% non-fat milk	1:250	WB
12-LOX (Abcam)	ab23678	75	Rabbit	Normal Horse serum	1:100 (2 hours)	IHC
	ab23678	75	Rabbit	5% non-fat milk	1:500	WB

5.2.3 Statistical analysis

Statistical analysis was performed using SPSS software version 22.0 (SPSS, Chicago, USA). Univariate analysis was carried out for 5-LOX and 12-LOX expression using Kaplan Meier survival curves with log rank analysis. Multivariate analysis was calculated using Cox regression analysis to take into account the histological subtypes which are known to be an independent prognostic variable in MPM (O'Kane et al., 2005).

5.2.4 Cell lines

The MPM cell lines NCI-H2452 (epithelial), NCI-H2052 (sarcomatoid) and MSTO-211H (biphasic/mixed) were obtained from the American Type Culture Collection (ATCC). In addition, the non-small cell lung cancer (NSCLC) cell line A549 was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) foetal bovine serum, 100 U/ml penicillin and 100 µg streptomycin in a humidified incubator with 5% CO_2 at 37^{o}C as discussed in section 5.4.1. Cell lines were passaged at 70-80% confluence and regularly checked for mycoplasma contamination.

5.2.5 Cell lysis and western blot

Cells were grown to 70-80% confluence then lysed in Laemmli buffer (65 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue) with the addition of 5% β-mercaptoethanol and 1% protease inhibitor mix (#80-6501-23, Amersham Biosciences). Protein lysates were quantified using the RCDC protein assay (# 500-0122, Biorad) and 50 µg of protein was analysed per lane on a 12% acrylamide gel (#25222, Pierce) under reducing conditions and transferred to a nitrocellulose membrane using a semi-dry iBlot system (Life Technologies). Membranes were blocked in 5% non-fat milk before samples were probed with antibodies as detailed in Table 5.2. To serve as a loading control, the anti α-tubulin antibody (#ab7291, Abcam) was applied at 1:3000 for 2 hours. Visualisation of protein bands was achieved using the SuperSignal West Pico Chemiluminescent Substrate kit (#34078, Pierce).

5.2.6 MTS assay

Commercially available inhibitors were purchased as detailed in Table 5.3 Licofelone (ML3000) was provided as a gift by Professor Stefan Laufer (Department of Pharmaceutical Chemistry, Eberhard Karls University, Tübingen, Germany) (Table 5.3). Licofelone is a dual COX/5-LOX inhibitor (Albrecht et al., 2008). All stock drug solutions were prepared in DMSO and stored at -20^{o}C for further use. Drugs were diluted in fresh media prior to each experiment. Cells were plated in 96-well plates at 1 x 10^3 cells/well and grown overnight in supplemented media as above. After 24 hours, cells were treated at concentrations of 0 – 300 µM in replicates of 6 and cell viability was measured after 72 hours using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (#G3581, Promega) as detailed in Section

4.7.1. Following the 3 hour labelling of metabolically active cells with MTS, results were measured at 492 nm using an absorbance plate reader (Multiskan FC Microplate photometer, Thermo Scientific). Values were normalised to untreated control cells in order to generate dose response curves. At least 3 independent experiments were carried out for each drug analysis before IC50 values were calculated using Graphpad Prism 5.0 software. Student's paired t test was used to assess the differences observed between single agent treatment and combinations ($p < 0.05$). To assess drug synergy between celecoxib and baicalein on cell growth inhibition of the cell lines, a combination index (CI) was calculated using data obtained from the MTS assay as described in section 5.7.1. Concentration-effect values were generated using the following CI equation: CI = (D1)/(D1a) + (D2)/(D2a) + (D1 x D2)/(D1a x D2a), where D1 and D2 are the concentrations of baicalein and celecoxib respectively that exhibited a determined effect when applied simultaneously to the cells and D1a and D2a are the concentrations of these drugs that exhibited the same determined effect when used as single agents. The CI values indicate a synergistic effect when <1, an additive effect when equal to 1 and an antagonistic effect when >1 (Chou & Talalay, 1984).

Table 5.3 COX-2 and lipoxygenase Inhibitors used and their clinically relevant doses

Inhibitor	Target	Cmax (µM)	Supplier	Catalogue number	Reference for cmax
Celecoxib	COX-2	3.0 - 6.2 µM	Tocris	3786	(Davies et al., 2000)
Licofelone	Dual COX/ 5-LOX	2.9 – 6.2 µM	Prof Laufer (Merck)	N/A	(Ding & Cicuttini, 2003)
Baicalein	12 LOX	Not yet achieved 400 nM has been well tolerated.	Tocris	1761	(Li et al., 2014)
MK886	5LOX activating protein	4.0 – 8.7 µM	Tocris	1311	(Depre et al., 1993)
Zileuton	5 LOX	14.1 – 22.81 µM	Tocris	3308	(Awni et al., 1995)

5.3 Results

5.3.1 Immunohistochemistry

5.3.1.1 Expression of 12-LOX protein

Positive 12-LOX protein expression was recorded in 69/83 (83%) of MPM tissue samples (Table 5.4). The total number of cases are different for the individual protein expression data due to the exemption of unscoreable tissue samples and some of the blocks that had run out of tissue. The COX-2 data for the same cases has been previously published (O'Kane et al., 2005). All 8 benign pleural samples exhibited strong nuclear and cytoplasmic staining patterns such that the mesothelial cells were stained in both reactive and non-reactive pleural tissues (Table 5.5; Figure 5.4 A & B). In the MPM samples the expression of the 12-LOX protein was again predominantly found in the nucleus and cytoplasm of the malignant cells, with varying intensity (Figure 5.4 C & D). The expression of 12-LOX was not associated with survival ($p = 0.455$).

Table 5.4 Clinicopathological variables of the MPM cohort assessed for 5-LOX, 12-LOX and COX-2 protein expression

Characteristics	**12-LOX Number of cases (%)**	**5-LOX Number of cases (%)**	**COX-2 Number of cases (%)**
Total	83 (100)	77 (100)	93 (100)
Age-median (range)	68 (42-94)	67 (42-88)	68 (42-94)
≤64	32 (34)	29 (38)	32 (34)
>64	61(66)	48 (62)	61(66)
Median survival (months)	7.2	7	6.9
Gender			
Female	7 (8.4)	7 (9)	7 (8)
Male	76 (91.6)	70 (91)	86 (92.5)
Histology			
Epithelioid	44 (53)	42 (54.5)	48 (51.6)
Biphasic/Mixed	25 (30.1)	22 (28.6)	27 (29)
Sarcomatoid	14 (16.9)	13 (16.9)	18 (19.4)
IHC score			
0 - negative	14 (16.9)	21 (27.3)	35 (37.6)
1 - positive	69 (83.1)	56 (72.7)	58 (62.4)
Reference			(O'Kane et al., 2005)

Table 5.5 Immunohistochemical analysis of 5-LOX and 12 LOX expression in benign pleura samples

Sample reactivity	Total	5-LOX Expression	12-LOX Expression
Non-reactive	2	0 (0)	2 (100%)
Mildly reactive	2	2 (100%)	2 (100%)
Very reactive	4	4 (100%)	4 (100%)

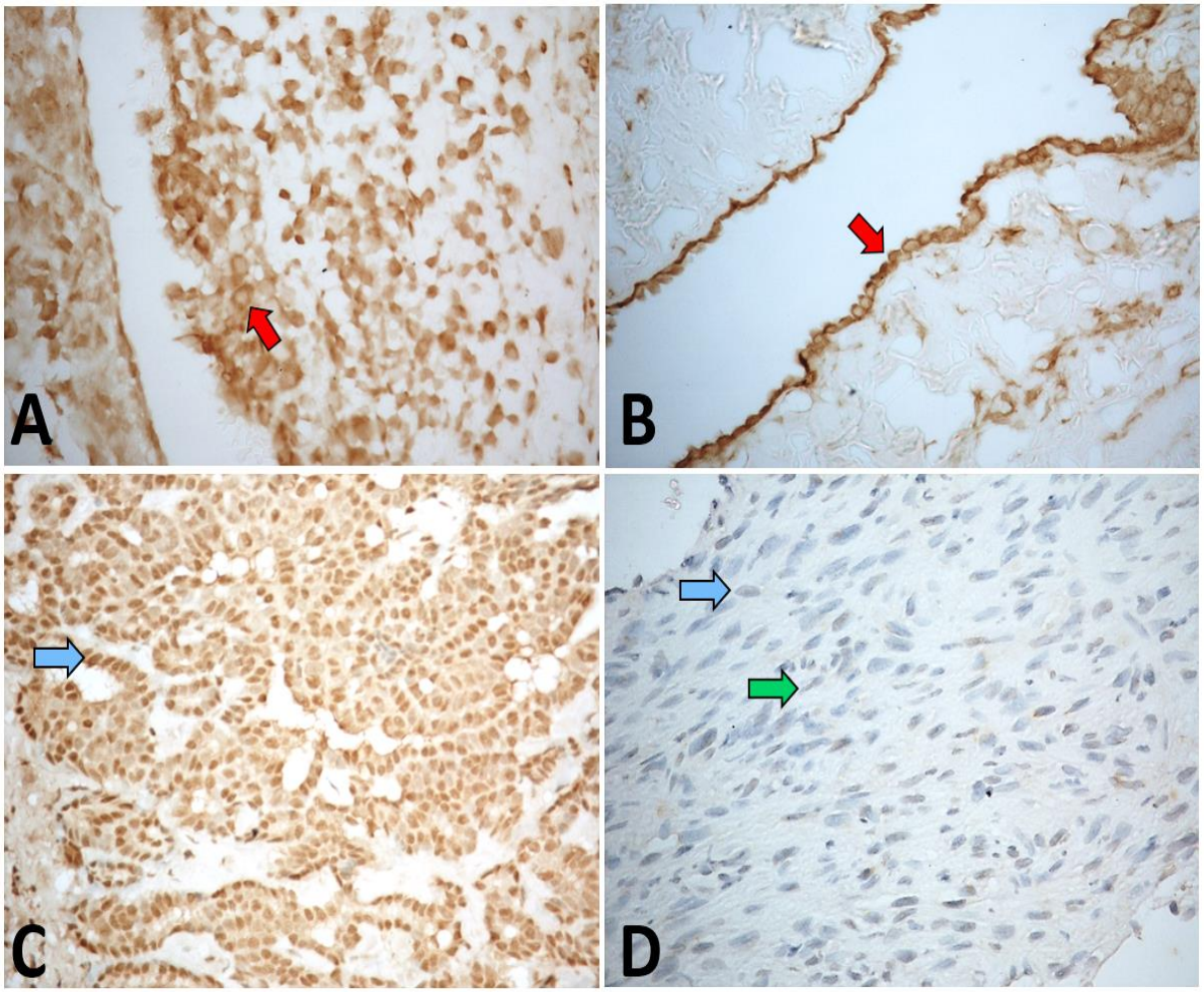


Figure 5.4 Expression of 12-LOX protein demonstrated by IHC. A: 12-LOX expression in a reactive pleural tissue sample. Red arrow shows the mesothelial cells infiltrating into the connective tissue (x 400). B: 12-LOX expression in a non-reactive tissue sample. A well-arranged thin layer of mesothelial cells (red arrows) are seen overlying the connective tissue (x400) similar to the H&E stain shown in Figure 1.7. C: Epithelial subtype of MPM demonstrating strong positive expression. Stained epithelial cells are indicated by the blue arrow (x 400). D: Biphasic/Mixed subtype of MPM demonstrating negative expression. The blue arrow shows negative epithelial cells and the green shows negative spindle cells within the same tissue (x 400).

5.3.1.2 Expression of 5-LOX protein

The 8 benign pleural samples exhibited differential expression of the 5-LOX protein based on their reactive status (Figure 5.5 A & B). Where staining was observed, 5-LOX protein was localised to the cytoplasm and nucleus. Few inflammatory cells were seen in the 2 non-reactive pleural samples and the mesothelial cells exhibited no expression of 5-LOX protein. Mildly reactive pleural samples (n = 2) exhibited weak staining for 5-LOX in the mesothelial cells and very reactive pleural samples (n = 4) exhibited strong positive staining for 5-LOX in the mesothelial cells (Table 5.5). Positive staining of lymphocytes and other inflammatory cells served as an internal positive control (Figure 5.5 A & B). Of the 83 MPM tissue samples, 77 samples were successfully scored. The immunohistochemical staining revealed nuclear and cytoplasmic expression of the 5-LOX protein in malignant cells with varying intensity (Figure 5.5 C & D). Positive 5-LOX expression was observed in 56/77 (73%) of MPM tissue samples (Table 5.6). Overall, the expression of 5-LOX was not associated with survival ($p = 0.640$), however when considering only the sarcomatoid subtype (n = 13) the positive expression of 5-LOX was significantly associated with improved survival (median survival 4.2 months *versus* 1 month in 5-LOX negative cases; $p = 0.028$) (Figure 5.6).

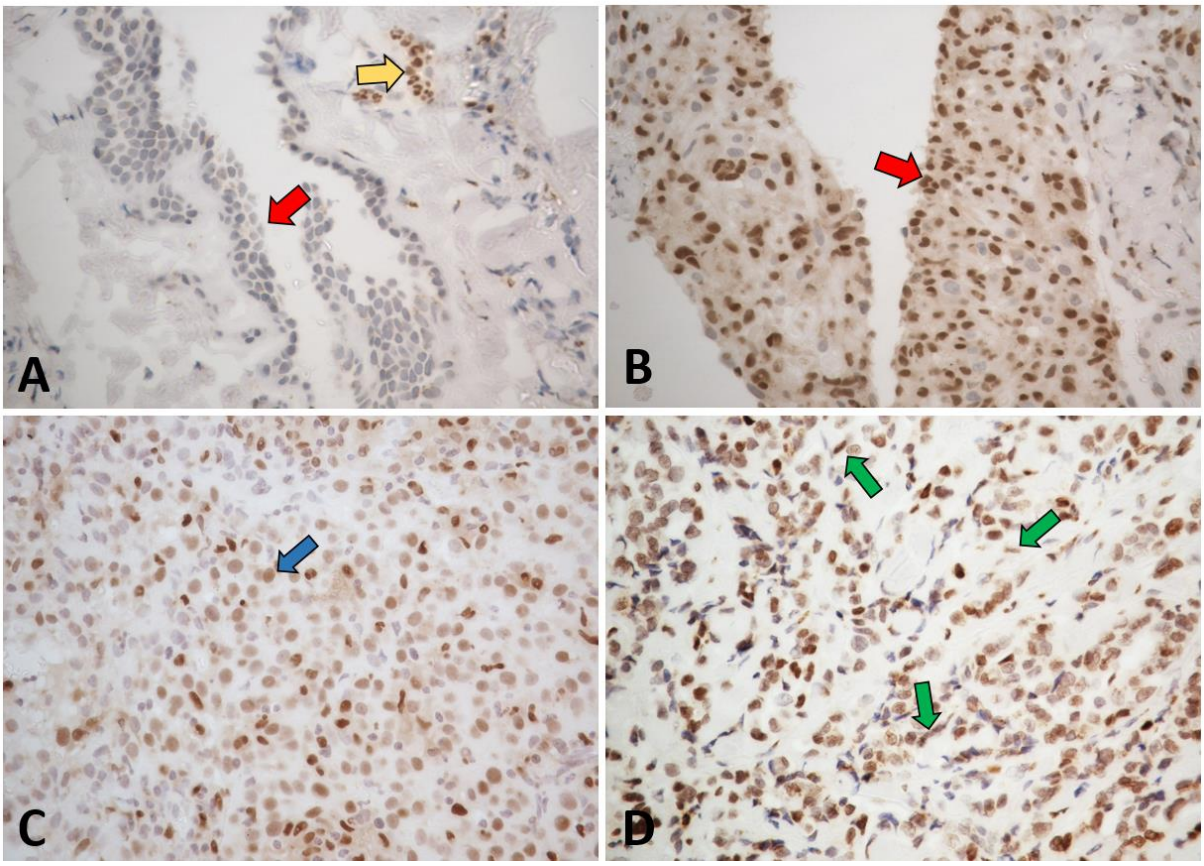


Figure 5.5 Expression of 5-LOX protein. A: Benign pleural tissue with non-reactive mesothelial cells, which can be seen as an organised strip of mesothelial cells on the surface (red arrows), exhibiting no expression of 5-LOX protein. Inflammatory cells (yellow arrow) can be seen in the connective tissue (x 400). B: Benign pleural tissue with reactive mesothelial cells demonstrating positive expression for 5-LOX. The mesothelial cells have lost their well-arranged pattern and can be seen within the connective tissue (x 400). E: Epithelial subtype of MPM demonstrating positive 5-LOX expression. The epithelial cells are shown by the blue arrow (x 400). F: Sarcomatoid subtype of MPM demonstrating positive 5-LOX expression. The spindle cells are highlighted by the green arrows (x 400).

Table 5.6 Immunohistochemical analysis of 5-LOX expression categorised by histological subtype.

Samples	**Total**	**5-LOX expression score**	
		Positive (1)	**Negative (0)**
All samples	77	56 (73%)	21 (27%)
Epithelial	42	30 (71.4%)	12 (28.6%)
Biphasic	22	18 (81.8%)	4 (18.2%)
Sarcomatoid	13	8 (62%)	5 (38%)

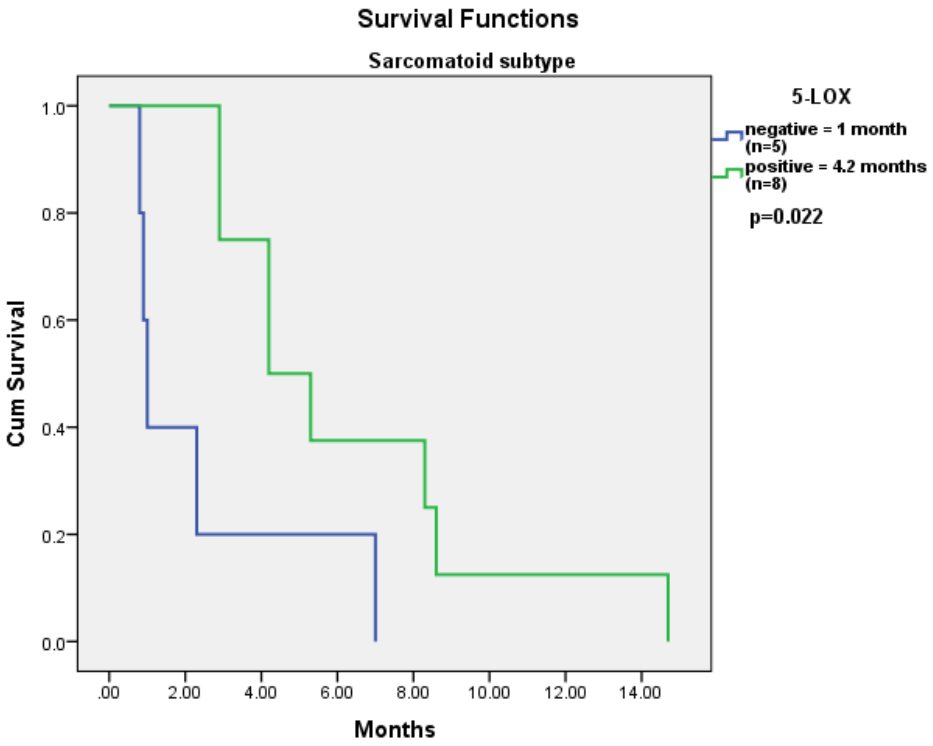


Figure 5.6 Survival analysis for 5-LOX protein expression in the sarcomatoid subtype (n=13. Kaplan Meier plot showing univariate analysis of 5-LOX expression (p = 0.028, log rank). The median survival was 4.9 months in 5-LOX positive cases (green line) *versus* 1 month in 5-LOX negative cases (blue line).

5.3.1.3 Correlations between 5-LOX, 12-LOX and COX-2 expression and clinicopathological variables.

Co-expression of 5-LOX with 12-LOX was seen in 46/78 (58%) of the MPM samples but this was neither statistically significant nor associated with survival. The Cawkwell group has previously published the COX-2 protein expression data for this cohort (O'Kane et al., 2005) (Table 5.4). A total of 41/77 (53%) of samples demonstrated co-expression of COX-2 with 5-LOX proteins and this status was significantly associated with improved survival when compared with cases which were negative for both proteins in univariate analysis (median survival 8.7 months versus 2.2 months; p = 0.011) (Figure 5.7).

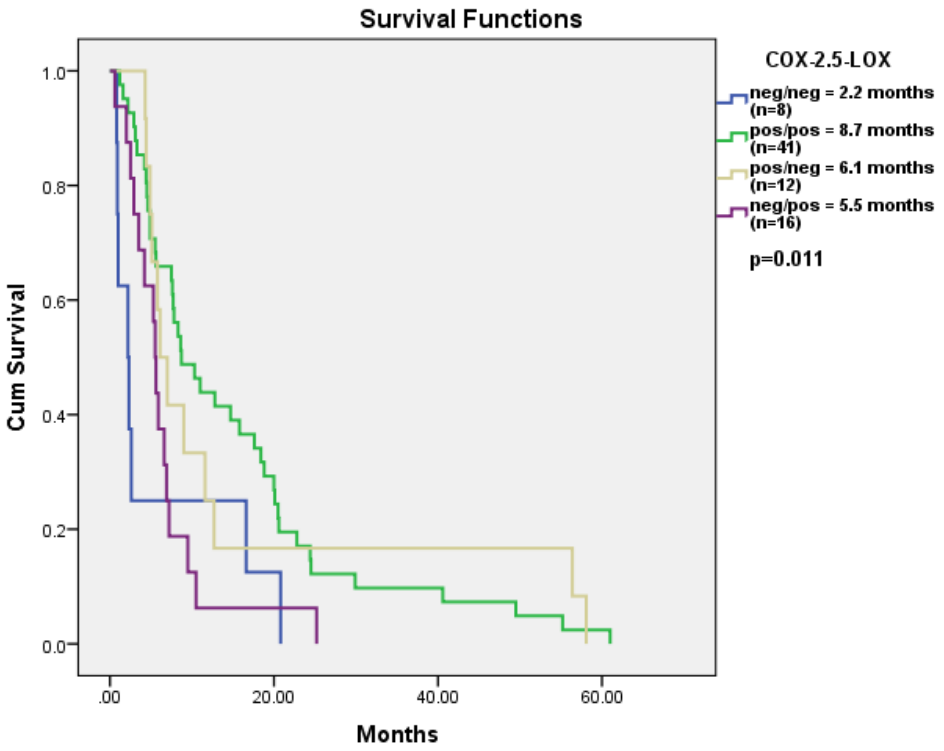


Figure 5.7 Kaplan Meier survival analysis for the co-expression of COX-2 and 5-LOX proteins. Median survival was 8.7 months in cases demonstrating co-expression of COX-2/5-LOX (green line) *versus* 2.2 months in cases demonstrating negative expression of both proteins (blue line) (p = 0.011, log rank).

Co-expression of COX-2 with 12-LOX was seen in 47/83 (56%) of the samples. In univariate analysis cases demonstrating a COX-2 positive /12-LOX negative status or co-expression of COX-2 with 12-LOX were associated with longer survival (Figure 5.8). Only LOX-12 showed significant positive correlation with a clinicopathological variable, age (p = 0.04, fisher's exact test).

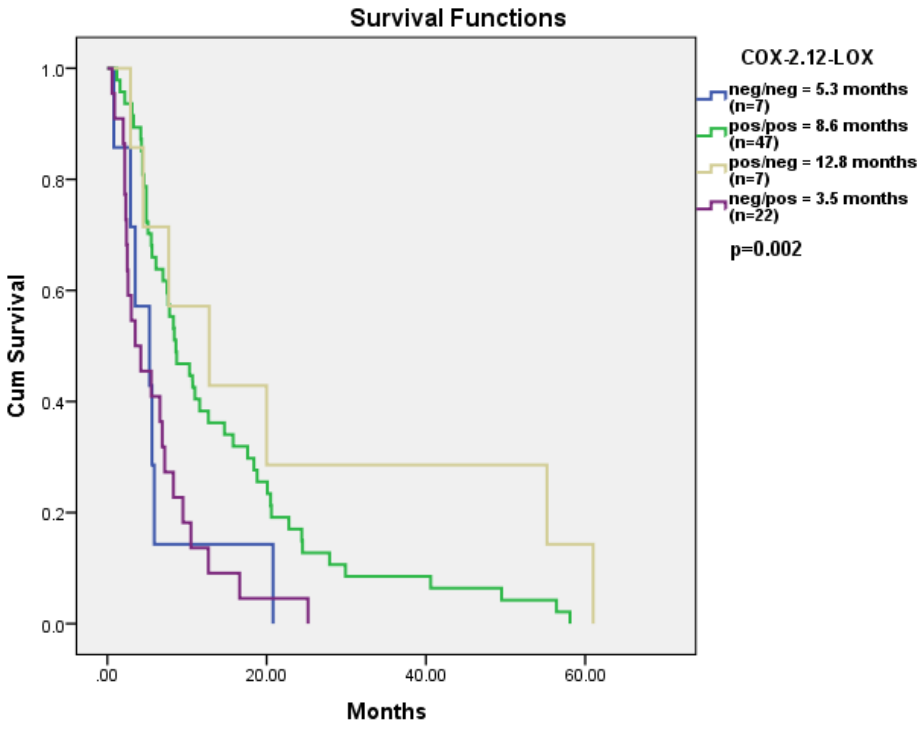
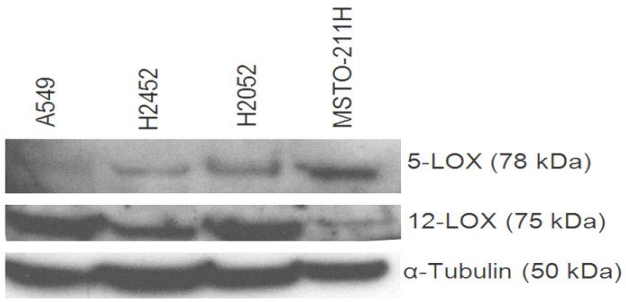


Figure 5.8 Kaplan Meier survival analysis for the co-expression of COX-2 and 12-LOX proteins. Median survival was 12.8 months in cases demonstrating the COX-2 positive /12-LOX negative status (yellow line) *versus* 3.5 months in cases demonstrating the COX-2 negative /12-LOX positive status (purple line) (p = 0.002, log rank). Median survival was 8.6 months in cases demonstrating co-expression of COX-2/12-LOX (green line).

5.3.2 Western blot analysis

The protein expression of 5-LOX and 12-LOX in MPM cell lines and their relative expression levels following normalisation, are shown in Figure 5.9. Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H high 5-LOX protein expression when compared to the A549 cells. The adjusted relative density of the mesothelioma cell lines relative to A549 is graphically represented in Figure 5.9 B. 12-LOX protein expression was lower in the mesothelioma cell lines than the A549 cells. MSTO-211H cells had low expression of 12-LOX but high 5-LOX expression. Expression of COX-2 protein in these cell lines has been previously published (O'Kane et al., 2010). Positive expression of 5-LOX, 12-LOX and COX-2 proteins was identified in all of the MPM cell lines and the NSCLC cell line A549.

(A)



(B)

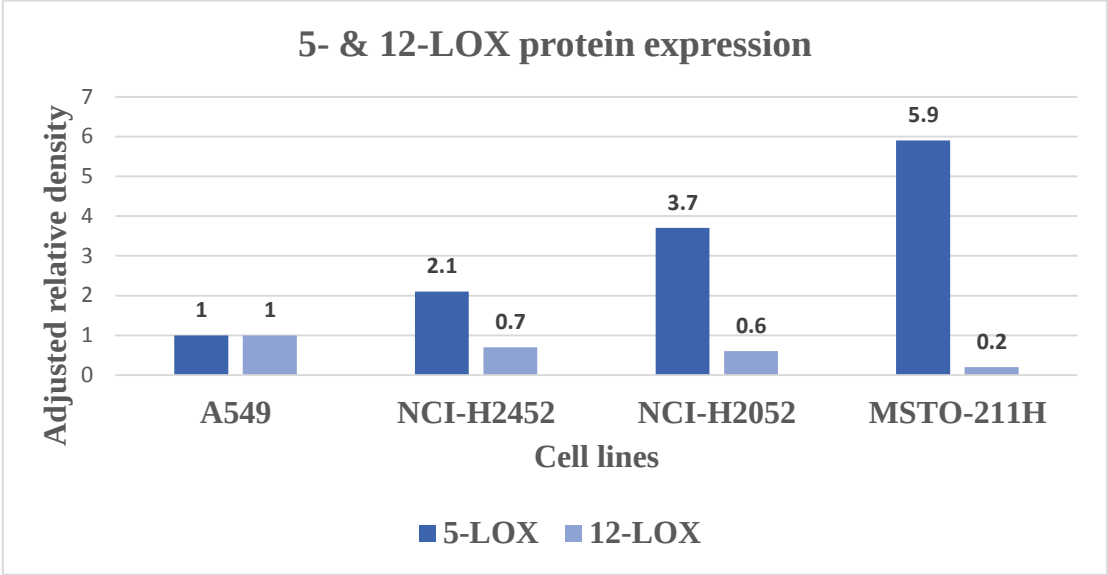


Figure 5.9: Immunoblotting analysis of 5-LOX and 12-LOX protein. (A) Western blots of 5-LOX and 12-LOX in A549 and mesothelioma cell lines. The image shows results with 5-LOX antibody (ab39347, Abcam) (1:250 dilution for 2 hours at room temperature) at 78 kDa and 12-LOX antibody (ab23678, Abcam) (1:500 dilution for 2 hours at room temperature) at 75 kDa. Alpha tubulin was used as a loading control at 50 kDa. (B) Graphical representation of adjusted relative density of mesothelioma cell lines relative to the A549 cells as determined by the densitometric analysis of western blots using the image J software.

5.3.4 Effect of LOX pathway inhibitors on cell viability

Cell viability was determined in all cell lines following single-agent treatment for 72 hours with increasing concentrations of the COX-2 inhibitor celecoxib and the LOX pathway inhibitors baicalein, MK-886, zileuton and licofelone (Figure 5.10 & Figure 5.11). Celecoxib (COX-2 inhibitor) demonstrated similar anti-proliferative effects in all MPM cell lines with

an IC50 range of 39.2 µM to 48.1 µM (Table 5.7). At low concentrations, zileuton (5-LOX inhibitor) and licofelone (dual COX/5-LOX) inhibitor did not demonstrate an effect in any of the MPM cell lines. MK-886 (FLAP inhibitor) exerted an effect at low concentrations in 2/3 of the MPM cell lines, however baicalein (12-LOX and 15-LOX inhibitor) was effective in 3/3 MPM cell lines at low concentrations with an IC50 range of 9.6 µM to 20.7 µM**.**

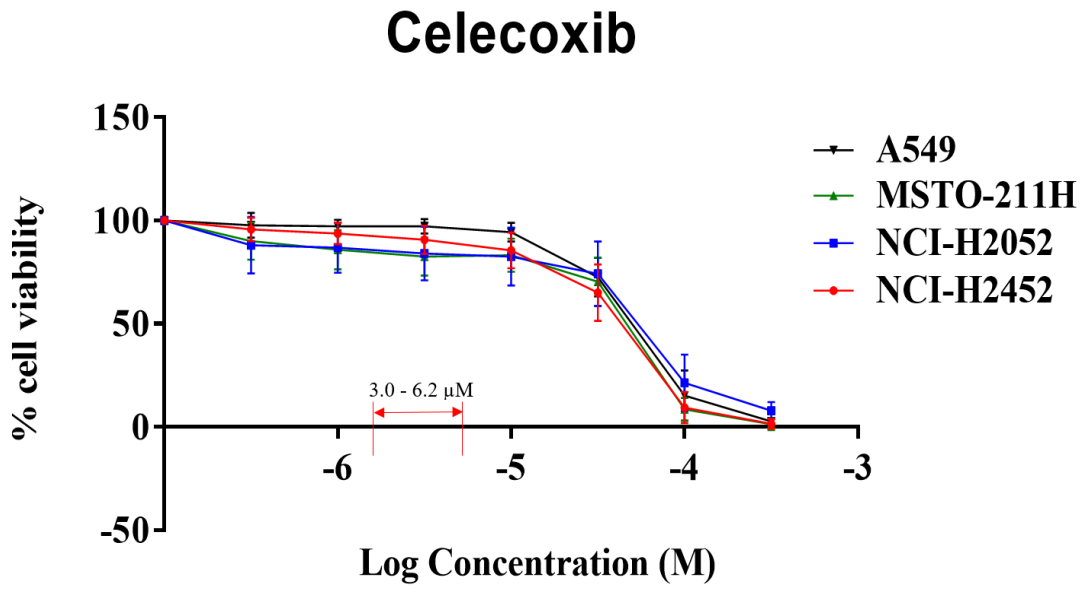


Figure 5.10 Antiproliferative effect of a selective COX-2 inhibitor on MPM cells. Cell proliferation (MTS) assays to investigate the single- agent effect in MPM and A549 cells of a COX-2 inhibitor (celecoxib). Following treatment for 72 hours, cell viability was determined using the MTS reagent and expressed as a ratio of cell viability in comparison to the relevant control (cells treated with <0.1% dimethyl sulfoxide). Each data point is the mean of 18 replicated and error bars represent the mean and standard deviation. The IC50 values generated for each cell line are shown in Table **5.7**. The cmax for celecoxib is 3.0-6.2 µM.

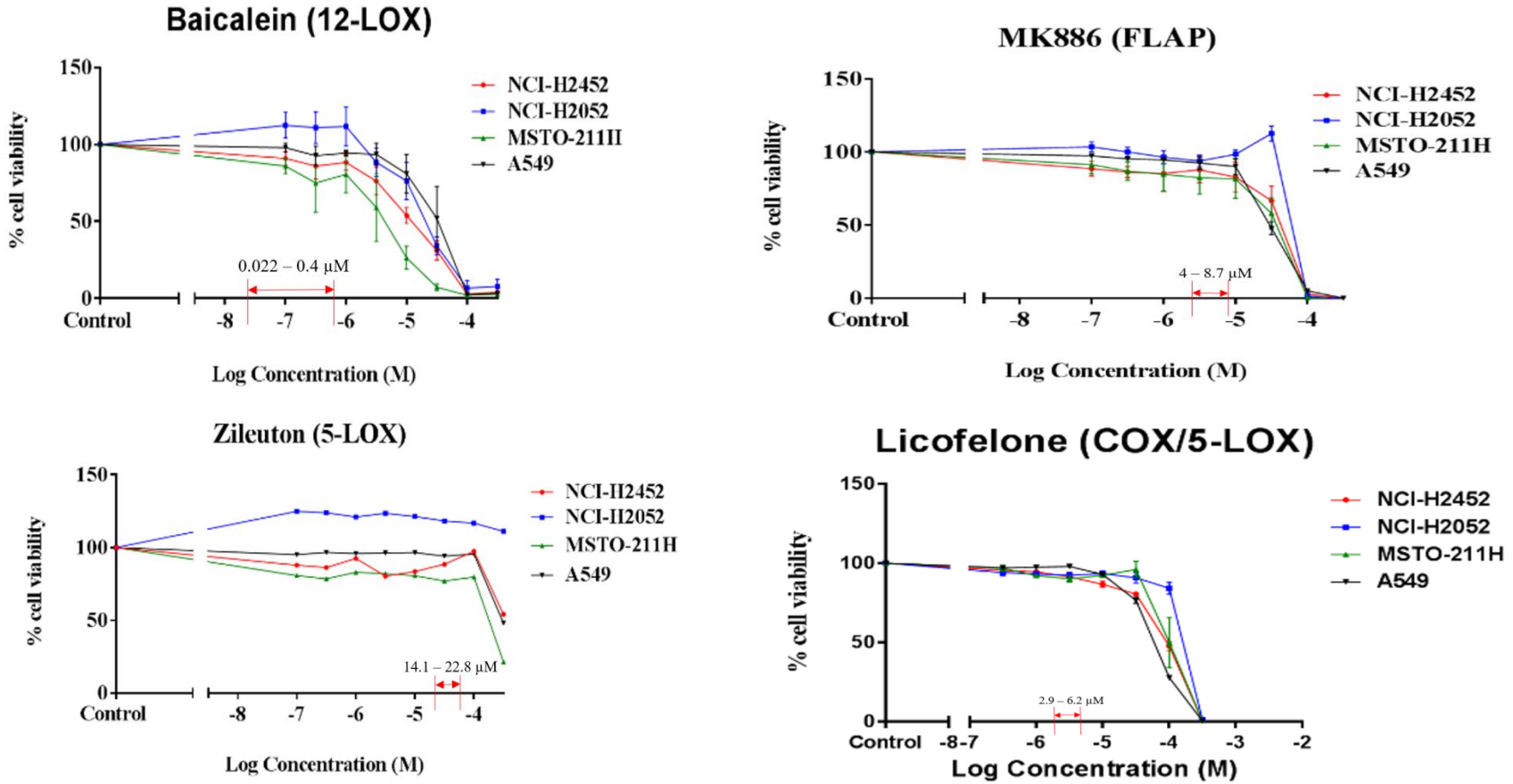


Figure 5.11 Antiproliferative effect of LOX and FLAP inhibitors on MPM cells. Cell proliferation (MTS) assays to investigate the single-agent effect of a 12-LOX/15-LOX inhibitor (baicalein), a FLAP inhibitor (MK-886), a 5-LOX inhibitor (Zileuton) and a dual COX/5-LOX inhibitor (licofelone) respectively in MPM and A549 cells. Following treatment for 72 hours, cell viability was determined using the MTS reagent and expressed as a ratio of cell viability in comparison to the relevant control (cells treated with <0.1% DMSO). Each data point is the mean of 18 replicates and error bars represent the mean and standard deviation. The IC50 values generated for each inhibitor and their cmax values are shown in Table **5.7**.

Table 5.7 IC50 and cmax values of arachidonic acid pathway inhibitors on MPM cells.

	Celecoxib	**Baicalein**	**MK-886**	**Zileuton**	**Licofelone**
Inhibitor of:	**COX-2**	**12-LOX**	**FLAP**	**5-LOX**	**Dual COX/5-LOX**
Published Cmax range:	3.0 – 6.2 µM at the recommended daily dose of 400 mg per day (Davies et al., 2000)	0.022 – 0.4 µM (Li et al., 2014)	4.0 – 8.7 µM (Depre et al., 1993)	14.1 – 22.8 µM at the recommended daily dose of 2400 mg per day (Awni et al., 1995)	2.9 – 6.2 µM (Ding and Cicuttini, 2003)
A549	47.9 µM	28.8 µM	29.7 µM	307.5 µM	58.6 µM
NCI-H2452	39.2 µM	10.7 µM	39.0 µM	342.7 µM	80.7 µM
NCI-H2052	48.1 µM	20.7 µM	84.0 µM	NA	140.0 µM
MSTO-211H	42.2 µM	9.6 µM	30.5 µM	137.3 µM	99.7 µM

NA: Not achieved

5.3.5 Effect of combined use of COX-2 and LOX pathway inhibitors on cell viability

As a proof of principle, we investigated the effect of combining celecoxib and baicalein on cell viability using a clinically achievable concentration of celecoxib (3 µM; Table 5.3). Baicalein was selected for combination with celecoxib because it was the most effective LOX inhibitor. The aim of this experiment was to evaluate the antiproliferative effect of these two inhibitors when combined in comparison to their single agent activity. Also to observe if their combination produces an additive or synergistic effect. A concentration of 10 µM was selected for baicalein based on the single-agent IC50 data for the MPM cells (Table 5.7). Cell viability was determined following combined treatment for 72 hours (Figure 5.12).

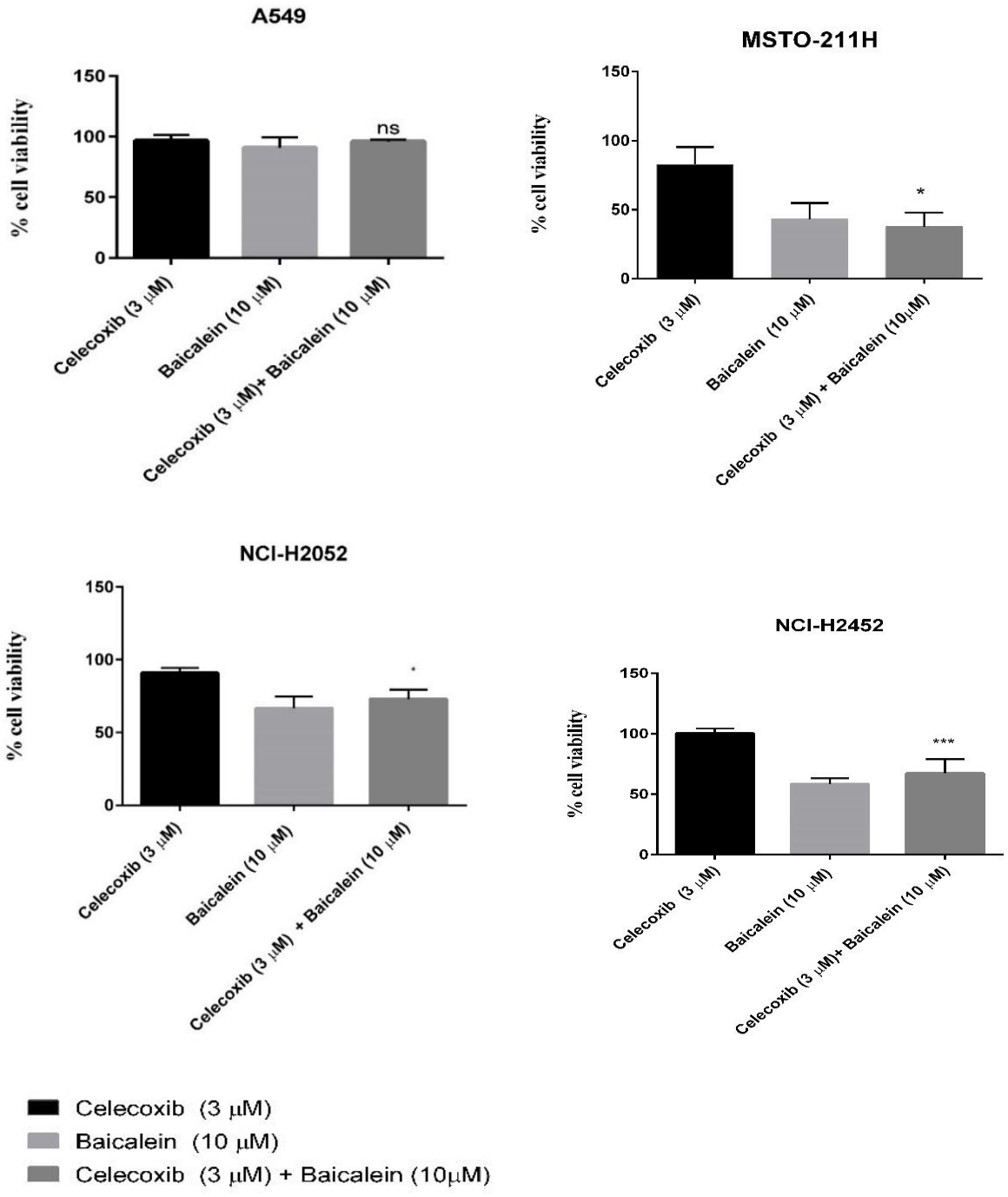


Figure 5.12 Effect of combining celecoxib with baicalein on the viability of MPM cells. Cell proliferation (MTS) assays were performed to investigate the combined effects of 3 μM celecoxib (COX-2 inhibitor) with 10 μM baicalein (12-LOX) in the NSCLC cell line A549 and the MPM cell lines NCI-H2452, NCI-H2052 and MSTO-211H. Following treatment for 72 hours, cell viability was determined using the MTS reagent. The data represents the mean and standard deviation of six replicates from at least three independent experiments and the statistical significance of the combination *versus* celecoxib-alone is shown (ns – not significant; * p = 0.01 to 0.05; *** p = 0.0001 to 0.001). The Chou-Talalay combination index (3.38 in A549; 1.72 in NCI-H2452; 1.36 in NCI-H2052; 0.73 in MSTO-211H) indicates a synergistic effect of celecoxib and baicalein in the MSTO-211H cells at the selected doses.

5.4 DISCUSSION

COX-2 has been found to be up regulated in many tumour types of epithelial origin (including mesothelioma) in response to pro-inflammatory signals and plays a major role in cancer development by inhibiting apoptosis and promoting hallmarks of cancer (Agarwal et al., 2009). Chronic inflammation is a major cascade in the pathogenesis of mesothelioma induced by the accumulation of insoluble asbestos fibres. The chronic inflammatory state maintains an elevated level of COX-2 and consequently results in the release of PGE_2 from inflammatory cells which results in the inhibition of mesothelial cell-mediated cytotoxicity (Bissonnette et al., 1990). 5-LOX and 12-LOX metabolites have also been reported to play a crucial role in the development of pancreatic, colorectal and prostate cancers. The overexpression of 5-LOX and 12-LOX proteins have been demonstrated in tissue samples of primary tumour cells and established cancer cell lines (Tang et al., 1996; Tong et al., 2005; Werz & Steinhilber, 2006; Chen et al., 2006). Previous studies have demonstrated the potential role of COX-2 as a therapeutic target in MPM (Edwards et al., 2002; Baldi, 2004; O'Kane et al., 2005; Mineo et al., 2010) and a selective COX-2 inhibitor has been reported to potentiate the effect of chemotherapeutic drugs (O'Kane et al., 2010).So far, there is only one published data that has demonstrated the expression of 5-LOX and 12-LOX mRNA in mesothelioma cell lines. To the best of our knowledge there is no published date on the presence of 5-LOX and 12-LOX proteins in mesothelioma tissue samples and the effect of co-inhibiting COX-2 and LOX pathways.

Immunohistochemistry

In the current study we have demonstrated the expression of 12-LOX protein in non-reactive and reactive benign pleural tissue samples whilst 5-LOX expression was evident only in cases with reactive mesothelial cells. The benign samples used in this study were from patients with an underlying condition which may result in the presence of reactive mesothelial cells due to inflammation. We have also demonstrated that the 5-LOX and 12-LOX proteins are expressed in a significant proportion of archival MPM samples (73% and 83% respectively) and may represent novel therapeutic targets in this disease (Table 5.4). The co-expression of COX-2 with 5-LOX or COX-2 with 12-LOX was associated with improved survival. In the sarcomatoid subtype, the expression of 5-LOX was associated with improved prognosis however these preliminary findings require further confirmation using a larger number of cases. To our knowledge, this is the first study to investigate 5-LOX and 12-LOX protein expression in MPM tissue samples.

Western Blot

5-LOX protein was expressed in all cell lines but the mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H showed high 5-LOX expression relative to the A549 cells. 12-LOX protein was also expressed in all cell lines but the mesothelioma cell lines expressed low 12-LOX protein compared to the A549 cells. A549 cells have been shown to express 5-LOX mRNA using RT-PCR (Avis et al., 1996). Our study is the first to demonstrate 5- & 12-LOX protein expression in NCI-H2452, NCI-H2052 and MSTO-211H cells. Romano et al (2001) previously reported the presence of 12-LOX mRNA in normal mesothelial and primary mesothelioma cell. 5-LOX mRNA was expressed in the primary mesothelioma cells but not in the normal mesothelial cells which is in keeping with our findings (Romano et al., 2001). There is a need for more studies to further evaluate the presence of these proteins in mesothelioma. The COX-2 expression in our panel of cell lines has been previously published by (O'Kane et al., 2010)

MTS assay

Our panel of MPM cell lines, which have exhibited expression of the COX-2, 5-LOX and 12-LOX targets, demonstrated no response to treatment for 72 hours with the 5-LOX inhibitor zileuton at the clinically relevant dose range. In each cell line, the IC50 for celecoxib was reached at a concentration which was outside the clinically relevant range. The dual COX/LOX-5 inhibitor, Licofelone showed minimal antiproliferative effects in the mesothelioma cell lines especially in the NCI-H2052 cell line. The dose required to inhibit 50% of the cells significantly exceeded the clinically relevant dose of the inhibitor. The maximum tolerated dose for baicalein and MK-886 in humans is not available since the clinically relevant doses that have been evaluated have so far been well tolerated (Table 5.7). Baicalein was more potent than other LOX inhibitors and to further examine the *in vitro* effects of simultaneous inhibition of the COX-2 and LOX pathways we examined baicalein in combination with celecoxib, which was used at the clinically relevant concentration. Baicalein, which is known to inhibit 12-LOX as well as 15-LOX (Deschamps et al., 2006), had demonstrated an effect in all 3 MPM cell lines at relatively low concentrations as single agent when compared to the other LOX pathway inhibitors. Baicalein has been reported to reduce cell proliferation *in vitro* in other cancer cell lines at concentrations of 5 – 80 μM (Lee et al., 2005; Chao et al., 2007; Takahashi et al., 2011; Chen et al., 2013) and was shown to induce cancer cell death, inhibit invasion and cell proliferation (Chao et al., 2007; Wu et al., 2011; Huang et al., 2012; Zhang et al., 2013; Chen et al., 2013; Aryal et al., 2014). There has only been one previous report of baicalein treatment of MPM cells and no effect was seen at

a single fixed concentration of 2 µM (Romano et al., 2001). This is in keeping with our result as the lowest IC50 achieved with baicalein was 9.6 µM in the MSTO-211H cells. The *in vitro* combination results indicated that celecoxib/baicalein treatment may be more effective than celecoxib alone in these cell lines; however 10 µM baicalein alone appears to be mainly responsible for this effect with drug synergy demonstrated in only one cell line (MSTO-211H). A possible explanation for the effect of baicalein on MSTO-211H cells could be as a result of reduced expression of 12-LOX protein in comparison to the A549 cells. Although, 5-LOX protein expression was higher in MSTO-211H cells than the A549 cells but zileuton was more effective in the MSTO-211H cells. MK886 and licofelone have been previously reported to inhibit tumour cells in dependent of lipoxygenase inhibition (Tavolari et al., 2008; Fischer et al., 2010), however zileuton suppressed 5-LOX enzymatic activity but had little or no effect on cell viability (Fischer et al., 2010). Baicalein might exert antitumour effect independent of LOX expression but this concept was not investigated within this study. The role of lipoxygenases in mesothelioma remain unclear; an understanding of the effect of inhibitors of the arachidonic acid pathway on COX-2 and LOX metabolites as well as other oncogenic pathways would help define the role of these inhibitors in mesothelioma. Although still controversial, there is an increase in the study of the role of 15-LOX and its metabolites in tumorigenesis and might be worth investigating in mesothelioma (Mashima & Okuyama, 2015). In summary, this study has shown that 5-LOX and 12-LOX proteins are expressed at high frequency in mesothelioma samples and may represent therapeutic targets. We have also demonstrated that the inhibition of cell growth using baicalein may be effective as a novel treatment for MPM, however further pharmacokinetic studies may be required in order to establish whether the concentration used *in vitro* is clinically achievable since the single pharmacokinetic study (Li et al., 2014) in humans did not establish a maximum tolerated dose. Further studies on the effect of baicalein in combination with chemotherapy drugs is warranted. CDC is another 12-LOX inhibitor with affinity for 5-LOX and 15-LOX that can be investigated in mesothelioma cells (Pergola et al., 2011).

CHAPTER SIX

EXPRESSION OF VEGFR-2 AND THE EFFECT OF VEGFR-2 INHIBITORS ON MESOTHELIOMA CELL LINES

Chapter 6 Expression of VEGFR-2 and the effect of VEGFR-2 inhibitors on mesothelioma cell lines

6.1 VEGF and VEGFR-2 expression in MPM

As previously discussed in section 2.2.2, the growth of a tumour is dependent on angiogenesis and this may be caused by an imbalance between proangiogenic and anti-angiogenic factors. In order to survive, a tumour requires a continuous supply of oxygen and nutrition. Malignant cells achieve this by stimulating the vascular endothelial growth factor receptors (VEGFRs) on the cell surface with their ligands most importantly (e.g. VEGF). VEGF is a major player in regulating angiogenesis and permeability of blood vessels essential for tumour growth and metastasis and it also promotes permeability in the mesothelial monolayer (Mohammed et al., 2001; Sack et al., 2005). VEGF levels have been found to be elevated in serum, cell lines and tissue of mesothelioma patients. Serum levels in MPM patients was significantly higher ($p < 0.0001$) than those with benign asbestos-related diseases or healthy individuals with a history of asbestos exposure. In addition, VEGF levels >460 pg/ml in the serum of MPM patients was shown to inversely correlate with survival (Yasumitsu et al., 2010). In MPM cells, VEGF stimulates growth in a dose-dependent manner. Addition of recombinant VEGF induced the phosphorylation of VEGFR-1, VEGFR-2 and increased cell proliferation in mesothelioma cell lines 1ST-Mes1, 1ST-Mes2, 1ST-Mes3 and MPP89 (Strizzi et al., 2001). Apart from inducing angiogenesis in MPM cells, VEGF expression also correlates with intratumoral microvessel density and expression of VEGFR-2 in MPM tissue samples (Ohta et al., 1999). Some findings suggest that the role of VEGF might extend beyond inducing angiogenesis but also stimulate MPM tumour cells directly in an autocrine/paracrine fashion via its receptors (Strizzi et al., 2001). Inflammatory mediators such as IL-6, 5-lipoxygenase and cyclooxygenase-2 are also able to induce VEGF production (Romano et al., 2001; Catalano et al., 2004; Adachi et al., 2006). VEGF has been shown to have a strong correlation with COX-2 levels in several cancers including colon, oesophageal, lung and gastric cancer (Cianchi et al., 2001; Kim et al., 2003; Huang et al., 2005; von Rahden, 2005). Romano and his colleagues also evidenced a direct effect of 5-LOX metabolite (5-HETE) on VEGF stimulation and gene expression in human mesothelioma cell lines (Romano et al., 2001). COX-2 is thought to be a key mediator of VEGF-independent tumour angiogenesis, although the mechanism is yet to be fully understood (Gately & Li, 2004). Prostaglandin E_2 produced by tumour cells can induce angiogenesis by directly stimulating the G-protein coupled receptors EP1-4 on endothelial or stromal cells which leads to the recruitment of more inflammatory cells and angiogenic

factors. Secreted VEGF is also able to induce COX-2 expression subsequently triggering the production of prostaglandins (Xu et al., 2014) (Figure 6.1). There is growing body of evidence on the involvement of cyclooxygenase 2 in tumour angiogenesis (Tsujii et al., 1998; Jones et al., 1999; Masferrer et al., 2000; Dermond & Rüegg, 2001; Iñiguez et al., 2003; Toomey et al., 2009). Few studies have directly evaluated the co-expression of VEGFR-2 and inflammatory mediators such as COX-2, 5-LOX and 12-LOX in MPM tissue and the effect of their co-expression on patient survival.

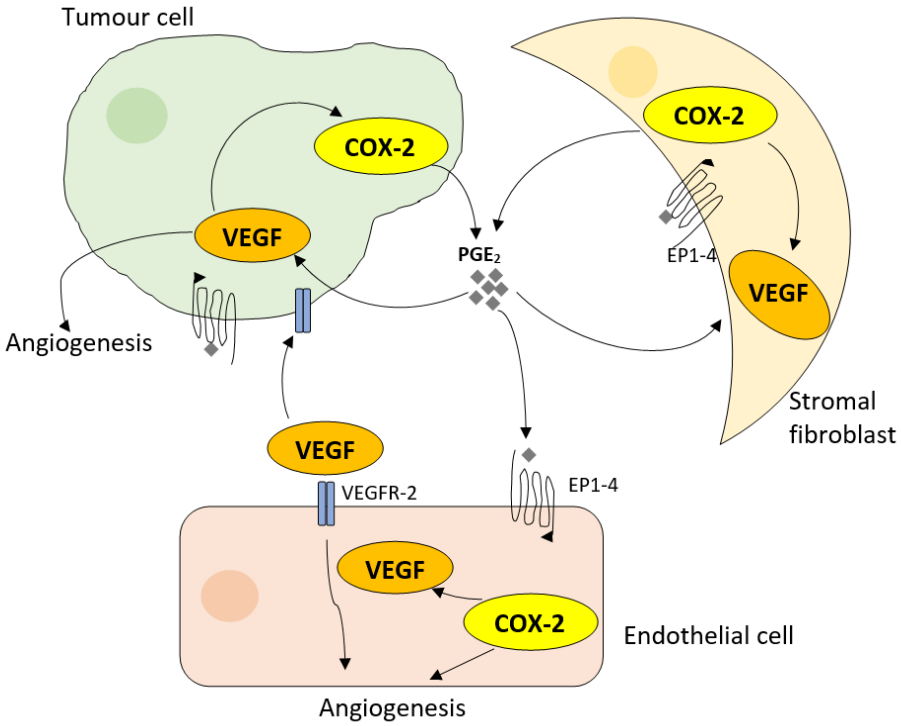


Figure 6.1 Schematic representation of COX-2 and VEGF mediated tumour angiogenesis. VEGF produced by tumour cells can stimulate angiogenesis directly. PGE_2 can also directly stimulate G-protein coupled receptors to induce angiogenesis. Prostaglandins can further induce VEGF production in a paracrine, autocrine and intracrine manner leading to the recruitment of more inflammatory cells and angiogenic factors. Adapted from (Iñiguez et al., 2003) and (Xu & Croix, 2014).

VEGFR-2 is known to be the most important mediator of VEGF induced signalling responses. Very few immunohistochemical studies with limited sample sizes have investigated VEGFR-2 expression in MPM tissue specimen and the six reports (in bold) have so far demonstrated that 71 – 100% of MPM tissue samples express VEGFR-2 (Table 6.1).

The established relationship between VEGF, its receptors and mesothelioma has led to several clinical trials discussed in section 6.1.1.

Table 6.1 Expression of VEGF and its receptors in MPM by immunohistochemistry

Author	No. of cases	Protein	Histological Subtype	Expression levels	Prognostic status
(König et al., 1999)	103	VEGF	Epithelial – 46 Biphasic – 11 Sarcomatoid – 19	76/103 (73.7%)	No
(Ohta et al., 1999)	54	VEGF VEGFC VEGFR-1 **VEGFR-2** VEGFR-3	Epithelial – 44 Biphasic – 6 Sarcomatoid – 4	41/54 (75.9%) 46/54 (85.2%) 40/54 (74.1%) 49/54 (90.7%) 52/54 (96.3%)	No
(König et al., 2000)	90	VEGF, VEGFR-1	Epithelial – 36 Biphasic – 41 Sarcomatoid – 13	62/90 (69% 57/60 (94.5%)	No
(Soini et al., 2001)	36	VEGF VEGFR-1 **VEGFR-2**	Epithelial – 24 Biphasic – 4 Sarcomatoid – 8	17/36 (47%) 25/36 (69%) 24/36 (67%)	No
(Strizzi et al., 2001)	12	VEGF VEGFR-1 **VEGFR-2**	Epithelial – 8 Biphasic – 3 Sarcomatoid – 1	12/12 (100%)	No
(Demirag et al., 2005)	40	VEGF	Epithelial – 30 Biphasic – 10	32/40 (80%)	Yes
(Aoe et al., 2006)	37	VEGF	Epithelial – 12 Biphasic – 10 Sarcomatoid – 14 Lymphohistiocytoid - 1	36/37 (97.3%)	No
(Filho et al., 2007)	29	VEGFR-3	Epithelial – 19 Biphasic – 3 Sarcomatoid – 3 Other - 4	14/29 (48.3%)	No
(Nutt et al., 2009)	17	**VEGFR-2**	Not specified	12/17 (71%)	No
(Loganathan et al., 2011)	38	VEGF **VEGFR-2**	Not specified	Not specified	Not specified
(Miettinen et al., 2012)	38	**VEGFR-2**	Not specified	35/38 (92%)	Not specified

6.1.1 Angiogenic therapies in MPM

Based on the growing body of evidence for the involvement of VEGF on mesothelioma cell growth, two drugs targeting the ligand (including one that targets VEGF and other immunomodulatory proteins) have been explored in clinical trials. Thalidomide, an antiangiogenic and oxidative stress-inducing drug was evaluated as maintenance therapy in MPM patients after the use of platinum based first-line therapy in a randomized phase II trial. There was no significant difference observed between the thalidomide and palliative treatment arms in terms of progression-free survival which was the primary endpoint of the study (Buikhuisen et al., 2013). A monoclonal antibody against VEGF, bevacizumab was also investigated in multiple phase II clinical trials. The combination of bevacizumab with gemcitabine and cisplatin was compared to a placebo arm. The median overall survival time for the combination arm (15.6 months) was not significantly different to the placebo arm (14.7 months) neither was there an improvement in progression free survival (Kindler et al., 2012). Two non-randomised phase II trials have also evaluated the administration of bevacizumab with either carboplatin or cisplatin in combination with pemetrexed. The addition of bevacizumab in both trials did not provide a significant benefit on survival (Dowell et al., 2012; Ceresoli et al., 2013). Recently a phase III randomized controlled trial conducted by Gérard Zalcman and colleagues delivered hope for the use of antiangiogenic therapy in mesothelioma. The trial randomized 448 patients with unresectable malignant pleural mesothelioma without prior treatment to treatment arms comparing the addition of bevacizumab to cisplatin and pemetrexed with cisplatin and pemetrexed only. The primary endpoint was overall survival and it was favoured by the bevacizumab arm (18.1 months *vs* 16.1 months; p=0.0167) (Zalcman et al., 2016).

Multi-targeted tyrosine kinase inhibitors such as axitinib, dasatinib, pazopanib, sunitinib, sorafenib and vatalanib with activity against VEGFR1/2/3, PDGFRs, c-Kit and other molecular targets have also been evaluated in phase II studies for the treatment of MPM (Figure 2.6). These inhibitor have shown very limited activity as monotherapy for first or second line therapy in unselected MPM patients (Laurie et al., 2011; Dudek et al., 2012; Jahan et al., 2012; Nowak et al., 2012; Papa et al., 2013; Buikhuisen et al., 2016). Cediranib, an oral, potent pan-VEGFR inhibitor was evaluated as a single agent in 47 pre-treated MPM patients at doses of 45 mg daily. There was modest activity observed with a disease control rate of 42% and limiting toxicities which required the dose to be lowered (Garland et al., 2011). Another phase II trial investigated cediranib as monotherapy in second line setting following standard chemotherapy. The primary endpoint (objective response rate) was not

met, although improved disease control was observed with higher cediranib dose (45 mg) but with grade 3 toxicities particularly hypertension (Campbell et al., 2012). An ongoing phase I/II study is investigating cediranib at low dose (20 mg) in combination with cisplatin and pemetrexed (NCT01064648). Nintedanib, a multi-target VEGFR, PDGFR and FGFR inhibitor is also in phase III trial in combination with standard chemotherapy in patients with unresectable mesothelioma (NCT01907100).

In this study, we aimed to investigate the expression of VEGFR-2 in a cohort of 80 samples and assess the anti-proliferative activity of MGCD265 (a VEGFR1/2/3 and c-MET inhibitor) and Cediranib (a VEGFR-1, 2 & 3 inhibitor) in mesothelioma cell lines. MGCD265 was selected based on its ability to target VEGFR-2 and c-MET proteins which are frequently upregulated in mesothelioma. We sought to compare the antiproliferative effect of the multitargeted inhibitor with Cediranib which solely a VEGFR inhibitor.

6.2 AIMS

In this study we aimed to:

- Assess the expression of VEGFR-2 in MPM and benign pleura tissue samples
- Assess the expression of VEGFR-2 protein expression in MPM cell lines using HUVEC and A549 cells as positive controls
- Investigate the co-expression of VEGFR-2 with COX-2, LOX-5 or LOX-12 and the effect of their co-expression on patient survival
- Evaluate the effect of Cediranib (a single kinase VEGFR1/2/3 inhibitor) and MGDC265 (a multitarget tyrosine kinase inhibitor of VEGFR1/2/3, Met, Tie and Ron) (Figure 2.6) in MPM cell lines and A549 cells

6.3 Materials and Methods

6.3.1 Immunohistochemistry

Eighty tissue specimens of patients diagnosed between 1995 and 2000 were analysed in this study: 43 epithelioid, 24 biphasic and 13 sarcomatous phenotypes. Benign pleura tissue sample was included as reference slide. Additional positive and negative controls were added using colorectal tissue samples. The negative control used all the reagents except for the primary antibody. The tissue samples had been fixed in formalin and embedded in paraffin wax.

The immunohistochemical study was performed using R.T.U. Vectastain Quick kit (#PK-7800, Vector Laboratories Inc., CA, USA) with standard techniques as detailed in Section 4.8. The anti-VEGFR-2 primary antibody (#2479, Cell signalling) was applied at a dilution of 1:75. All slides were scored by two independent scorers including a consultant histopathologist (Dr. Anne Campbell), as detailed in Section 4.8.9.

6.3.2 Western blot

MPM cell lines NCI-H2452, NCI-H2052 and MSTO-211H were obtained from the American Type Culture Collection (ATCC) and the non-small cell lung cancer cell line A549 was obtained from the European Collection of Cell Cultures (ECACC). Human umbilical endothelial cells (HUVECs) were also used as control in this experiment. These were a gift from Dr. Laura Sadofsky, University of Hull. Mesothelioma and A549 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin and fungizone as detailed in section 4.4.1. HUVECs were maintained in an endothelial cell basal medium with supplements (#C-22210, PromoCell). Western blot analysis was performed to analyse the status of the VEGFR-2 protein in the cell lines as detailed in section 4.9. Whole cell lysates were prepared using 1x Laemmli buffer. Briefly, proteins (20 µg per lane) was loaded onto a precise 4-20% Tris-HEPES gradient protein gel (#25524, Thermo scientific) and separated by one dimension gel electrophoresis. The separated protein were transferred on to a 0.2 µm nitrocellulose membrane (#IB301001, Thermo scientific) and incubated with anti-VEGFR-2 primary antibody (#2479, Cell signalling) at a 1:250 dilution overnight at $4^{o}C$ (Table 4.2).

6.3.3 MTS assay

Cediranib (#S1017) and MGCD265 (#S13161) were purchased from Selleckchem as discussed in Sections 4.3.5.1 and 4.3.5.2 respectively. Cell lines were cultured and counted as detailed in Section 4.4.1 and Section 4.5 respectively. Each cell line was seeded onto a 96 well plate at 1000 cells per well as detailed in Section 4.6 and the MTS assay was performed as detailed in Section 4.7.1.1. In each experiment, six replicate wells were used for each drug concentration and the experiment was repeated 3 times. Cells were exposed to varying concentrations of Cediranib and MGCD-265 from day 2 for 72 hours. The control wells were incubated with 0.01% DMSO diluted in media. Percentage cell growth and percentage of viable cells for each drug concentration was calculated as detailed in Section 4.7.1.2. The

average of the three experiments was taken and loaded onto Graphpad prism 6.0 to calculate the 50% cell growth inhibition.

Statistical analysis was carried out using SPSS software version 22.0 (SPSS, Chicago, USA). Univariate analysis for VEGFR-2 protein expression was performed using Kaplan Meier curves with log rank analysis. Multivariate analysis was calculated using COX regression analysis to take into consideration histological subtype, which is an established independent prognostic variable in MPM. The Chi-squared test was used to analyse the association between the expression of VEGFR-2 and clinipathological parameters. A level of $p<0.05$ was accepted as significant.

6.4 Results

The characteristics of the 80 MPM archival samples in this study are described in Table 6.2. Histologically, MPM was of the epithelial histotype in 43 patients, biphasic/mixed in 24 patients and sarcomatoid type in 13 patients. The median age was 68 years (range 42-88 years). Seventy-three patients were males and 7 were females. Median survival for all the cases was 7.4 months.

Table 6.2 Clinicopathological variables of the MPM cohort assessed for VEGFR-2 protein expression

Characteristics	**Number of cases (%)**
Total	80 (100)
Age-median (range)	68 (42-88)
≤64	27
>64	53
Median survival (months)	7.4
Gender	
Female	7 (9)
Male	73 (91)
Histology	
Epithelioid	43 (54)
Biphasic/Mixed	24 (30)
Sarcomatoid	13 (16)
VEGFR-2 score	
0-negative	5 (6)
1-positive	75 (94)

6.4.1 Immunohistochemistry

6.4.1.1 VEGFR-2 expression in MPM and benign pleura

Eighty MPM tissue samples were adequately stained for VEGFR-2. Figure 6.2A & B shows the negative control (antibody omitted) colorectal tissue sample and VEGFR-2 expression in benign pleura respectively. Figure 6.2C & D shows positive (1) and negative (0) VEGFR-2 expression in the epithelial subtype respectively. Positive and negative VEGFR-2 expression in the biphasic/mixed subtype can be seen in Figure 6.2E & F respectively. Figure 6.2G & H shows positive and negative VEGFR-2 expression in the sarcomatoid subtype respectively. VEGFR-2 protein is mostly expressed in the cytoplasm in endothelial cells in most tissues as observed in Figure 6.2B. VEGFR-2 was mainly present in the cytoplasm of the MPM tumour cells and no nuclear staining observed. Mesothelial cells in all benign pleura (n=8; 100%) were also immunoreactive for VEGFR-2 together with the endothelial cells within the tissue sample (Figure 6.2B). Each tissue sample was scored as positive (1) or negative (0) based on the staining intensity when compared to the reference slides. The reference slides used were the positive benign pleural samples and the negative control (antibody omitted) colorectal tissue sample. The MPM tissue samples were scored as positive if the tumour cells expressing VEGFR-2 are >25% and the intensity is similar or higher than that of the benign pleural tissue. Negative expression was scored as (0) when <25% of the tumours cells express VEGFR-2. The endothelial cells also served as internal positive control.

In total, 75 (93.8%) of the 80 specimens showed positive VEGFR-2 immunoreactivity in tumour cells (Table 6.3). In the epithelial mesotheliomas 93% of the specimens were positive for VEGFR-2 and only 7% showed no immunoreactivity for the protein. In the biphasic subtypes 96% of samples were positive and 4% negative. In the sarcomatoid subtypes 92.3% of samples were positive for VEGFR-2 and 7.7% were negative.

Table 6.3 VEGFR-2 expression in MPM samples

Samples	**Total**	**VEGFR-2 expression score**	
		Positive (1)	**Negative (0)**
All samples	80	75 (93.8%)	5 (6.3%)
Epithelial	43	40 (93%)	3 (7%)
Biphasic	24	23 (95.8%)	1 (4.2%)
Sarcomatoid	13	12 (92.3%)	1 (7.7%)

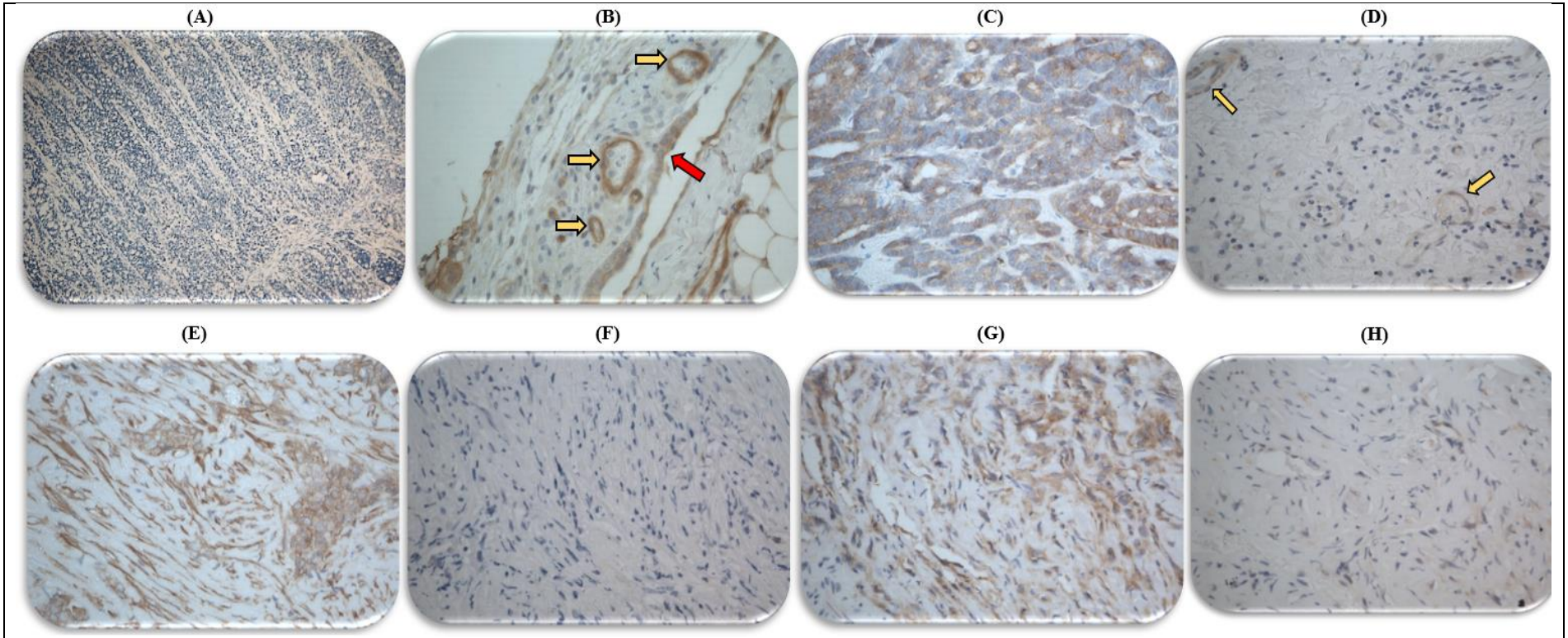


Figure 6.2 VEGFR-2 protein expression in MPM. (A) Negative control (antibody omitted) colorectal tissue sample (x100). (B) VEGFR-2 positive expression in mesothelial cells (red arrow) in the benign pleural sample. The endothelial cells can also be seen in the benign pleura (yellow arrow). (C) VEGFR-2 positive epithelial mesothelioma in the cytoplasm. (D) VEGFR-2 negative epithelial mesothelioma but endothelial cells within the tumour are positive (yellow arrows). (E) Biphasic/mixed mesothelioma positive for VEGFR-2 and (F) a negative biphasic specimen. (G) Sarcomatoid mesothelioma positive for VEGFR-2 and (H) a negative sarcomatoid specimen. Magnification (x400)

6.4.1.2 Survival analysis

There was no significant correlation observed between VEGFR-2 expression and age (p=1.00), gender (p=0.375) or histological subtype (p=0.877). Median survival for VEGFR-2 positive cases was 7.5 months *versus* 5.5 months in the VEGFR-2 negative cases. There was no significant association observed between VEGFR-2 expression status and survival time as indicated in univariate analysis using Kaplan Meier survival curves (p=0.087) (Figure 6.3).

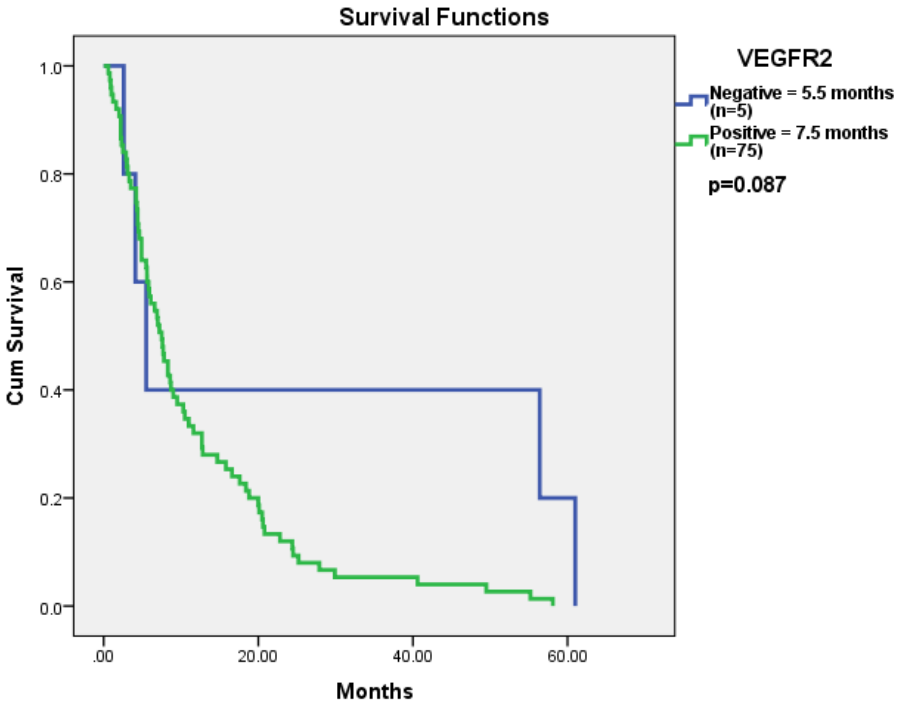


Figure 6.3: Kaplan-Meier survival analysis of VEGFR-2 protein expression in MPM. The plot shows univariate analysis of VEGFR-2 expression (p=0.087; log rank). The median survival was 7.5 months in VEGFR-2 positive cases (green line) versus 5.5 months for VEGF-2 negative cases (blue line).

In the epithelial mesothelioma cases, the absence of VEGFR-2 was associated with improved survival (median survival 56.4 months versus 10.3 months in VEGFR-2 positive cases; p=0.045) (Figure 6.4). However, when multivariate COX regression analysis was carried out taking histological subtype into consideration, VEGFR-2 expression was not an independent prognostic variable (HR 0.45; 95% CI 0.15 - 1.30; p=0.14) (Table 6.4).

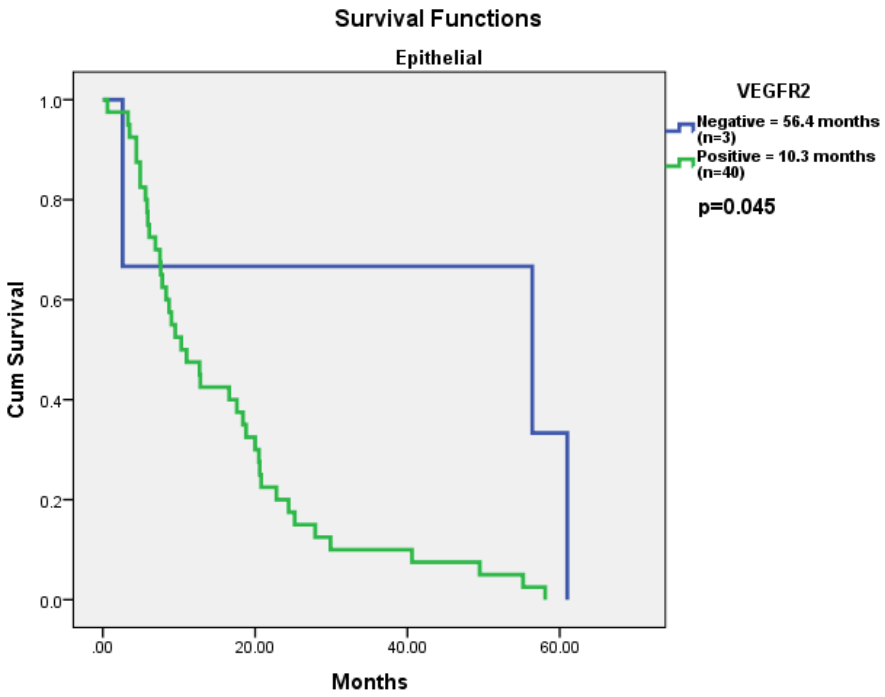


Figure 6.4: Survival analysis of VEGFR-2 protein expression in epithelial mesothelioma. The plot shows univariate analysis of VEGFR-2 expression in the epithelial subtypes (p=0.045; log rank). The median survival was 10.3 months in VEGFR-2 positive cases (green line) versus 56.4 months for VEGFR-2 negative cases (blue line).

Table 6.4 Multivariate analysis of VEGFR-2 expression using histological subtype as a confounding factor.

	HR	95% confidence interval		P-value
		Lower	Upper	
VEGFR-2	0.45	0.15	1.30	0.14
Histological subtype				<0.001
epithelial	0.23	0.12	0.46	<0.001
biphasic	0.61	0.31	1.21	0.159
sarcomatoid	1.00			

6.4.1.3 Co-expression of VEGFR-2 with either COX-2, 5-LOX or 12-LOX protein expression

Co-expression of VEGFR-2 with COX-2, 5LOX or 12LOX was further investigated in the entire cohort. COX-2 data has been previously reported to be an independent prognostic factor (S.L. O'Kane et al., 2005). Cross tabulation did not show any correlation between COX-2 and VEGFR-2 (p=0.337). The co-expression of VEGFR-2 and COX-2 in relation to

survival was evaluated such that the absence of both protein was (0), the presence of both proteins was (1), VEGFR-2 positive/COX-2 negative (3) and VEGFR-2 negative/COX-2 positive (4). Eighty samples were successfully analysed for both VEGFR-2 and COX-2. Of the 80, 50 (62.5%) were positive for both VEGFR-2 and COX-2; 25 (31.3%) were positive for VEGFR-2 but negative for COX-2; 2 (2.5%) were negative for VEGFR-2 but positive for COX-2 while 3 (3.8%) were negative for both proteins. There was a significant difference in the median survival times in the different sub-categories. Median survival was 8.6 months in VEGFR-2 positive/COX-2 positive cases, 4.2 months in VEGFR-2 positive/COX-2 negative cases, 56.4 months in VEGFR-2 negative/COX-2 positive cases and 4.1 months in VEGFR-2 negative/COX-2 negative cases (Figure 6.5). Multivariate Cox regression analysis showed that co-expression of VEGFR-2 and COX-2 was independent of histological subtype (both positive *vs* both negative; HR, 3.02; 95% CI: 0.9 – 10.3; p=0.002).

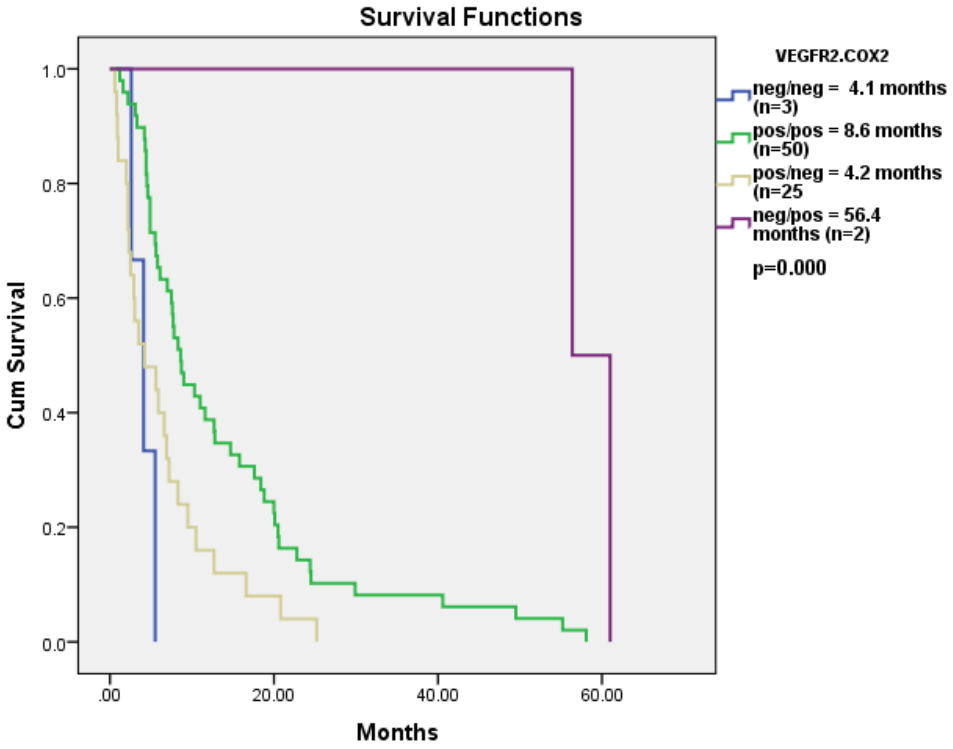


Figure 6.5 Survival analysis for the co-expression VEGFR-2 and COX-2 protein expression. Kaplan Meier plot showing univariate analysis of combined VEGFR-2/COX-2 expression status (p<0.001, log rank). The median survival was 8.6 months in VEGFR-2 positive/COX-2 positive cases (green line), 4.2 months in VEGFR-2 positive/COX-2 negative cases (yellow line), 4.1 months in VEFGR-2 negative/COX-2 negative cases (blue line) and 56.4 months in VEGFR-2 negative/COX-2 positive cases (pink line).

Seventy-four samples were analysed for the co-expression of VEGFR-2 and 5-LOX. The 5-LOX protein results have been previously discussed in chapter 6. There was no significant correlation between VEGFR-2 and 5-LOX expression (p=0.058). Of the 74 tissue samples, 4.1% (3/74) were negative for both VEGFR2 and 5-LOX; 71.6% (53/74) were positive for both VEGFR-2 and 5-LOX; 22.9% (17/74) were positive for VEGFR-2 but negative for 5-LOX and 1.4% (1/74) was negative for VEGFR-2 but positive for 5-LOX. Median survival was 7.8 months in VEGFR-2 positive/5-LOX positive cases; 5.8 months in VEGFR-2 positive/5-LOX negative cases; 5.5 months in VEGFR-2 negative/5-LOX positive cases and 56.4 months in VEGFR-2 negative/5-LOX negative cases (Figure 6.6). There was no significant difference observed between the sub-categories however, cases that had no immunoreactivity for both proteins (n=3) showed improved median survival of 56.4 months.

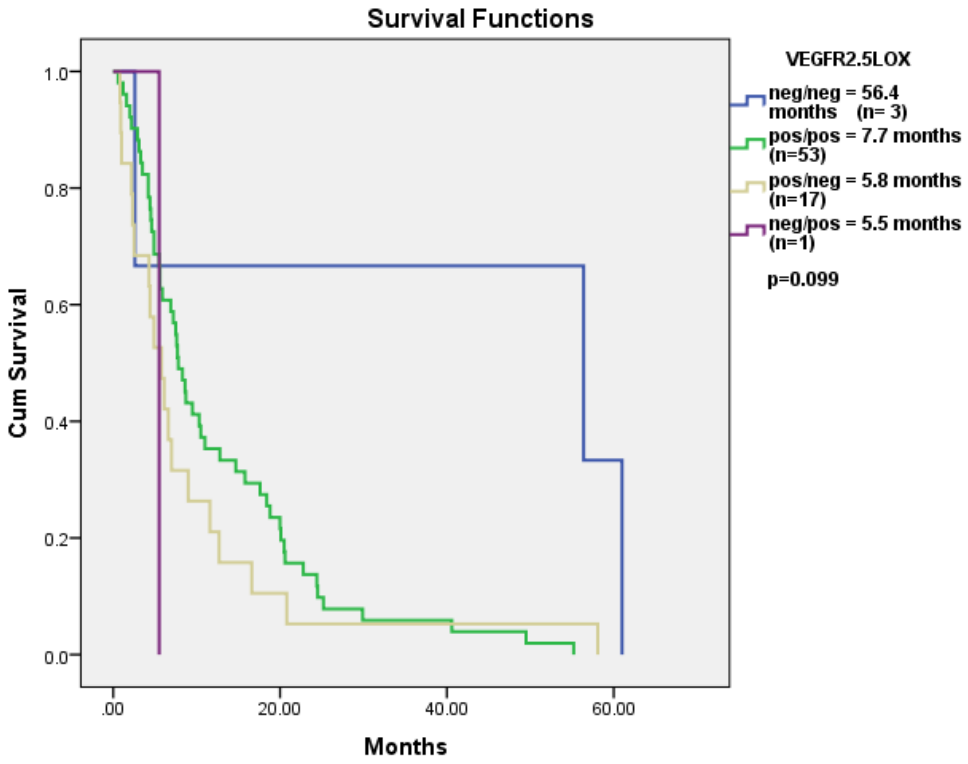


Figure 6.6 Survival analysis for the co-expression of VEGFR-2 and 5-LOX. Kaplan Meier plot showing univariate analysis of combined VEGFR-2/5-LOX expression status (p=0.076, log rank). The median survival was 7.7 months in VEGFR-2 positive/5-LOX positive cases (green line), 5.8 months in VEGFR-2 positive/5-LOX negative cases (yellow line), 56.4 months in VEFGR-2 negative/5-LOX negative cases (blue line) and 5.5 months in VEGFR-2 negative/5-LOX positive cases (pink line).

Similar results were obtained when VEGFR-2 and 12-LOX co-expression was evaluated. 81.6% (62/76) of the samples were positive for both VEGFR-2 and 12-LOX but no significant correlation was observed (p=0.472). There was also no significant difference in survival times however the absence of both proteins favoured improved survival (61 months) in only one sample (Figure 6.7). Significant correlation was observed between age and co-expression of VEGFR-2 and 12-LOX (p=0.031). 90% (45/50) of tissue samples of patients that were more than 64 years expressed both VEGFR-2 and 12-LOX (Table 6.5).

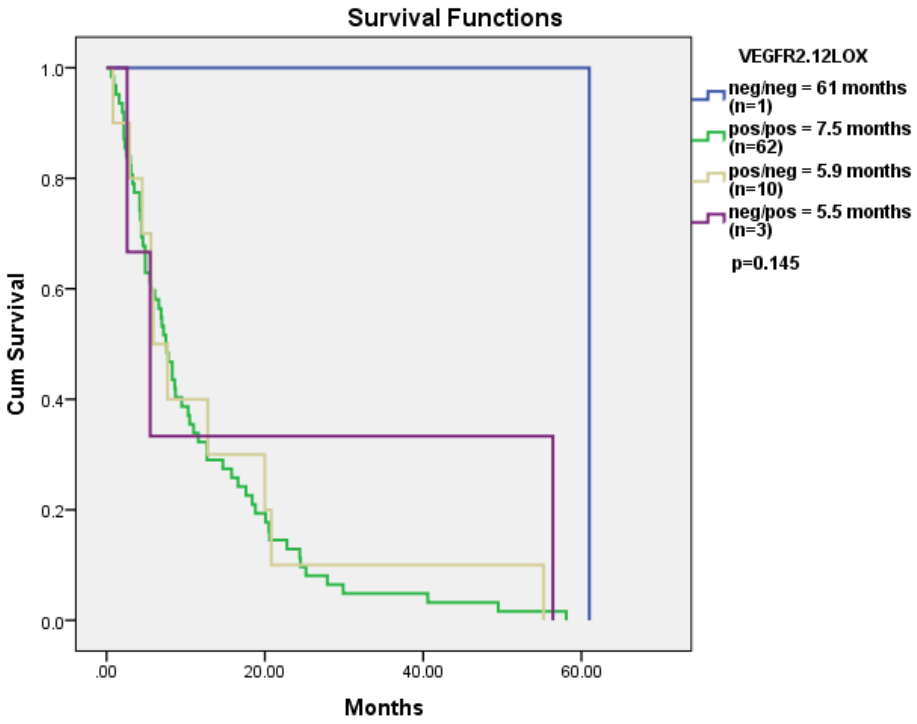


Figure 6.7 Survival analysis for the co-expression VEGFR-2 and 12-LOX. Kaplan Meier plot showing univariate analysis of combined VEGFR-2/12-LOX expression status (p=0.145, log rank). The median survival was 7.5 months in VEGFR-2 positive/12-LOX positive cases (green line), 5.9 months in VEGFR-2 positive/12-LOX negative cases (yellow line), 61 months in VEFGR-2 negative/LOX-12 negative cases (blue line) and 5.5 months in VEGFR-2 negative/12-LOX positive cases (pink line).

Table 6.5 Cross tabulation of age and samples that expressed both VEGFR-2 and12-LOX

Chi-square Test (2 sided) = 0.031	VEGFR-2.12LOX				Total
	Both absent	Both present	VEGFR-2 positive/12-LOX negative	VEGFR-2 negative/12-LOX positive	
Age ≤ 64	1	17	7	1	26
Age >64	0	45	3	2	50

6.4.2 Western blot analysis

VEGFR-2 protein was strongly expressed in the HUVEC cell line with an expected molecular weight of 230 kDa but absent in A549, NCI-H2452, NCI-H2052 and MSTO-211H cell lines (Figure 6.8). HUVEC cells are positive for VEGFR-2 and were recommended as a positive control by the antibody manufacturer. The loading control (α-tubulin) indicates that protein was correctly loaded onto the gel. To optimize the antibody, 20 µg and 40 µg cell lysates were loaded in different experiments.

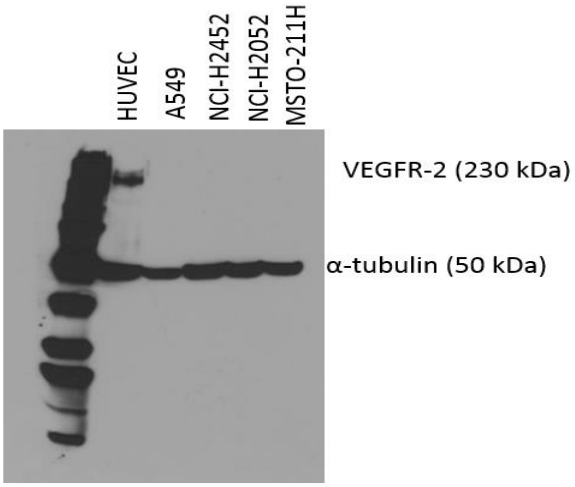


Figure 6.8: Western blot image of VEGFR-2 protein expression. The western blot showed the expression of VEGFR-2 protein (230 kDa) in the human umbilical vein endothelial cells (HUVECs) used as positive control. A nitrocellulose membrane with 20 µg of protein from mesothelioma cells, A549 and HUVEC was incubated with anti-VEGFR2 (#2479, Cell signalling) antibody at 1:250 dilution overnight at 4^0C. VEGFR-2 expression was not observed in the A549 and mesothelioma cell lines with this antibody.

6.4.3 MTS ASSAY

To determine the effective dose for 50% cell growth inhibition in A549, NCI-H2452, NCI-H2052 and MSTO-211H, a cell proliferation assay was carried out using the MTS assay with a VEGFR-2 inhibitor (cediranib) and a multitarget inhibitor (MGCD265). The inhibitory dose of each inhibitor was determined based on individual IC50 values after 72 hour treatment. Both inhibitors reduced cell growth in a dose-dependent manner (Figure 6.9 and Figure 6.10). Cediranib reduced cell growth by 50% with IC50 values ranging from 0.75 µM -3.6 µM. MGCD-265 reduced cell viability by 50% with IC50 values of 12.4 µM, 13 µM, 15.5 µM and 19 µM in MSTO-211H, A549, NCI-H2452 and NCI-H2452 respectively (Table 6.6).

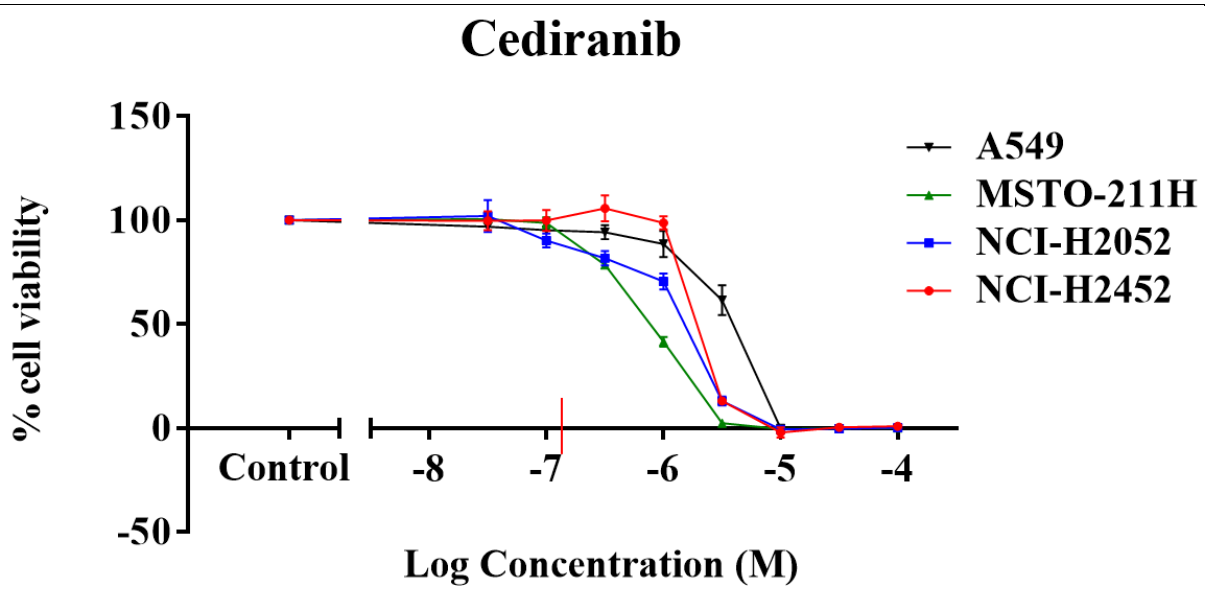


Figure 6.9 Antiproliferative effect of Cediranib (VEGFR-1/2/3 inhibitor). The data presented is the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate the mean and standard error of the mean of six replicates. The IC50 value for cediranib was achieved in each cell line. The cmax for cediranib is 155 nM (70 ng/mL) (red line) (Table 6.6).

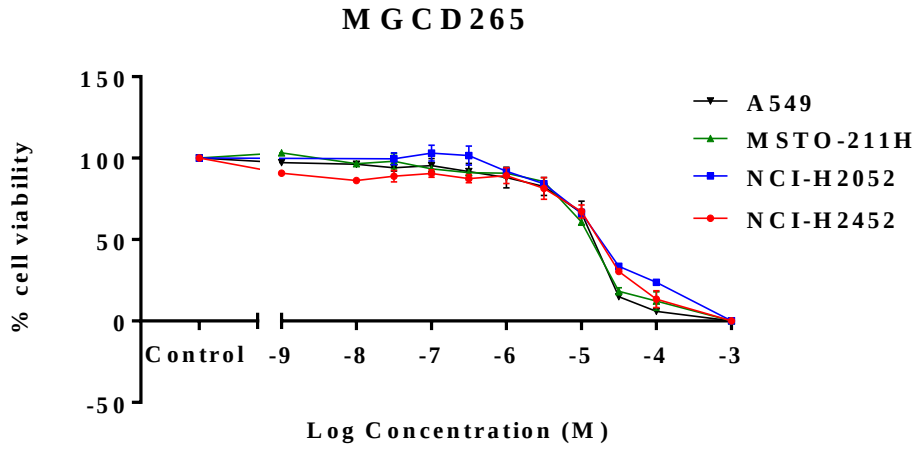


Figure 6.10: Antiproliferative effect of MGCD-265 (VEGFR, c-MET, Tie and Ron inhibitor). The data presented is the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate the mean and standard error of the mean of six replicates. The IC50 value for MGCD-265 was achieved in each cell line. The cmax for MGCD-265 is not known (Section 4.3.5.2).

Table 6.6 IC50 Values of Cediranib and MGCD-265 in MPM and A549 cells.

	Cediranib **cmax = 155 nm** (Zhu et al., 2013)	**MGCD265** **Cmax unknown**
Cell line	**VEGFR-2 inhibitor**	**VEGFR1/2/3, MET, Ron, Tie inhibitor**
A549	3.6 µM	13 µM
NCI-H2452	2.2 µM	15.5 µM
NCI-H2052	1.4 µM	19 µM
MSTO-211H	0.75 µM	12.4 µM

6.5 DISCUSSION

Immunohistochemistry

In the present study, immunohistochemistry revealed cytoplasmic staining in the mesothelioma and benign pleura biopsies. We demonstrated that VEGFR-2 is expressed in all benign pleura samples and the presence of endothelial cells served as internal control. RT-PCR and immunohistochemical analysis previously demonstrated the presence of VEGFR-2 mRNA and protein in normal pleural tissue samples in line with our observation (Ohta et al., 1999). In contrast, Soini et al (2001) reported the absence of VEGFR-2 in non-neoplastic pleural mesothelial cells (Soini et al., 2001). These result variations may be attributable to difference in antibody and immunohistochemistry techniques. We also demonstrated that VEGFR-2 protein is expressed in 93.8% (75/80) of all MPM tissue samples. Previous studies have also reported VEGFR-2 expression in 67-100% of MPM (Ohta et al., 1999; Soini et al., 2001; Strizzi et al., 2001; Nutt et al., 2009; Loganathan et al., 2011; Miettinen et al., 2012). The presence of VEGFR-2 protein expression was not found to be significant in univariate and multivariate analyses. All three histological types of mesothelioma tissue showed positive staining. The positive rate was 95.8% (23/24) in the biphasic/mixed, 93% (40/43) in the epithelial and 92.3% (12/13) in the sarcomatoid types (Table 6.3). Overall, there was no significant difference observed in median survival times between VEGFR-2 negative and positive cases (7.5 months in VEGFR-2 positive cases *vs* 5.5 in VEGFR-2 negative cases; p=0.087). However, in the epithelial subtype, the absence of VEGFR-2 was significantly

associated with improved survival (p=0.045) (Figure 6.4), although the number of negative cases in this subtype was only three therefore conclusions are drawn with caution and a larger series would be required to validate this finding. Cytoplasmic and membranous expression patterns for VEGFR-2 in MPM have been previously described in MPM and protein expression was found to be predominant in the epithelial subtype. 92% of epithelial samples was VEGFR-2 positive but all 6 sarcomatoid mesotheliomas were negative (Miettinen et al., 2012). VEGFR-2 expression level was also observed to be higher in MPM tumours than in normal pleura tissue, this was in accordance with our findings (Ohta et al., 1999). Similarly, Loganathan et al (2011) also reported that VEGFR-2 and its ligand VEGF, were strongly expressed in the epithelial subtype but moderate in the sarcomatoid subtypes when compared with the control tissue (Loganathan et al., 2011). Our study did not reveal any difference in staining intensity between the three histological subtypes in VEGFR-2 positive samples.

We next determined whether VEGFR-2 expression was associated with the expression of inflammatory mediators COX-2, 5-LOX and 12-LOX previously discussed in chapter 6. We also investigated whether their co-expression has an association with survival. COX-2 has been reported to be involved in angiogenesis and influence the expression of VEGF and its receptor VEGFR2 via the stimulation of prostaglandins specifically PGE_2, PGI_2 and thromboxane A_2 (TXA_2). Direct correlation has been reported between COX-2 and VEGFR2 in biopsies of colon, cervical and hepatocellular carcinomas (Xie & Yuan, 2006; Nagy et al., 2011). To the best of our knowledge, this is the first study that has evaluated the co-expression of VEGFR-2, 5-LOX and 12-LOX within MPM tissue samples. This study showed that 62.5% (50/80) of the samples were positive for both COX-2 and VEGFR-2; 71.6% (53/74) expressed VEGFR-2 and 5-LOX and 81.6% (62/76) were immunoreactive for both VEGFR-2 and LOX-12. There was a significant difference observed in survival times when the samples were immunoreactive for COX-2 but VEGFR-2 negative compared to when both proteins were absent (Figure 6.5); although the limitation of this result is the small sample number in the VEGFR-2 negative/COX-2 positive subgroup. A significant correlation was observed between age and co-expression of VEGFR-2 and 12-LOX. Patients less than 64 years had improved survival (7.6 months) than older patients (6 months) (p=0.03) and 90% (45/50) of patients in the older age group (age > 64) had a co-expression of both proteins. This could suggest that both proteins are associated with poor survival in patients older than 64 but it is not possible to draw firm conclusion as only one sample was negative for both

proteins. Further studies on the expression and correlation of these proteins in mesothelioma are warranted in larger tissue, serum and pleura effusion samples.

Western blot

We were unable to show VEGFR-2 expression in mesothelioma cell lines and A549 using the monoclonal antibody purchased. The NCI-H2052, MSTO-211H and NCI-H2452 cell lines have previously been reported to express VEGFR-2 protein using a different antibody and larger quantity of protein lysates (80µg) (Loganathan et al., 2011). Other authors have also demonstrated the expression of VEGFR-2 in the same cell lines using antibodies different from ours at protein and mRNA levels. VEGFR-2 mRNA expression was reported in NCI-H2052 and MSTO-211H cells but was not found in NCI-H2452 (Ogino et al., 2008). Similarly, VEGFR-2 mRNA expression was observed in MSTO-211H and NCI-H2052 cells but only the NCI-H2052 cells expressed VEGFR-2 at protein level (Masood et al., 2003). Loganthan et al (2011) demonstrated the expression of VEGFR-2 in all three mesothelioma cells. Likewise, A549 has also been shown to express VEGFR-2 at protein level. Both studies that showed VEGFR-2 protein expression used 50-80 µg of protein lysates (Masood et al., 2003; Loganathan et al., 2011). The high quantity of protein load could result in gel saturation particularly for loading controls resulting in inconsistent densitometric data analysis. Several factor could result in the variations observed between these studies and ours for example, the use of different antibodies, quantity of lysates and techniques across research groups, the condition and source of the cell lines.

MTS assay

We have also evaluated the antiproliferative effect of the multiple tyrosine kinase inhibitor MGCD265 (VEGFR, c-MET, Tie-2 & Ron inhibitor) in mesothelioma cells and A549. MGCD265 was selected based on its broad inhibitory effect and to the best of our knowledge there is no published preclinical data evaluating the effect of MGCD265 in mesothelioma. In other cancers, little is known of its antiproliferative effect however, it was well tolerated in all doses administered in the Phase I study in patients with advanced malignancies (Kollmannsberger et al., 2009). Cediranib (VEGFR1-3 inhibitor) had significantly lower IC50 values in all cell lines compared to MGCD265. However, the IC50 of cediranib was up to 14-fold greater than its maximum tolerated dose of 155 nM. This could be attributed to the absence of VEGFR-2 in the cell lines such that cediranib is unable to bind to the extracellular domain of VEGFR-2 in order to inhibit VEGFR-2 signalling and cell growth. There are no preclinical studies in mesothelioma to compare our results with although cediranib was very

effective in colon, lung, prostate, ovary and breast tumour xenografts (Wedge et al., 2005). The high IC50 observed could be a reason for the limited activity seen in the phase II clinical trials that evaluated its effect as a single agent in mesothelioma since there was no preceding *in vitro* data. Multikinase inhibitors are thought to have a broad spectrum of antitumour activity over single kinase inhibitors although majority of the time the full molecular mechanism of action of inhibitors are usually not studied in order to fully understand their affinity for specific targets. Phase II trials using multikinase angiogenenic inhibitors have been futile not yielding much benefit in the overall survival and progression free survival time in mesothelioma patients. Vatalanib, sorafenib and sunitinib have shown limited activity in mesothelioma (Dubey et al., 2010; Jahan et al., 2012; Nowak et al., 2012). To the best of our knowledge there has been no published work to evaluate the effect of a VEGFR and c-MET multikinase inhibitor in mesothelioma. Although our result shows that mesothelioma cells are more sensitive to cediranib than MGCD265, we cannot draw firm conclusions because VEGFR-2 was not expressed in the cell lines and the maximum tolerated dose for MGCD265 is yet to be established. Downstream targeting of VEGFR-2 poses a lot of challenges since the signalling cascades are similar to those used by other growth factors. Future studies will evaluate expression of VEGFR-2, 5-LOX, COX-2 and 12-LOX in larger tissue and serum samples and also investigate the effect of co-inhibiting VEGFR-2 and COX-2 or 12-LOX or 5-LOX in mesothelioma cells. The absence of VEGFR-2 and 12-LOX was observed in one patient with a survival time of 61 months. Likewise the absence of VEGFR-2 and 5-LOX was observed in three patients with a survival time of 56.4 months. On the contrary, the absence of COX-2 and VEGFR-2 showed poor survival (4.1 months; n=3) while better survival (56.4 months; n=2) was observed in patients with negative VEGFR-2 and positive COX-2 expression. COX-2 expression in our cohort of sample was a prognostic factor for improved survival however other studies have shown that it is associated with poor survival (Edwards et al., 2002; Baldi, 2004; O'Kane et al., 2005). Catalano et al (2004) demonstrated the ability of mesothelioma cells to evade celecoxib-induced apoptosis and Akt dephosphorylation when exposed to VEGF, the combination of celecoxib with a VEGF inhibitor (SU-1498) however restored the chemosensitivity of mesothelioma cells to celecoxib and the combination of both inhibitors yielded a synergistic effect *in vitro* (Catalano et al., 2004). Celecoxib treatment significantly reduced metastasis, growth and tumour angiogenesis in human pancreatic adenocarcinoma *in vitro* and *in vivo* by suppressing the activity of Sp1-binding sites on the VEGF promoter hence lowering the expression of

VEGF (Wei et al., 2004). In colon and breast cancer experimental models, dual COX-2/VEGF pathway inhibition was significantly more effective than monotherapy. Initiating dual therapy after surgical resection of the primary orthotopic breast tumours reduced metastasis and improved overall survival suggesting the possibility of the combination in an adjuvant setting (Xu & Croix, 2014; Xu et al., 2014). Similarly, in MCF-7 and MDA-MB-231 breast cancer cells lines, combination of tamoxifen with celecoxib decreased VEGF levels by 2-folds compared to treatment with tamoxifen alone (Kumar et al., 2013).

In conclusion, we demonstrated that a significant proportion of MPM tissue samples express VEGFR-2 in addition to COX-2, 5-LOX and 12-LOX proteins and mesothelioma cells and A549 cells were more sensitive to cediranib than MGCD265 based on the IC50s observed. There is still a need for a better understanding of the role of the VEGF family in the pathogenesis of mesothelioma and a need to identify biomarkers that will predict response to antiangiogenic therapies.

CHAPTER SEVEN

HER2 EXPRESSION IN MESOTHELIOMA AND A POSSIBLE ROLE FOR AFATINIB IN THE TREATMENT OF MPM

Chapter 7 HER2 expression in mesothelioma and a possible role for Afatinib in the treatment of MPM

7.1 EGFR and HER2 expression in MPM

As discussed in Chapter 2 of this report, there is substantial evidence indicating the role of the EGFR superfamily (particularly EGFR or HER2) in promoting tumour proliferation and metastasis. In addition, aberrant expression of EGFR or HER2 has been linked to resistance to chemotherapy and poor survival in patients with lung, oesophageal, breast and ovarian cancers (Laskin & Sandler, 2004; Nguyen & Schrump, 2004). Epidermal Growth Factor Receptor (EGFR) protein is frequently over-expressed in malignant mesothelioma and has been reported in 44 to 97% of mesotheliomas (Table 7.1) (Agarwal et al., 2011). Although EGFR is upregulated, there are conflicting reports on the correlation of EGFR expression and patient survival. Using immunohistochemistry Destro et al (2006), Okuda et al (2008) and Gaafar et al (2010) reported the absence of a relationship between patient outcome and EGFR over-expression (Destro et al., 2006; Okuda et al., 2008; Gaafar et al., 2010). Employing the same technique, Dazzi et al (1990), O'Byrne et al (2004), Edwards et al (2006) and Kothmaier et al (2008) reported conflicting evidences that EGFR over-expression has a correlation with improved survival but was not seen as an independent prognostic indicator (Dazzi et al., 1990; J G Edwards et al., 2006; Kothmaier et al., 2008) (Table 7.1).

EGFR up regulation is frequently observed in epithelioid mesotheliomas which are known to be associated with favoured outcome and result variations could be attributed to the subjective nature of the immunohistochemical technique applied ranging from antibody disparity to scoring systems. Studies have also shown inconsistent results for the expression of ERBB2 (HER2) in mesothelioma. Horvai et al (2003) reported the expression of HER2 protein in 70% (26/37) of mesothelioma cases however increased expression did not correlate with histologic subtype. There was also no significant difference in HER2 mRNA expression between tumours that showed strong cytoplasmic staining for HER2 and negative tumours. Irregularities were observed between the two antibodies used for immunohistochemical analysis and increased immunoreactivity did not correlate with gene amplification (Horvai et al., 2003). A study by Thirkettle et al also demonstrated HER2 protein expression in 97% (28/29) of malignant mesothelioma samples and in keeping with the studies of Horvai et al gene expression was not associated with genomic DNA amplification. (Thirkettle et al., 2000) (Table 7.2).

Table 7.1 EGFR protein overexpression in MPM by immunohistochemistry

Study	No. of cases	Protein	Histological Subtype	Antibody used	Expression levels	Prognostic status
(Enomoto et al., 2012)	22	EGFR	Epithelial – 16 Biphasic – 5 Sarcomatoid - 1	EGFR pharmDx (clone 2-18C9) (K1492 Dako-Cytomation)	21/22 (95.4%)	No
(Rena et al., 2011)	83	EGFR	Epithelial – 57 Biphasic – 20 Sarcomatoid - 6	EGFR antibody clone H11 (Dako, Denmark)	70/83 (84%)	Yes
(Gaafar et al., 2010)	71	EGFR	Epithelial – 39 Biphasic – 19 Sarcomatoid – 7 Not specified - 2	EGFR pharmDx (clone 2-18C9 Dako-Cytomation)	53/71 (74.65%)	No
(Okuda et al., 2008)	25	EGFR	Epithelial – 12 Biphasic – 8 Sarcomatoid – 4 Desmoplastic - 1	EGFR pharmDx (clone 2-18C9 Dako-Cytomation)	17/25 (68%)	No
(Garland et al., 2007)	57	EGFR	Not indicated	EGFR antibody clone 31G7 (Zymed Laboratories Inc)	43/57 (75%)	No
(Edwards et al., 2006)	168	EGFR	Epithelial – 98 Biphasic – 37 Sarcomatoid – 33	EGFR 113 (Novocastra Laboratories Ltd., Neswcastle, UK)	74/168 (44%)	No
(Destro et al., 2006)	61	EGFR	Epithelial – 50 Biphasic – 9 Sarcomatoid - 2	EGFRAb-10 (clone 111.6) (Neomakers, Union City, CA)	34/61 (55.7%)	No
(Dazzi et al., 1990)	34	EGFR	Epithelial – 16 Biphasic – 9 Sarcomatoid - 9	F4 monoclonal antibody	23/34 (68%)	No

Table 7.2 HER2 protein overexpression in MPM by immunohistochemistry

Study	No. of cases	Histological Subtype	Antibody used	Expression levels	Prognostic status
(Horvai et al., 2003)	37	Epithelial – 19 Biphasis – 15 Sarcomatoid – 3	NCL-CB11 (Novocastra, UK) and Herceptin (Dako)	26/37 (70%) 2/37 (9%)	No No
(Thirkettle et al., 2000)	29	Epithelial	NCL-CB11 (Novocastra, UK)	28/29 (97%)	No

7.1.1 EGFR therapies in MPM

Targeting EGFR with small tyrosine kinase inhibitors (TKIs) or anti-EGFR antibodies has been effective in the treatment of several solid tumours such as lung, pancreatic, head and neck, thyroid, breast and colon cancers. Drawing from the large body of evidence for the overexpression of EGFR in mesothelioma and the success of targeted therapies in the treatment of other cancers, two phase II non randomised clinical trials were conducted by two independent research groups using two 1st generation EGFR tyrosine kinase inhibitors (Gefitinib and Erlotinib) as first line therapy in MPM. The CALGB 30101 Phase II trials enrolled 43 untreated mesothelioma patients (42 pleural, 1 peritoneal) with good performance status to evaluate the effect of Gefitinib as a single agent. The primary endpoint was the percentage of patients who remained alive and progression-free in three months. The expected percentage was ~60% but the observed 3-month progression-free survival was 40% (95% CI, 25-56%) (Govindan et al., 2005). The second Phase II trial enrolled 63 chemotherapy-naïve MPM patients with performance status 0-1 who were treated with Erlotinib. The primary objective was to measure survival outcomes. Thirty-three patients had measurable disease, among which four patients had inadequate assessments. There were no objective responses however 42% (14/33) had stable disease and 45% (15/33) had disease progression (Garland et al., 2007). Results from these two trials dampened the hope for EGFR TKIs in the treatment of mesothelioma indicating that EGFR inhibitors might not be useful as single agents in this malignancy (Govindan et al., 2005; Garland et al., 2007). EGFR protein expression was evaluated in both studies using immunohistochemistry but did

not predict response to the inhibitors. Mutations in the EGFR TKI domain and amplification of the EGFR gene were not assessed in both trials (Lynch et al., 2004; Paez et al., 2004) since they have been shown to be uncommon in mesothelioma (Section 2.2.1.3); this and other study limitations could be plausible reasons for the disappointing results observed.

Recently, second and third generation EGFR inhibitors have been developed and have been shown to overcome the limitations of first-generation EGFR TKIs by irreversibly binding to the catalytic domain of the receptors hence increasing their efficacy and preventing resistance (Hirsh, 2011). Afatinib was recently approved for the treatment of NSCLCs with EGFR mutation. *In vitro* studies have demonstrated that Afatinib is not only effective in lung cancer cells harbouring EGFR mutations alone but also in the wild type isoforms. In addition, Afatinib was observed to be more effective that 1st generation EGFR inhibitors (Li et al., 2008).

The RAS pathway is frequently dysregulated in several cancers including mesothelioma (Figure 7.1) (de Melo et al., 2006). MEK1 and MEK2 are essential downstream effectors of the RAS/MAPK pathway which regulate protein acetylation, translation, transcription, lipid metabolism, proliferation and survival via ERK1/2 substrates. Since the emergence of the first MEK inhibitor in 1995 only few have progressed into clinical trials (Akinleye et al., 2013). MEK inhibitor U0126, has been shown to induce cell cycle arrest and apoptosis in MPM *in vitro* and in *vivo* (Miyoshi et al., 2012). In addition studies have shown that this inhibitor also repress proangiogenic factors in MPM cells (Cole et al., 2006). Selumetinib, a MEK1/2 inhibitor is currently in phase II trials for several cancers including NSCLC as single therapy and in combination with other chemotherapeutic agents but little or no preclinical studies have been reported in mesothelioma.

Currently only two studies have examined the expression of HER2 in MPM tissue samples using immunohistochemistry and with relatively small numbers (Table 7.2). Second-generation EGFR inhibitors have also not been well studied in mesothelioma. Investigating HER2 expression in a larger cohort of mesothelioma samples might reveal possible correlations with clinicopathological factors. Furthermore, second-generation EGFR inhibitors or targeting downstream signalling proteins of the MAPK pathway might confer enhanced cytotoxic effect on mesothelioma cells than first generation inhibitors (Figure 7.1).

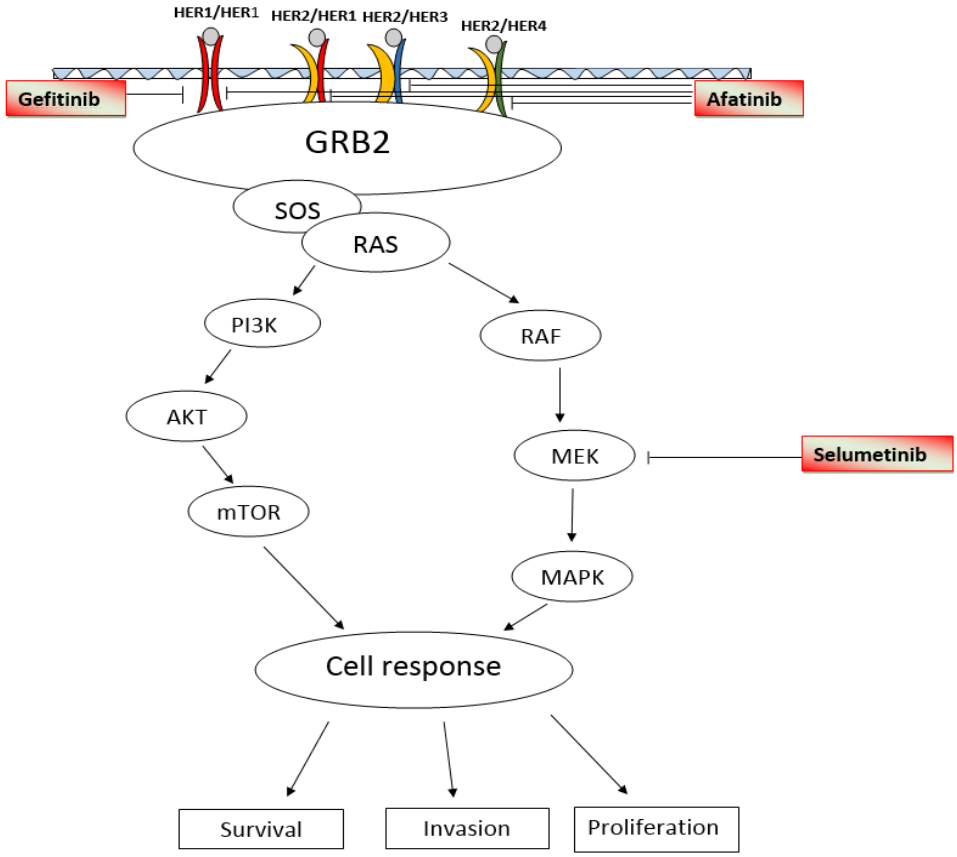


Figure 7.1: EGFR signalling and inhibitors. Upon ligand binding, the EGF receptors form homo or hetero dimers with other members of the EGFR (HER) family resulting in the activation of the two major downstream signalling cascades; the PI3K/mTOR and RAS/MAPK pathways. Gefitinib is a first generation EGFR inhibitor, Afatinib is a second generation EGFR inhibitor with strong affinity for EGFR 1&2 but also inhibits EGFR 3&4. Selumetinib is a MEK inhibitor.

7.2 Aims

In this study, we aimed to:

- Assess the expression of HER2 protein in our archival MPM tissue samples using immunohistochemistry and evaluate its effect on patient survival
- Assess the expression of EGFR protein by western blotting in MPM cell lines using A549 cells as positive control
- Examine the effect of Gefitinib, Selumetinib and Afatinib on mesothelioma cell lines using MTS assay

7.3 Materials and Methods

Eighty archival tissue samples as described in section 5.1 were used to determine the expression of the HER2 protein. Benign pleura tissue samples were also included in the cohort.

MPM cell lines NCI-H2452, NCI-H2052 and MSTO-211H were obtained from the American Type Culture Collection and the non-small cell lung cancer cell line (A549) was obtained from the European Collection of Cell Cultures. All cell lines were maintained in RPMI 1640 medium as detailed in section 5.4.1. Afatinib (HER2/EGFR inhibitor) and Selumetinib (MEK inhibitor) were purchased from Selleckchem as discussed in Section 4.3.2.1 and Section 4.3.2.3 respectively. Gefitinib (cat no. 3000) was purchased from Tocris Bioscience (Section 4.3.2.2). MTS reagent was obtained from Promega (Cell Titre 96® Aqueous Solution Cell Proliferation Assay; G582; Promega. Madison, WI).

7.3.1 Immunohistochemistry

Immunohistochemistry was performed using the R.T.U. Vectastain Quick Kit (#PK-7800, Vector Laboratories Inc., CA, USA) as described in section 5.8 with monoclonal anti-HER2/neu primary antibody (15811A (554299), clone 3B5; Becton Dickinson) at a dilution of 1:35. Positive controls included in each run were a specimen of colorectal tissue samples which was known to be positive for HER2. Negative controls had the primary antibody omitted.

All slides were inspected at up to x400 magnification using light microscopy by two independent observers including a consultant histopathologist (Dr. Anne Campbell) blinded to clinicopathological data and outcome. Presence of cytoplasmic or membranous HER2 expression was noted if present in at least 25% of tumour cells. In cases where the observers differed in their assessment of HER2 expression, consensus was determined using a dual headed microscope and the assessment was based on the consultant's description as detailed in section 5.8.9.

7.3.2 Statistical analysis

Statistical analysis was performed using the IBM SPSS statistics software version 22 (SPSS, Chicago, USA). Survival curves were estimated using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between groups. Cox

proportional hazards models were used to identify statistically significant differences in survival and estimate hazard ratios and 95% Confidence Intervals.

7.3.3 Western blotting

Western blotting was performed as detailed in Section 4.9 to assess the status of the EGFR, p-ERK and ERK proteins using anti-EGFR (ab2430, Abcam), anti-p-ERK (sc-7383), anti-ERK (Sc-154) antibodies respectively. Briefly, 40 µg protein for each cell line was electrophoresed for 60 minutes alongside a standard western C marker on 4-20% gradient gels. The protein was then blotted onto 0.2 µm nitrocellulose membranes by using the iBlot system for 6 minutes. The membranes were blocked overnight in 5% milk solution (appendix B) and probed for each of the proteins (EGFR, p-ERK & ERK) at a 1:150, 1:500 and 1:200 dilution respectively for 16 hours at 4^{o}C. The membranes were washed 6 times for 5 minutes and a relevant secondary antibody (#SC-2030 or #SC-2031, Santa Cruz biotechnology) was applied at 1:1000 for 1 hour at room temperature. Bands were visualized using the supersignal west pico chemiluminescent substrate kit (#34080, Pierce) and x-ray films (#34090, Thermo scientific).

7.3.4 MTS assay

All cell lines were cultured as described in Section 4.4.1 and counted as detailed in Section 4.5. Cells were seeded onto a 96 well flat-bottom plate at 1000 cells per well in 100 µl of RPMI media as previously described in Section 4.6. MTS assay was performed as detailed in section 4.7.1.1. In each experiment, 6 replicate wells were used for each drug concentration and the experiment repeated 3 times. The inhibitors were diluted to 50 mM in 100% DMSO and further diluted to concentrations of 0 – 300 µM with RPMI media. The cells were subsequently exposed to the varying concentrations of Afatinib, Gefitinib and Selumetinib in different assays for 72 hours from Day 2. The final DMSO concentration in each well was maintained at 0.01%. Drugs and media were inspected daily to ensure optimum condition of growth for the cell lines. The percent growth of treated cells was calculated as a percentage of control cells (Section 5.7.1.2). Results from individual experiments were uploaded onto graphpad prism 6.0 and IC50 values were generated for each inhibitor.

7.4 Results

The characteristics of the 80 MPM archival tissue samples used within this study are shown in Table 7.3. The median age for the patients was 68 years (range 42-88 years). Seventy-three patients were males and 7 were females with a male to female ratio of 10.4:1. Median survival for all the cases was 6.9 months.

Table 7.3 Clinicopathological variable of MPM archival tissue sample assessed for HER2 protein expression

Characteristics	Number of cases (%)
Total	80 (100)
Age-median (range)	68 (42-88)
Median survival (months)	6.9
Gender	
Female	7 (9)
Male	73 (91)
Histology	
Epithelioid	43 (54)
Biphasic	22 (27)
Sarcomatoid	15 (19)

7.4.1 Immunohistochemistry

Colorectal tissue samples were used as positive and negative (antibody omitted) controls Figure 7.2 A&B). The benign pleural samples were used as reference slides for scoring. All benign pleura samples (n=8; 100%) were positive for HER2 with a granular cytoplasmic stain (Figure 7.2 C&D). Two scoring methods were used as a result of variation in staining intensity. A three category method was used to classify the specimens into negative, weak or strong cases for statistical analysis in this chapter and was later classified as negative or positive (inclusive of weak or strong expression). The three category method was carried out such that:

- 0 – negative (no staining or staining observed in <25% of tumour cells)
- 1+ – weak (staining present in > 25% of tumour cells with intensity less than that of the positive reference slide)
- 2+ – strong (staining present in > 25% of tumour cells with intensity equal to or greater than that of the positive reference slide)

HER2 expression was negative (0) in 14% (11/80), weak (1+) in 54% (43/80) and strong (2+) in 32% (26/80) (Table 7.4). The staining was also a granular cytoplasmic pattern in all positive cases (Figure 7.3). No membranous staining was observed. In the biphasic/mixed subtypes, majority of the spindle cell population were negative for HER2. The scoring was later categorised as positive (1) or negative (0) for further analysis in chapter 11. In the two category scoring method, weak (1+) and strong (2+) was classified as positive (1) and negative remained as (0). Based on the two category scoring, HER2 immunostaining was identified in 69 cases out of 80 (86.2%).

Table 7.4: Frequency of HER2 protein expression in MPM tumour samples

Samples	Total	HER2 staining score		
		0	1+	2+
All samples	80	11 (13.8%)	43 (53.7%)	26 (32.5%)
Epithelial	43	1 (2.3%)	22 (51.2%)	20 (46.5)
Biphasic	22	2 (9.1%)	16 (72.7%)	4 (18.1%)
Sarcomatoid	15	8 (53.3%)	5 (33.3%)	2 (13.3%)

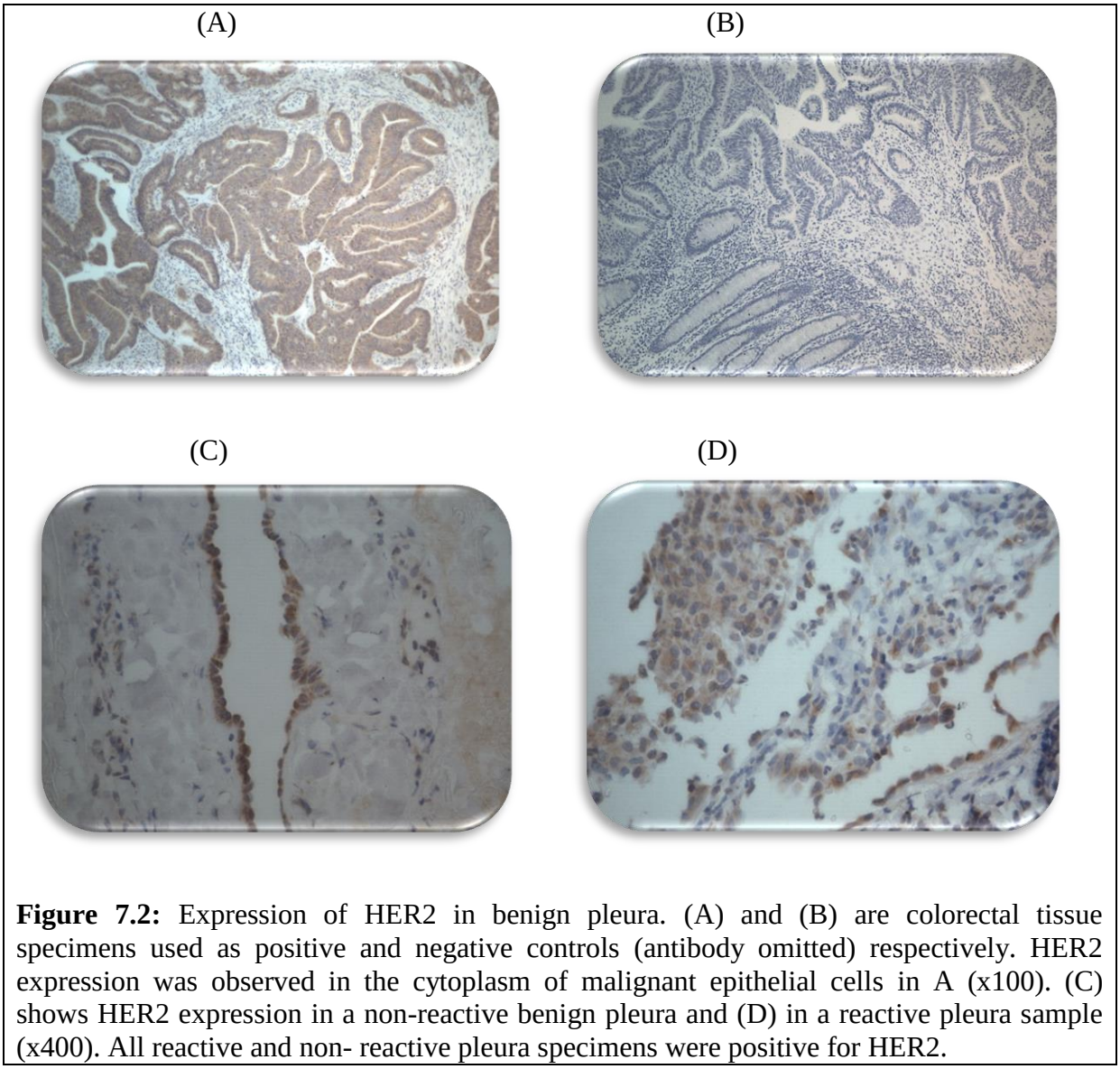


Figure 7.2: Expression of HER2 in benign pleura. (A) and (B) are colorectal tissue specimens used as positive and negative controls (antibody omitted) respectively. HER2 expression was observed in the cytoplasm of malignant epithelial cells in A (x100). (C) shows HER2 expression in a non-reactive benign pleura and (D) in a reactive pleura sample (x400). All reactive and non- reactive pleura specimens were positive for HER2.

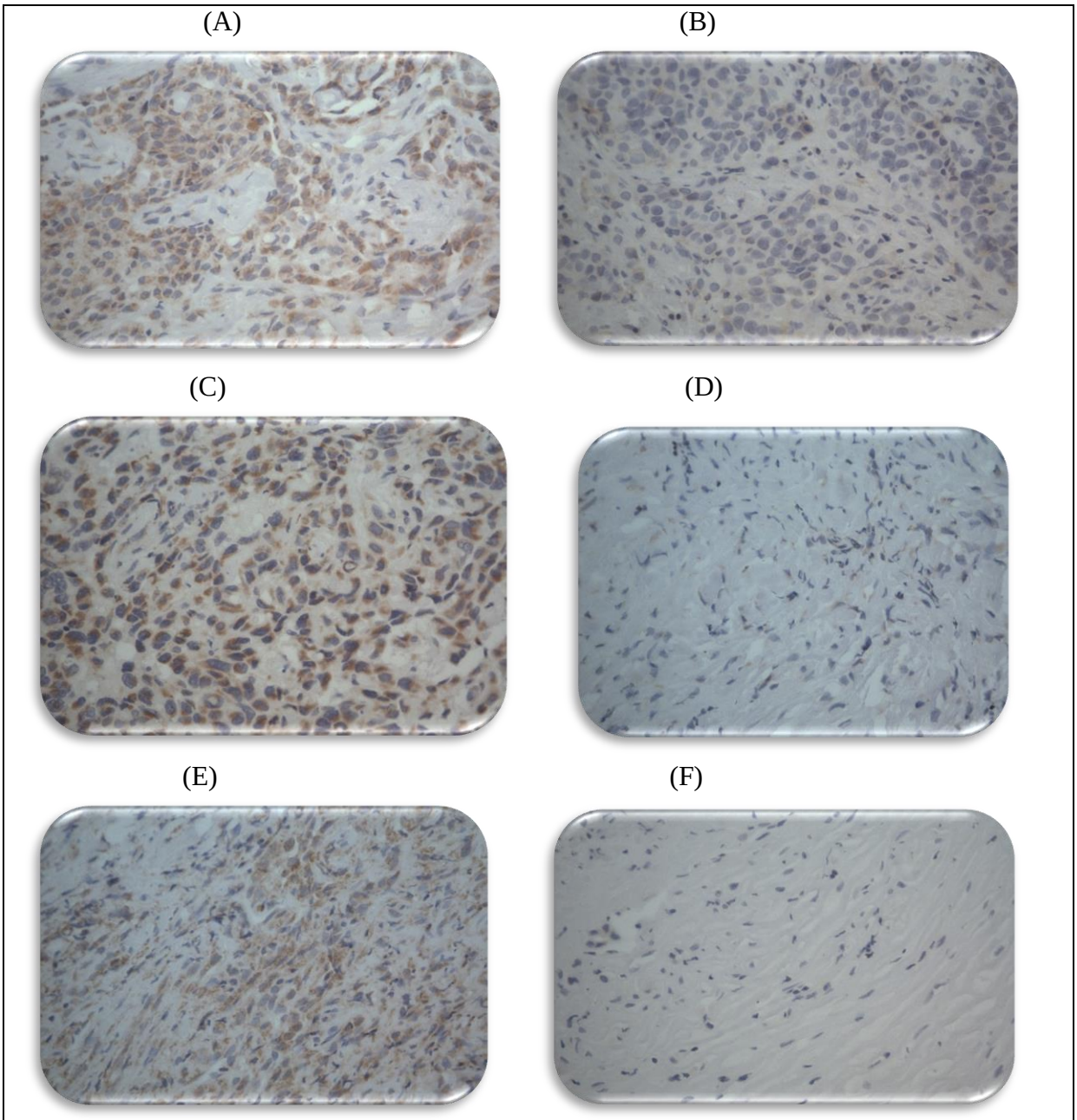


Figure 7.3: Expression of HER2 in mesothelioma tissue samples. HER2 positivity was observed in the cytoplasm. (A) Epithelioid mesothelioma subtype positive (2+) and (B) is negative for HER2; (C) biphasic mesothelioma positive (2+) and (D) is negative for HER2; (E) sarcomatoid mesothelioma positive (2+) and (F) negative for HER2. Immunoreactivity in <25% of malignant cells were scored as negative (0) and those >25% were scored positive for HER2. Magnification (x400)

7.4.1.1 Survival

Patients with strong (2+) HER2 immunopositive tumours had a median survival of 11.6 months (95% CI, 7-16), patients with weak (1+) HER2 immunoreactive tumours had a median survival of 6.1 months (95% CI, 4-8) and those with no (0) HER2 immunoreactivity had a median survival of 4.2 months (95% CI, 2-6) (p=0.006) (Figure 7.4). Multivariate analysis did not indicate that HER2 is an independent prognostic variable.

There was a significant difference observed in HER2 expression in the epithelial and sarcomatoid subtypes. Strong and weak expression of HER2 in epithelial mesotheliomas favoured improved survival by 17.6 months (n= 20) and 7.6 months (n=22) respectively. Only one case was negative for HER2 with a survival time of 2.6 months (Figure 7.5). In the sarcomatoid subtype, strong HER2 expression was significantly associated with poor survival (0.9 months; n=2) (Figure 7.6). As a result of the small number of cases (n=15) definite conclusions cannot be drawn on the role of HER2 expression in the sarcomatoid subtype but further studies are warranted in larger sample size.

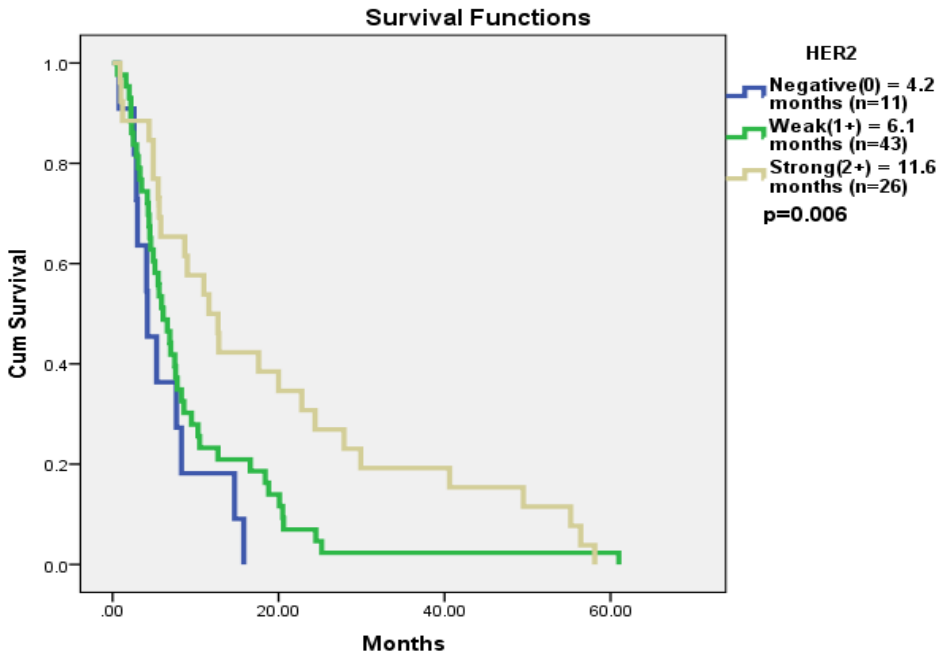


Figure 7.4: Survival analysis for HER2 protein expression in MPM cohort**.** Kaplan-Meier plot showing that strong (2+) HER2 expression (yellow line) was associated with improved survival (p=0.006).

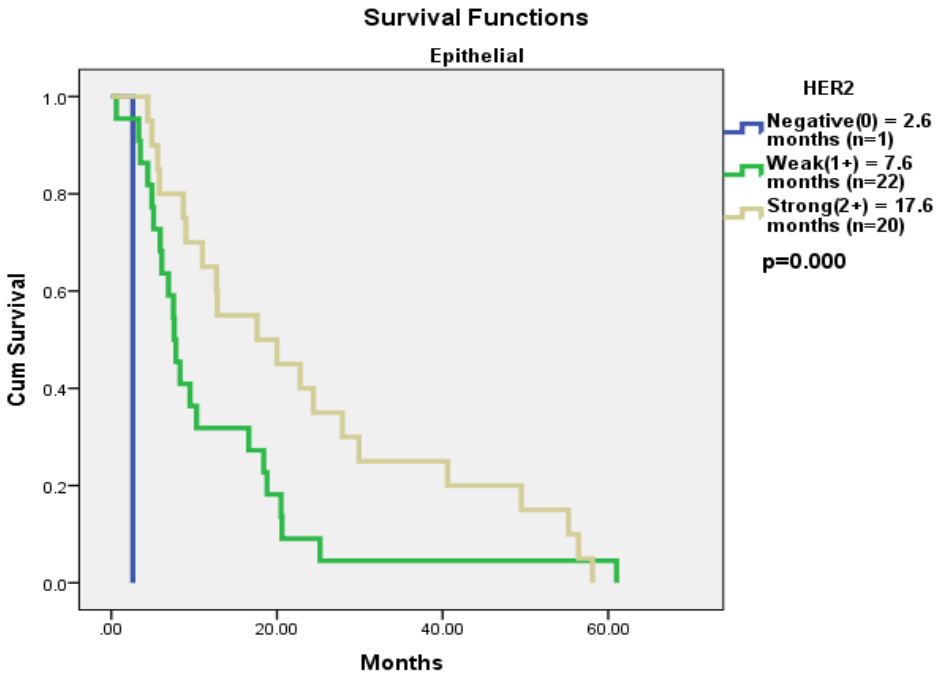


Figure 7.5: Survival analysis for HER2 protein expression in epithelial mesothelioma. Kaplan-Meier plot showing that strong (2+) HER2 expression improved survival in epithelial mesothelioma. Only one case was negative (blue line) in the epithelial subtype (p<0.001).

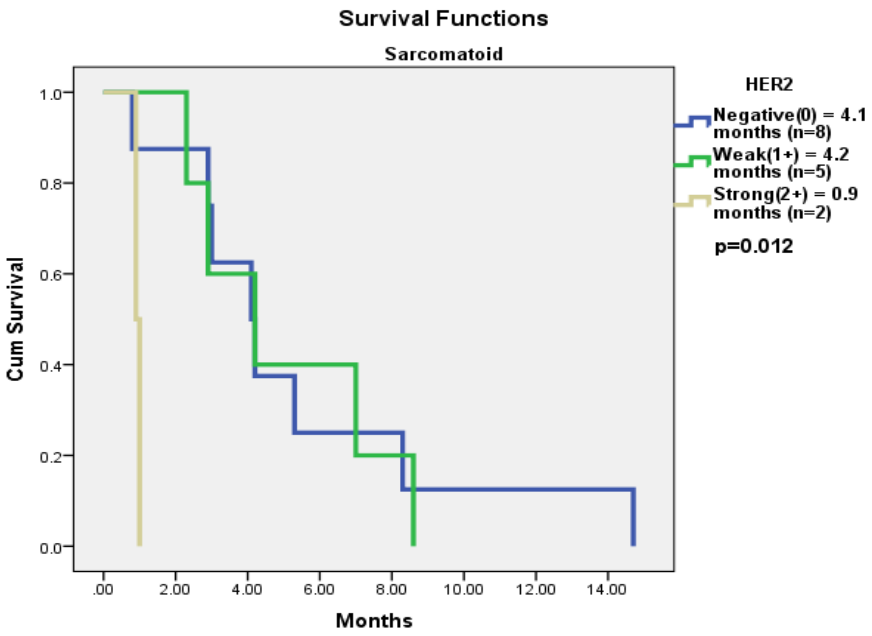


Figure 7.6: Survival analysis for HER2 in sarcomatoid mesothelioma. Kaplan-Meier plot showing that strong (2+) HER2 expression is associated with poor survival in sarcomatoid mesotheliomas. However only two cases showed strong immunoreactivity for HER2 protein (p=0.012).

7.4.2 Western blotting

Prior to subjecting the cells to treatment with selected EGFR inhibitors, each cell was assessed for the presence of EGFR, ERK2 and p-ERK protein. Alpha tubulin was also used as a loading control.

EGFR protein was present in A549 and the mesothelioma cell lines. MSTO-211H showed abundant EGFR expression in comparison with the other cell lines. Similar expression levels was observed for ERK2 in all cell lines. A549 and NCI-H2452 cells strongly expressed p-ERK 1/2 but the NCI-H2052 and MSTO-211H showed weak expression (Figure 7.7). ERK was also expressed in all cell lines but less abundant in MSTO-211H. The density of each band was calculated using the image J software (NIH) (Figure 7.8).

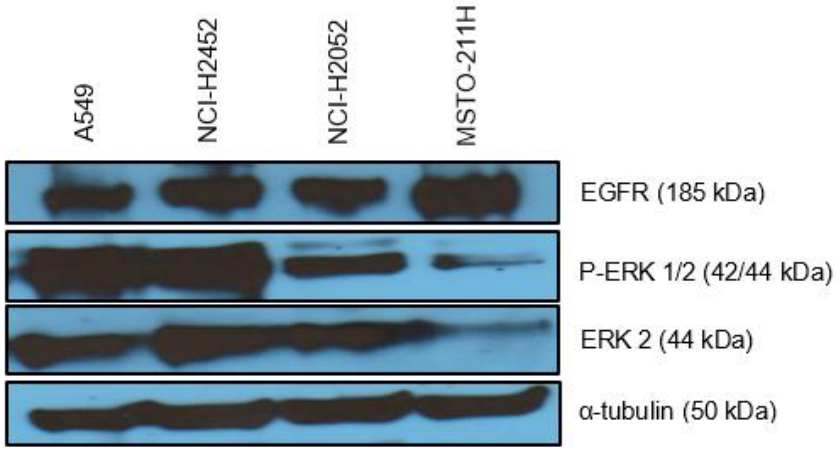


Figure 7.7 Western blot analysis of EGFR, P-ERK and ERK in MPM cells and A549. The western blots demonstrates the presence of EGFR protein in A549, NCI-H2452, NCI-H2052 and MSTO-211H cell lines. P-ERK 1/2 was observed to be abundant in A549 and NCI-H2452 cells. MSTO-211H showed a weak expression of ERK in its basal and phosphorylated form. The image shows results with anti-EGFR antibody (ab2430, Abcam) (1:150 dilution over 16 hours at 4^0C) at 185 kDa; anti-p-ERK 1/2 antibody (sc7383, SantaCruz) (1:500 dilution over 16 hours at 4^0C) at 42/44 kDa; anti-ERK2 antibody (sc154, SantaCruz) (1:200 dilution over 16 hours at 4^0C) at 44 kDa. Alpha tubulin (ab7291, Abcam) was used as loading control 50 kDa indicating equal loading**.**

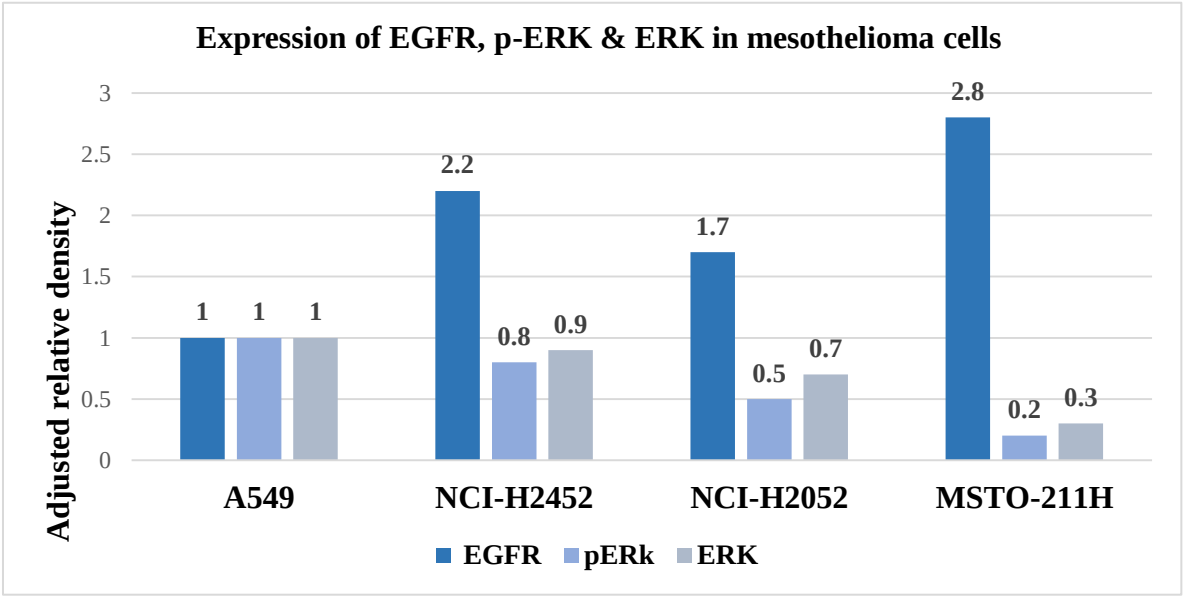


Figure 7.8 Relative density analysis of western blot bands by image J. The adjusted relative density of EGFR, p-ERK and ERK were calculated using A549 cells as control. The loading control was α-tubulin.

7.4.3 MTS assay

Following the confirmed activation of the EGFR pathway in the cell lines via western blotting, each cell line was subjected to varying doses of three inhibitors of the EGFR pathway using an MTS assay for 72 hours. The three inhibitors Gefitinib (EGFR), Selumetinib (MEK) and Afatinib (EGFR/HER2) were observed to reduce cell growth in all cell lines in a dose-dependent manner (Figure 7.9; Figure 7.10; Figure 7.11). Gefitinib reduced cell growth by 50% with IC50 values ranging from 3.9 µM – 16.8 µM. Selumetinib (MEK inhibitor) reduced cell viability by 50% with IC50 values of 25.3 µM, 36.1 µM, 84.2 µM and 40.4 µM in NCI-H2052, A549, NCI-H2452 and MSTO-211H cells respectively. Afatinib was more potent than Gefitinib and Selumetinib with IC50 values ranging from 1 µM – 3.1 µM. MSTO-211H cells were the most sensitive to Gefitinib and also showed high levels of EGFR protein expression. Response to Selumetinib and Afatinib was not dependent on the expression of EGFR or the downstream proteins. NCI-H2452 and MSTO-211H were the most sensitive to Afatinib and NCI-H2052 showed increased sensitivity to Selumetinib (Table 7.5).

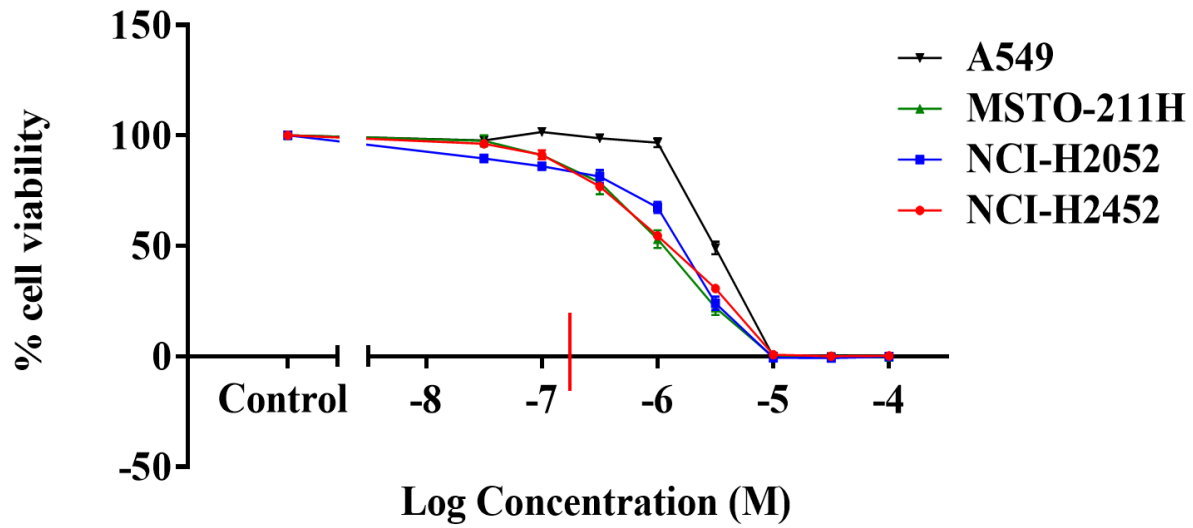


Figure 7.9 The antiproliferative effect of Afatinib. The data presented represents the mean of three independent experiments after 72 hours treatment. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. The cmax for afatinib is 131.7 nM (red line) (Table 7.5).

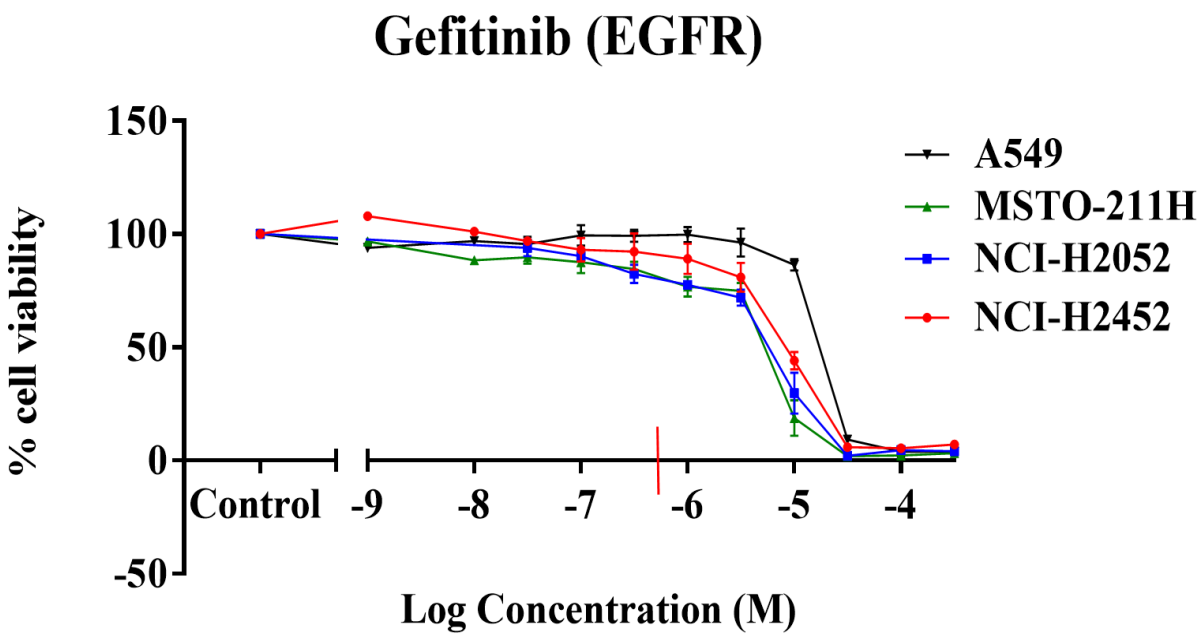


Figure 7.10 The antiproliferative effect of gefitinib. The data presented represents the mean of three independent experiments 72 hours treatment. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. The cmax for gefitinib is 340.5 nM (red line) (Table 7.5).

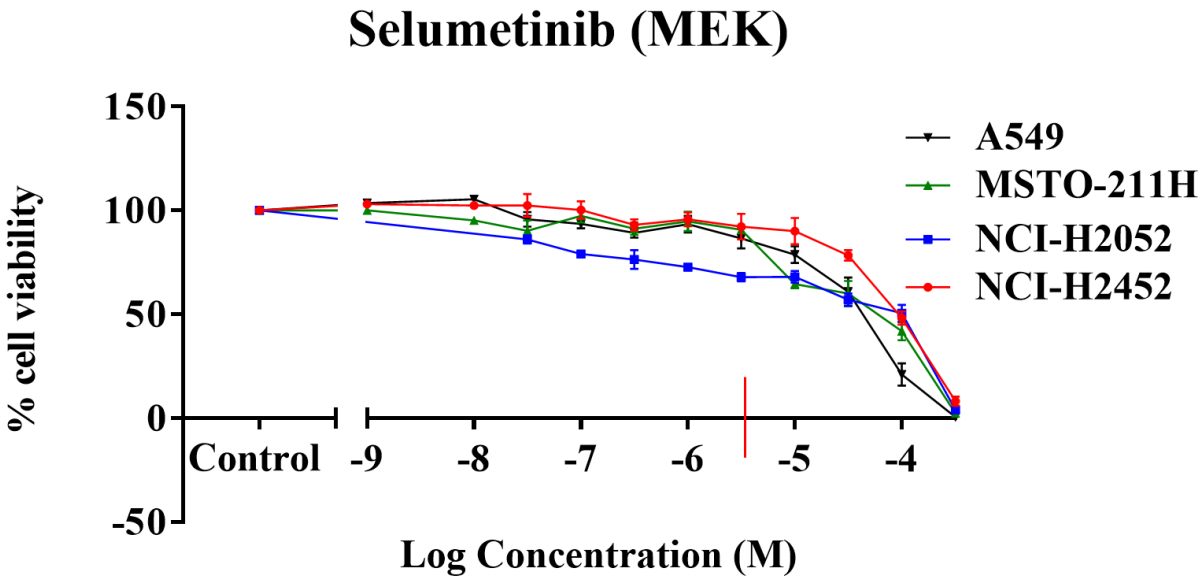


Figure 7.11 The antiproliferative effect of selumetinib. The data presented represents the mean of three independent experiments 72 hours treatment. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. The cmax for selumetinib is 3.5 µM (red line) (Table 7.5).

Table 7.5: IC50 values of Gefinitib, Selumetinib and Afatinib in MPM and A549 cells.

Cell line	Gefitinib **EGFR inhibitor** **cmax = 340.5 nM** (Swaisland et al., 2005)	Selumetinib **MEK inhibitor** **cmax = 3.5 µM** (O'Neil et al., 2011)	Afatinib **EGFR/HER2 inhibitor** **cmax =131.7 nM** (Gordon et al., 2013)
A549	16.8 µM	36.1 µM	3.1 µM
NCI-H2452	7.8 µM	84.2 µM	1 µM
NCI-H2052	4.6 µM	25.3 µM	1.4 µM
MSTO-211H	3.9 µM	40.4 µM	1 µM

Table 7.6 EGFR, ERK and p-ERK expression status in A549 and mesothelioma cells.

Cell lines	EGFR protein	ERK protein	p-ERK protein
A549	Expressed	Expressed	Expressed
NCI-H2452	Expressed	Expressed	Expressed
NCI-H2052	Expressed	Expressed	Expressed
MSTO-211H	Expressed	Expressed	Expressed

7.5 Discussion

Immunohistochemistry

In this study, we demonstrated that all benign pleura samples (n=8; 100%) expressed HER2 protein and granular cytoplasmic stain was observed in each one. We also demonstrated that HER2 protein expression was weakly expressed in 53.7%, strongly expressed in 32.5% and absent in 13.8% of MPM tissue samples when compared to the reference slides which were the benign pleura samples (Figure 7.2). We did not observed any membranous staining in any of the tissue samples. Our scoring system was similar to the one previously described by Horvai et al (2003) as the Herceptest score however, we chose 25% cytoplasmic positivity in

tumour cells as our cut off. The Cawkwell research group have also published using similar scoring system (Agarwal et al., 2013). Previous immunohistochemical studies have demonstrated HER2 expression in MPM tissue samples. A study by Thirkettle et al (2000) reported HER2 expression in 97% (28/29) of malignant mesothelioma. A large proportion of their tissue samples displayed surface membrane staining (76%) and internal granular or vesicular staining was observed in nearly all samples (93%; 27/29) (Thirkettle et al., 2000). Horvai et al (2003) investigated HER2 expression in 37 MPM tissue samples using two antibodies; NCL-CB11 (Novocastra) and Herceptin (Dako). HER2 protein was observed in the cytoplasm of 70% (26/37) MPM cases using the NCL-CB11 antibody. Membranous staining was only seen in the positive control breast carcinoma tissue. The Herceptin antibody was immunoreactive in 7% (2/30) of the MPM tissue samples and demonstrated cytoplasmic staining. Cytoplasmic granular or vesicular staining was observed in HER2 positive cases with both antibodies. Gene amplification studies were carried out by fluorescence in situ hybridization on three samples that had demonstrated significantly high HER2 protein expression using the NCL-CB11 antibody but there was no HER2 gene amplification observed in either HER2 positive and negative tumours. RT-PCR analysis was also carried out to qualitatively compare HER2 mRNA expression in MPM cases with high HER2 protein expression and cases with no HER2 staining however, there was no significant increase of HER2 mRNA in cases that showed HER2 immunoreactivity with the NCL-CB11 antibody (Horvai et al., 2003). Other authors have reported conflicting evidence of little or no HER2 protein staining in mesothelioma cells (Ascoli et al., 1993; Suzuki et al., 1995; Garland et al., 2007). Variation in techniques might account for the differences observed in those studies since immunocytochemistry was used which distorts the original architecture of the surrounding tissue since the extracellular matrix and stroma components are eliminated.

In our study, HER2 protein expression was examined in 80 MPM tissue samples and correlated with clinicopathological variables (Table 7.3). Using the HER2 monocloncal antibody clone 3B5, we observed cytoplasmic granular staining in tissue samples that were HER2 positive and the staining pattern is in keeping with previous immunohistochemical reports using a different antibody. In the entire sample cohort, strong HER2 expression significantly improved survival (11.6 months vs 4.2 months in negative samples; p=0.006) (Figure 7.4). HER2 expression was predominant in the epithelial mesothelioma subtype (98%) in addition, 91% of samples in the biphasic subtype and 47% in the sarcomatoid subtypes were also HER2 positive. We observed that 32.5% of the tissue sample showed equal or high intensity when compared to HER2 expression in the normal pleura (Table 7.4).

HER2 expression in the epithelial subtype favoured improved survival however, the two sarcomatoid samples with strong HER2 expression had very poor prognosis (0.9 months vs 4.2 months in weak or negative samples) (Figure 7.6). The use of different antibodies in independent studies has showed similar expression patterns in mesothelioma except for the Herceptin antibody used by Horvai et al. HercepTest is the gold standard semi-quantitative IHC assay used in the determination of HER2 overexpression in breast cancer tissues based on its specificity and it is characterized by a strong membranous staining pattern (Jacobs et al., 1999). The antibody used in this study is the mouse monoclonal anti-human c-ErbB-2 (HER2) clone 3B5 antibody that recognizes the c-terminal domain of HER2 corresponding to amino acids 1242-1255 (TAENPEYLGLDVPV). The HER2 protein show sequence homology to other members of the HER family in the c-terminal region particularly HER1 (TAENAEYLRVAPQ; 1191-1203aa; access number X00588) and HER4 (VAENPEYLSEFSL; 1278-1290aa; access number L07868) (Benson et al., 2009). In the intracellular region of these proteins, it is observed that there is an uninterrupted homologous sequence of seven amino acids (underlined) in HER2 and HER4. As a result of these alignments, there is a likelihood of cross-reactivity occurring which might be a reason for the difference in staining patterns observed with other antibodies in comparison to Herceptin. Herceptin might be more specific for the HER2 epitope than other antibodies. Differences in antibody specificity are presumably the main cause for differences between studies. Cross-reactivity as a result of alignments might also support reports on the role of HER4 in the pathogenesis of mesothelioma although not explored in this study.

Western blot

Western blot analysis revealed the expression of the EGFR pathway in all the three MPM cell lines and A549 cells used (Figure 7.7). Basal and phosphorylated ERK was more strongly expressed in the NCI-H2452, NCI-H2052 and A549 cells when compared with the MSTO-211H cells although the presence of EGFR was increased in this cell line (Figure 7.8). The expression of the EGFR protein has also been previously reported in these cell lines by other authors using western blot analysis and flow cytometry. (Jänne et al., 2002; Tracy et al., 2004; Nutt et al., 2009; Nayak et al., 2011). RT-PCR analysis of MPM cells showed significantly increased HER2 mRNA expression in the MSTO-211H cell line in comparison with the MCF-7 breast cancer cell line and other patient-derived mesothelioma cells (Toma et al., 2002). Phospho-RTK array and immunoblot assays have also demonstrated the activation of multiple tyrosine kinases including EGFR and HER3 in two MPM cell lines (MESO924 and MESO428) derived from untreated patients of different histological subtypes (Ou et al.,

2011). Okita et al (2015) further confirmed the expression of HER2 and EGFR but not HER3 in NCI-H2052 and MSTO-211H cell lines by flow cytometry (Okita et al., 2015). Western blot analysis of MPM cell lines (H2461 and H226) demonstrated that addition of EGF to growth medium results in the phosphorylation of EGFR and downstream signalling proteins such as AKT and ERK1/2 (Jänne et al., 2002; Nutt et al., 2009). Previous unpublished work in our lab by Dr. Agarwal analysed each cell line for EGFR, KRAS and BRAF mutations. Only the A549 cell line showed a single KRAS mutation (Gly12Ser) in keeping with previous studies (Tracy et al., 2004; Krypuy et al., 2006). Mutation in the KRAS gene may result in the activation of the RAS/RAF/MAPK pathway thus induce resistance to EGFR inhibition by Gefitinib (Section 2.2.1.3). The absence of EGFR activating mutation in the A549 cell line also demonstrates resistance to EGFR TKIs (Tracy et al., 2004; Krypuy et al., 2006). There were no EGFR, KRAS or BRAF mutations identified in the NCI-H2452, NCI-H2502 and MSTO-211H cell lines and there are currently no published studies reporting the mutational status of these genes in the NCI-H2452, NCI-H2052 and MSTO-211H cell lines.

To best of our knowledge, very few studies have investigated HER2 protein expression using immunohistochemistry and gene amplification by RT-PCR in mesothelioma patients with relatively small numbers and no HER2 mutations have been described. On the other hand, EGFR protein expression and gene amplification has been extensively studied but only recently were actionable mutations identified in MPM patient samples. Studies have shown that mutations in the EGFR gene and some of its downstream signalling proteins predicts response of NSCLC patients to first generation EGFR inhibitors such as gefitinib (Lynch et al., 2004; Paez et al., 2004; Pao & Miller, 2005). The absence of activating mutations in mesothelioma might be a possible reason for the failure of gefitinib and erlotinib as single agents in the two phase II trials. Evidence of EGFR activating mutations was not identified in 66 and 32 mesothelioma patients when investigated by (Cortese et al., 2006) and (Velcheti et al., 2009) respectively. In congruence with their findings, other authors have also reported the absence of EGFR mutations using sensitive methods such as RT-PCR combined with direct sequencing (Lam et al., 2015; Schildgen et al., 2015; Mäki-Nevala et al., 2016). In contrast, Enomoto et al (2012), identified five EGFR missense mutations in six out of 38 pleural mesothelioma Japanese patients. T725M and Q787Q had been previously reported in NSCLC (Jia & Chen, 2011; U et al., 2014), T785T (Foster et al., 2010) in malignant peritoneal mesothelioma but N816K and G875 were novel suggesting that a subset of MPM patients could benefit from EGFR therapy.

MTS assay

Due to the absence of activating mutation in EGFR we expected A549 and the mesothelioma cells to be resistant to Gefitinib. The presence of a KRAS mutation in the A549 cell line could result in constitutive activation of the RAS/RAF/MAPK pathway which can be inhibited by selumetinib which targets MEK1 and MEK immediately downstream of RAF. All cells expressed EGFR and downstream proteins therefore inhibiting the proteins with tyrosine kinase inhibitors might result in the inhibition of cell proliferation. In this study, we investigated the antiproliferative effect of Afatinib (a dual HER2/EGFR inhibitor also know to inhibit ERBB4), Selumetinib (a MEK inhibitor) and Gefitinib (EGFR inhibitor) in NCI-H2452, NCI-H2052, MSTO-211H and A549 cells. Each inhibitor was effective in a dose-dependent manner in all the cell lines.

Selumetinib was the least toxic with IC50 ranging from 25.3 – 84.2 µM which is significantly higher than its average plasma concentrations achieved in patients at the maximum tolerated dose (3.5 µM) when 100 mg is administered twice daily (O'Neil et al., 2011). A549 (KRAS mutant G12S) was resistant to selumetinib with an IC50 of 36.1 µM in keeping with the study by Li et al (2015) where the IC50 of selumetinib for A549 was reported as >10 µM which is approximately 3.6-folds lower than what we obtained in our study (Li et al., 2015). Troiani et al (2012) also previously reported that A549 was resistant to selumetinib with an IC50 of 5 µM. The authors treated the cells for 96 hours using an MTT assay which might have led to further inhibition/reduction of the cells (Troiani et al., 2012). A study by Ihle et al (2012) demonstrated the heterogeneous nature of KRAS mutations such that tumours with KRAS mutations G12C or G12V had worse progression free survival than other mutant KRAS or wildtype KRAS (p=0.016). In addition, mutant KRAS proteins was reported to alter downstream signalling transducers in a dissimilar manner which might influence sensitivity to therapeutic interventions (Ihle et al., 2012). Preliminary results from a randomised Phase II trial with selumetinib plus docetaxel in KRAS mutant NSCLC patients showed that KRAS G12C or G12V mutations might have greater sensitivity to selumetinib. The findings are being evaluated in a larger ongoing study (SELECT-1; NCT01933932) (Jänne et al., 2015). The NCI-H2452 cell line was the least sensitive with high p-ERK expression, twice that of A549 (Figure 7.8). The NCI-H2052 cell line was the most sensitive and expressed weak p-ERK expression lower than that of A549. There is currently no published data on the effect of selumetinib on mesothelioma cells.

Gefitinib demonstrated antiproliferative effects in all cell lines but the IC50s were significantly higher than its maximum tolerated dose of 340.5 nM. In NSCLC cell lines with

EGFR activating mutation (HC3255, DFCILU-011 and PC-9), Gefitinib was significantly more effective at inhibiting cell growth using MTS assay with IC50 ranging from 10 to 63 nM. These values are significantly lower than that observed in our study (Mukohara et al., 2005). A549 cells with wild type EGFR has been reported to be resistant to Gefitinib with IC50 >10 µM similar to that seen in our study (16 µM) (Tracy et al., 2004). Resistance of MPM cell lines and A549 cells to Gefitinib may be due to the absence of mutations in the EGFR tyrosine kinase domain or the presence of mutations downstream of the EGFR signalling pathway. The dose of gefitinib required to reduce cell proliferation by 50% as reported by Giovannetti et al (2011) using MTT assay was; 4.83 µM in NCI-H2452, 5.22 µM in NCI-H2052, 4.91 µM in MSTO-211H and 3.99 µM in NCI-H28 (Giovannetti et al., 2011). The IC50s were slightly similar to those observed in our study NCI-H2452 (7.8 µM), NCI-H2052 (4.6 µM) and MSTO-211H (3.9 µM).

There is little or no published study evaluating the antiproliferative effect of Afatinib in MPM cell lines (NCI-H2452, NCH-H2052 and MSTO-211H). The IC50 values observed in all cell lines was significantly higher than the mean steady state plasma concentration at FDA approved dose levels (131.7 nM). The A549 cell line was the least sensitive with an IC50 of 3.1 µM and the MPM cell lines (NCI-H2452 and MSTO-211H) were the most sensitive with IC50s of 1 µM. In the study by Li et al (2008), the dose of Afatinib required to inhibit 50% of the NSCLC cell lines with wild type EGFR H1666 and A549 was 60 nM and 1437 nM respectively (Li et al., 2008). Suzawa et al (2016) also reported that A549 cells were resistant to the effect of Afatinib with an IC50 of 5.3 µM (Suzawa et al., 2016). The resistance of the A549 cells to the effect of Afatinib could be as a result of the presence of an activating KRAS mutation which confers resistance to anti-EGFR/HER2 therapy. Okita et al (2015) reported the first published study evaluating the effect of a dual EGFR/HER2 inhibitor (Lapatinib) in combination with trastuzumab to enhance antibody dependent cellular cytotoxicity in MPM cell lines (MESO1, MESO4, NCI-H28, NCI-H2052 and MSTO-211H). The MPM cell lines were more sensitive to the antiproliferative effects of Lapatinib and Afatinib than Gefitinib. Both inhibitors strongly inhibited the phosphorylation of EFGR and HER2 in the NCI-H28 and NCI-H2052 cells and lapatinib enhanced trastuzumab-mediated antibody dependent cellular cytotoxicity (Okita et al., 2015).

The effect of Afatinib in NSCLC patients with wild type EGFR is yet to be full understood. A Phase II single-arm trial evaluated the effect of Afatinib as a third-line treatment in advanced NSCLC patients whose tumours harboured wildtype EGFR. 42 patients were enrolled who had relapsed from two previous lines of chemotherapy. Afatinib was administered at 40 mg

per day until disease progression or occurrence of intolerable adverse event. Overall, 24% (9/38) of patients experience stable disease with a median duration of 19.3 weeks. No objective tumour response rate or partial response was reported which was the primary endpoint. Third-line afatinib was shown to be tolerated in this patients with manageable adverse events however, the absence of a comparable arm limits the conclusions that can be drawn from the study (Ahn et al., 2014). The LUX-LUNG 8 a Phase III randomised controlled trial also evaluated the effect of afatinib or erlotinib as second-line treatment in patients with advanced squamous cell carcinoma. EGFR mutations are rare in this type of cancer and mutations are sometimes associated females and non-smokers. (Zhang et al., 2015). Afatinib (40 mg per day) and Erlotinib (150 mg per day) was administered to the 795 eligible patients (398 to afatinib, 397 to erlotinib) who had progressed after at least four cycles of platinum based chemotherapy. The primary endpoint was progression free survival and the secondary end point was overall survival. Preliminary results showed that progression free survival (median 2.6 months *vs* 1.9 months; p=0.0103) and overall survival (median 7.9 months *vs* 6.8 months; p=0.008) was significantly improved with afatinib than erlotinib suggesting that afatinib could be an additional therapeutic option for patients with squamous cell carcinoma of the lung. The trial is still on going to identify the influence of EGFR mutation on patient outcome (NCT01523587) (Soria et al., 2015).

In conclusion, we have demonstrated that HER2 is expressed in the different histological subtypes of mesothelioma, with a trend toward poor survival in the sarcomatoid subtype however a larger cohort of sample is required for validation. We also showed that mesothelioma and A549 cells are more sensitive to afatinib than gefitinib and selumetinib as indicated by the lower IC50 values. In chapter 10, we further examined the antiproliferative effect of afatinib when combined with selected tyrosine kinase inhibitors and standard chemotherapy.

CHAPTER EIGHT

EXPRESSION OF c-MET AND THE EFFECT TO MET INHIBITORS IN MESOTHELIOMA CELL LINES

Chapter 8 Expression of cMET and the effect of MET inhibitors in mesothelioma cell lines

8.1 c-MET and HGF expression in MPM

In section 2.2.3 the role of the hepatocyte growth factor and its receptor (c-MET) in oncogenesis was discussed. Activation of c-MET by the binding of mature HGF secreted by stromal cells leads to a cascade of intracellular signal transduction pathways, including PI3K, Ras-MAPK, Ras-Rac/Rho, Ras-CDC42 and phospholipase Cγ pathways (Figure 2.8). c-MET activation is not exclusive to several physiological cellular processes such as cell mitosis or tissue regeneration but also controls cell proliferation, metastasis and migrations (Birchmeier et al., 2003). In normal mesothelial cells, strong polarized staining for c-MET was reported to be seen along the apical plasma membrane (Harvey et al., 1996). By contrast, c-MET expression was not observed at protein or RNA level by Tolnay et al (1998) and Klominek et al (1998) respectively in mesothelial cells. Similarly, a study by Jagadeeswaran et al (2001) reported minimal c-MET expression in a normal mesothelial cell line MeT-5A but none in 21 non-malignant pleural tissue samples (Jagadeeswaran et al., 2006). The expression of HGF has been reported in 38% to 100% of mesothelioma tissue specimens (Table 8.1). In addition, c-MET protein expression has also been detected in 74% to 100% of mesothelioma tumour specimens by immunohistochemistry (Table 8.1). Recently, c-MET was reported to be predominant in the epithelial subtype (78%) and c-MET confined to the plasma membrane localization was associated with improved prognosis in multivariate analysis with median overall survival of 25 months *versus* 13 months for other c-MET localization in MPM patients (Levallet et al., 2012). The co-expression of c-MET and HGF in tumour cells suggest an autocrine role in mesotheliomas. Fluorescence in situ hybridisation showed that mesotheliomas with epithelial differentiation had increased c-MET transcripts than the sarcomatoid subtype in keeping with the pattern of protein expression observed by immunohistochemistry (Tolnay et al., 1998). The majority of pleural effusion samples from mesothelioma patients have also been observed to express HGF (Eagles et al., 1996; Harvey et al., 1998, 2000; Klominek et al., 1998). HGF stimulation in MPM cell lines leads to increased invasiveness, migration, proliferation and the adhesion and synthesis of matrix metalloproteinases as discussed in Section 2.2.5.

Table 8.1 **HGF and c-MET expression in MPM by immunohistochemistry**

Study	**No. of cases**	**Protein**	**Histological Subtype**	**Expression levels**	**Prognostic value of c-MET expression**
(Harvey et al., 1996)	9	HGF/SF c-MET	Not indicated	9/9 (100%) 9/9 (100%)	No
(Tolnay et al., 1998)	39	HGF/SF c-MET	Epithelial – 14 Biphasic – 17 Sarcomatoid - 8	30/39 (77%) 29/39 (74%)	No
(Thirkettle et al., 2000)	29	HGF/SF c-MET	Not indicated	9/24 (38%) 29/29 (100%)	No
(Jagadeeswaran et al., 2006)	66	c-MET	Epithelial – 47 Biphasic – 16 Sarcomatoid - 3	54/66 (82%)	No
(Kawaguchi et al., 2009)	35	c-MET	Epithelial – 22 Biphasic – 6 Sarcomatoid – 5 Other - 2	28/35 (80%)	No
(Levallet et al., 2012)	157	c-MET	Epithelial – 119 Biphasic – 15 Sarcomatoid – 19 Desmoplastic - 4	119/157 (76%)	Yes, good prognosis; ($p = 0.043$)

An *in vitro* study also showed that the epithelial MPM cell line BT, demonstrated enhanced mitogenesis, increased metastasis and intact cell-cell contact in response to HGF but the fibroblastoid cell line (BR) showed reduced cell contacts and increased cell motility suggesting a significant role for HGF in metastasis (Harvey et al., 1998). Transfection of mesothelial cells with SV40 DNA stimulates the HGF/cMET autocrine loop ans is subsequently accompanied by epithelial-mesenchymal-transition and G1-S cell cycle progression (Cacciotti et al., 2001). Furthermore, exposure of rat pleural mesothelial cells to crocidolite asbestos results in increased c-MET expression regulated by early response of the proto-oncogene fos-related antigen 1 (fra-1) suggesting the involvement of a PI3K/ERK5/Fra-1 feedback mechanism that results in tumour specific effects of c-MET inhibitors in malignant mesothelioma (Ramos-Nino et al., 2003, 2008).

Mutations in the c-MET gene within the semaphorin (N375S, M431V and N4541) and juxtamembrane (T1010I and G1085X) domains (Figure 2.7) have been reported in approximately 9% (4/43) of mesothelioma patients and 2 MPM cell lines (H2596 and H513) of sarcomatoid and epithelioid origin respectively (Jagadeeswaran et al., 2006). Similarly the germline polymorphism in the juxamembrane region T1010I, was also reported in 4% of MPM cases (section 2.2.3.2) (Brevet et al., 2011). In contrast, no activating c-MET mutation was in identified in a series of 30 MPM cell lines (Mukohara et al., 2005; Kawaguchi et al., 2009). Genetic factors such as inactivation of tumour suppressor genes NF2, CDKN2A and p53 have been shown to enhance tumour aggressiveness associated with increased c-MET activation and expression and also enrichment of cancer stem population (Menges et al., 2014).

8.1.1 HGF/c-MET targeting in mesothelioma

Preclinical studies have demonstrated the antitumour effects of small molecule MET kinase inhibitors and decoy ligands such as NK4. Mukohara et al (2005) was the first to evaluate the therapeutic effect of a c-MET selective tyrosine kinase inhibitor PHA-665752 on mesothelioma cells. PHA-665752 decreased cell growth, migration and invasion but increased cell-cell contact, and resulted in G1-S cycle arrest in MPM cell lines (H2461 and JMN-1B) that demonstrated a HGF/cMET autocrine loop (Mukohara et al., 2005). Subsequent studies have investigated the effect of other selective and non-selective inhibitors (SU11274, Crizotinib) as well as an ATP non-competitive inhibitor Tivantinib. SU11724 and a c-MET siRNA reduced cell growth and migration remarkably in mesothelioma cells however, SU11274 was more effective in cell lines (H513 and H2596) harbouring the T1010I polymorphism (Jagadeeswaran et al., 2006). Preclinical studies have also shown that NK4, a fragment of HGF consisting of an N-terminal hairpin domain and 4 kringle domains of the α-chain of HGF, can inhibit mesothelioma cell growth and migration through its inability to phosphorylate c-MET because it is devoid of HGF-related biological activities (Date et al., 1997; Suzuki et al., 2010). There are currently no published data on the effect of a c-MET monoclonal antibody on mesothelioma cells. Onartuzumab (MetMAb) was developed by Genentech as an anti-c-MET monovalent monoclonal antibody with an engineered monovalent Fab fragments that binds to c-MET but does not agonize the receptor rather functions as an antagonist. MetMAb functions as a receptor antagonist by competing with HGF for binding to c-MET (Merchant et al., 2013). MetMAb demonstrates good specificity

for the c-MET receptor, it is generally well tolerated as a single agent and in combination with other agents therefore yielding promising reports in NSCLC patients (Yu et al., 2011; Spigel et al., 2012, 2014). It is also under investigation in breast (NCT01186991) and gastroesophageal (NCT01590719) cancers. We aimed to investigate the expression of c-MET in archival tissue samples from the Hull and East Yorkshire hospitals and assess the in vitro effect of c-MET tyrosine kinase inhibitors and onartuzumab on mesothelioma cells.

Aims:

- To assess the expression of c-MET in archival MPM tissue samples using immunohistochemistry and its association with patient survival
- To assess the expression of c-MET protein by western blotting in MPM cell lines NCI-H2452, NCI-H2052 and MSTP-211H using A549 cells as positive control
- To examine the effect of SU11274, Crizotinib, Tivantinib and MetMab (a monoclonal c-MET antibody) in mesothelioma cell lines (NCI-H2452, NCI-H2052 and MSTO-211H).

8.2 Materials and Methods

8.2.1 Tissue sample collections

Seventy one archival MPM tissue samples of patients diagnosed with MPM at Hull Royal Infirmary, Hull, UK from 1995 to 2000 were obtained. Clinicopathological data for all patients were available. Immunohistochemical analysis was performed in all of the 71 MPM tissue samples (36 epithelial, 21 biphasic, 14 sarcomatoid) to determine the expression of c-MET protein. Eight benign pleura samples from male pneumothorax patients were also included in the cohort of the slides as reference slides.

8.2.2 Immunohistochemistry

Immunohistochemistry was performed using the R.T.U. Vectastain Quick kit (#PK-7800, Vector Laboratories Inc., CA, USA) as described in section 5.8. Anti-c-MET primary antibody (C-12) (#sc-10, Santa Cruz) was applied at a dilution of 1:100 for 2 hours at room temperature. All slides were scored by two independent scorers including Dr Anne Campbell a consultant histopathologist (Section 4.8.9). Colorectal tissue samples stained with antibody were used as a positive control and slide with antibody omitted was the negative control.

8.2.3 Western blot

MPM cell lines NCI-H2452, NCI-H2052 and MSTO-211H and lung cancer cell line A549 were grown to 70-80% confluence then lysed in Laemmli buffer (65 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue) with the addition of 5% β-mercaptoethanol and 1% protease inhibitor mix (#80-6501-23, Amersham Biosciences). Protein lysates were quantified using the RCDC protein assay (# 500-0122, Biorad) and 50 µg of protein was analysed per lane on a 4-20% acrylamide gel (#25222, Pierce) under reducing conditions and transferred to a nitrocellulose membrane using a semi-dry iBlot system (Life Technologies). Membranes were blocked in 5% non-fat milk before samples were probed for 16 hours with the rabbit polyclonal anti-cMET primary antibody (C-12) (#sc-10, SantaCruz) at a final concentration of 1:200. Secondary anti-rabbit antibody was then applied after three washes at a final concentration of 1:10,000 for 1 hour at room temperature. To serve as a loading control, the anti α-tubulin antibody (#ab7291, Abcam) was applied at 1:3000 for 2 hours. Visualisation of protein bands was achieved using the SuperSignal West Pico Chemiluminescent Substrate kit (#34078, Pierce) as described in Section 4.9.

8.2.4 MTS assay

Commercially available inhibitors were purchased as follows crizotinib (#4368, Tocris), which is a c-MET/ALK inhibitor, SU11274 (#4101, Tocris) an ATP competitive and selective c-MET inhibitor, Tivantinib (#S2753, Selleckchem) a non-ATP competitive and selective c-MET inhibitor (Table 8.2). In addition, onartuzumab (MetMab), a monoclonal c-MET antibody was supplied by Genentech on a material transfer agreement (appendix D). The tyrosine kinase inhibitors were prepared in 100% DMSO and stored at -80^{o}C for further use. The MetMab monoclonal antibody was supplied in 75 µl of PBS at a concentration of 600 µM. Drugs were diluted in fresh media prior to each experiment. Cells were plated in 96-well plates at 1 x10^{3} cells/well and grown overnight in supplemented cell culture medium (appendix B). After 24 hours, cells were treated in replicates of 6 and cell viability was measured after 72 hours using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (#G3581, Promega) as detailed in Section 4.7.1. Following the 3 hour labelling of metabolically active cells with MTS, results were measured at 492 nm using an absorbance plate reader (Multiskan FC Microplate photometer, Thermo Scientific). Values were

normalised to untreated control cells in order to generate dose response curves. At least 3 independent experiments were carried out for each drug analysis before IC50 values were calculated using Graphpad Prism 6.0 software.

Table 8.2 Inhibitors used and their clinically relevant doses

Inhibitor	Target	Cmax	Supplier	Catalogue number	Reference for cmax
Tivantinib	c-MET	4.5 µM	Selleckchem	S2753	(Goldman et al., 2012)
Crizotinib	c-MET/ALK	300 – 569 nM	Tocris	4368	(M. P. Curran, 2012; H. Xu et al., 2015)
SU11274	c-MET	Not available	Tocris	4101	
MetMab	c-MET monoclonal antibody	Not yet published	Genentech		Appendix D

8.2.5 Statistical analysis

Statistical analysis was performed using SPSS software version 22.0 (SPSS, Chicago, USA). Univariate analysis was carried out for c-MET expression using Kaplan Meier survival curves with log rank analysis. Multivariate analysis was calculated using Cox regression analysis to take into account the histological subtypes which are known to be an independent prognostic variable in MPM (O'Kane et al., 2005).

8.2.6 Results

The characteristics of the 71 MPM archival tissue samples used in this study are described in Table 8.3. Histologically, MPM was of the epithelial subtype in 36 patients, biphasic in 21 patients and sarcomatoid type in 14 patients. The median age for the patients was 67 years (range 42-88 years). Sixty-five patients were males and 6 were females. Median survival for all the cases was 6.6 months (Table 8.3).

Table 8.3 Clinicopathological characteristcs of MPM archival tissue sample assessed for c-MET protein expression

Characteristics	Number of cases (%)
Total	71 (100)
Age-median (range)	67 (42-88)
Median survival (months)	6.6
Gender	
Female	6 (9)
Male	65 (91)
Histology	
Epithelioid	36 (51)
Biphasic	21 (29)
Sarcomatoid	14 (20)

8.2.6.1 Immunohistochemistry

Colorectal tissue samples were used as positive and negative (antibody omitted) controls (photos not included). Positive c-MET expression was observed in the nucleus and cytoplasm of reactive and non-reactive mesothelial cells in benign pleura samples (Figure 8.1A). All benign pleura samples (n=8; 100%) were positive for c-MET. Nuclear and cytoplasmic staining was also observed in the MPM tissue specimens that showed reactivity for c-MET but cytoplasmic staining was dominant. There was no distinct spectrum of intensities in the mesothelioma tissues hence they were scored based on a two-tier scoring system as positive or negative. Tissue samples were scored as positive (1) when >25% of tumour cells expressed c-MET protein and negative (0) when <25% of tumour cells expressed c-MET protein. c -MET expression was found in 58 MPM cases (82%) but predominant in the epithelioid subtype 89% (32/36). 81% (17/21) and 64% (9/14) were immunoreactive in the biphasic and sarcomatoid subtypes respectively. (Table 8.4). c-MET expression was independent of age (p=0.754), gender (p=1.00) and histological subtype (p=0.129) when assessed by chi-square test.

Table 8.4 c-MET expression in MPM samples

Samples	Total	c-MET expression score	
		Positive (1)	**Negative (0)**
All samples	71	58 (82%)	13 (18%)
Epithelial	36	32 (89%)	4 (11%)
Biphasic	21	17 (81%)	4 (19%)
Sarcomatoid	14	9 (64%)	5 (36%)

8.2.6.1.1 c-MET expression and survival

Univariate analysis using Kaplan Meier curves demonstrated that c-MET expression was significantly associated with improved survival. Median survival for c-MET positive cases was 7 months versus 4.5 months in the c-MET negative cases (p=0.019, log rank) (Figure 8.2). In the epithelial subtype, poor survival was associated with the absence of c-MET (median survival was 9.5 months in c-MET positive cases *versus* 4.9 months in c-MET negative cases (n=4); p=0.010, log rank) (Figure 8.3). In multivariate analysis, c-MET was not found to be prognostic when histological subtype was confounding factor (p=0.314; HR=1.4; 95%CI 0.73-2.7), chi-square analysis had also shown that the expression of c-MET was not associated with histological subtype (p=0.129).

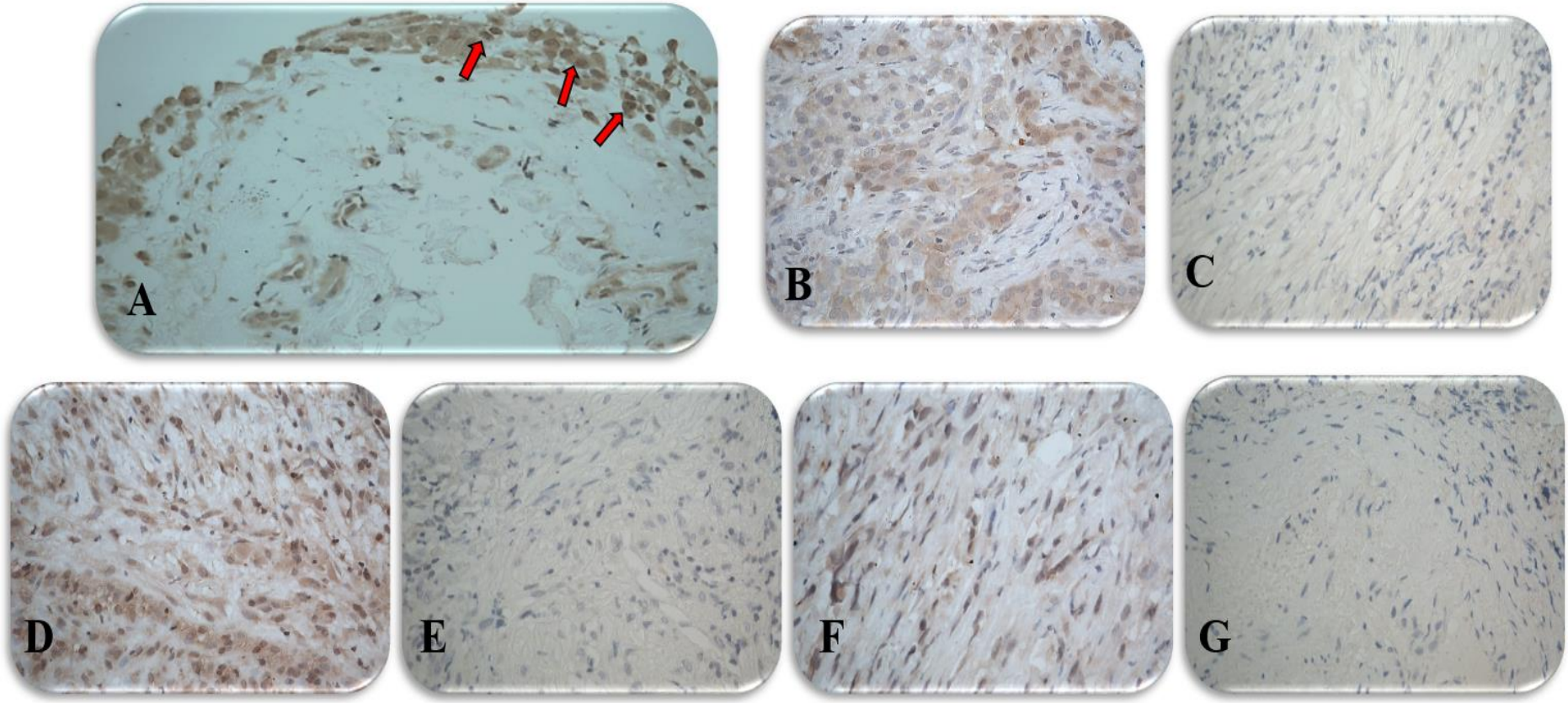


Figure 8.1 Expression of c-MET protein demonstrated by IHC. (A) c-MET expression in reactive mesothelial cells (red arrows) in benign pleura tissue. (B) Positive (1) cytoplasmic c-MET expression in epithelial MPM (C) Negative (0) c-MET expression in epithelial MPM tissue. (D) Biphasic/mixed subtype demonstrating positive (1) c-MET nuclear and cytoplasmic expression. (E) Negative (0) c-MET expression in biphasic/mixed MPM tissue. (F) Sarcomatoid subtype demonstrating positive (1) c-MET cytoplasmic expression. F: Negative (0) c-MET expression in sarcomatoid MPM tissue. (x400)

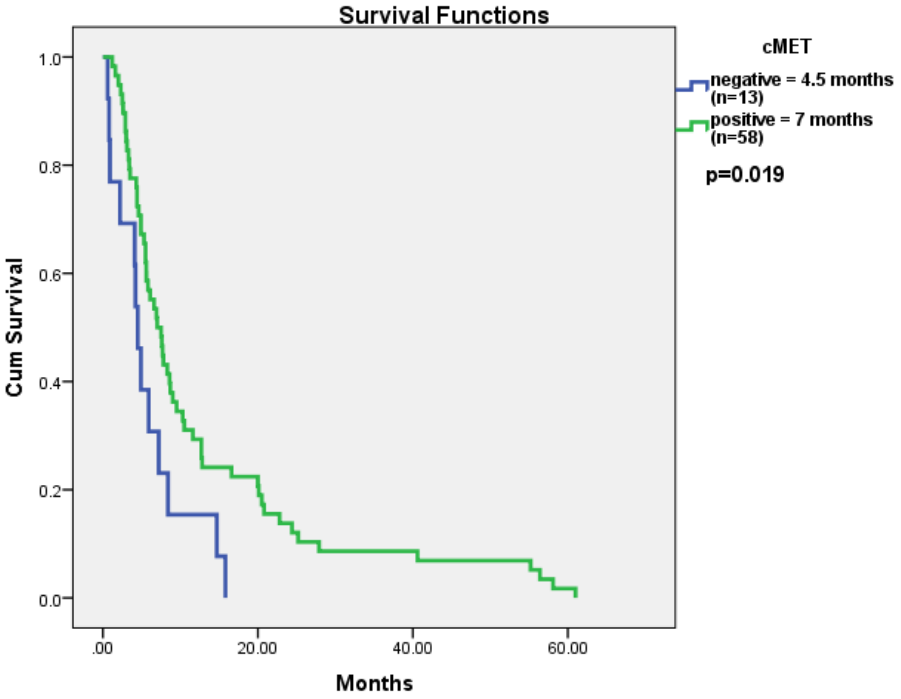


Figure 8.2 Survival analysis of c-MET protein in all MPM cases. Kaplan Meier plot showing univariate analysis of c-MET expression (p=0.019, log rank). The median survival was 7 months in c-MET positive cases (green line) *versus* 4.5 months in c-MET negative cases (blue line).

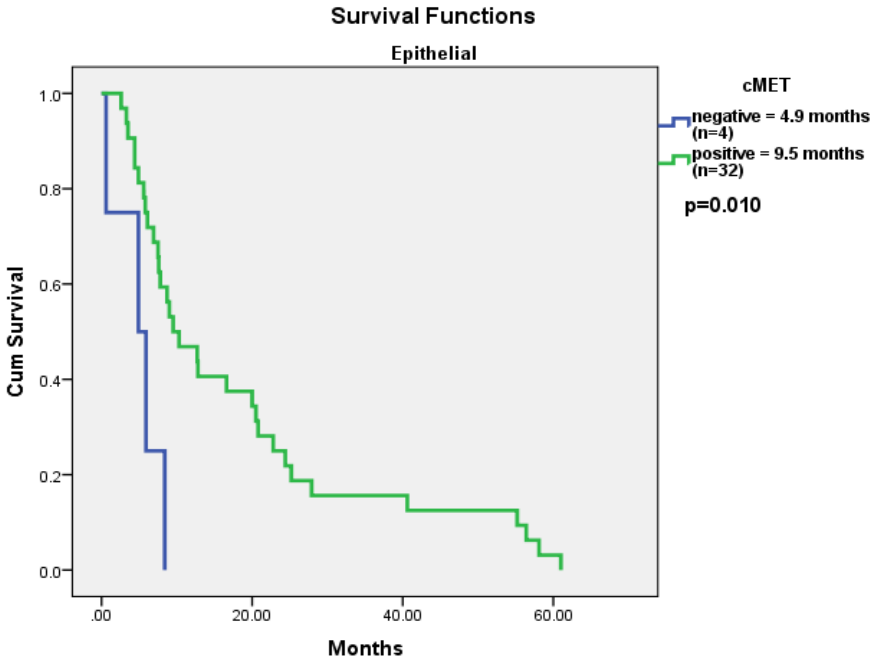


Figure 8.3 Survival analysis for c-MET protein expression in the epithelial subtype. Kaplan Meier plot showing univariate analysis of c-MET expression (p=0.010, log rank). The median survival was 4.9 months in c-MET negative cases (blue line) *versus* 9.5 months in c-MET positive cases (green line).

8.2.6.1.2 Correlations between HER2 and c-MET expression

Based on several reports on the co-activation of c-MET and EGFR in MPM tissues, we carried out a Fisher's exact test to determine if there is a significant association between c-MET and HER2 expression. Using updated survival data, we correlated the HER2 protein expression results from chapter 7 with the c-MET results in the same cohort of MPM patients. Sixty-six samples were eligible for this test of independence. Positive c-MET expression was significantly more likely in the presence of positive HER2 (p=0.037, Fisher's exact, Table 8.5). Of the 66 samples which were successfully analysed for c-MET and HER2, 76% (50/66) expressed both protein. Univariate analysis demonstrated that the co-expression of c-MET and HER2 (n=50) was significantly associated with improved prognosis when compared with samples that did not express either c-MET or HER2 (n=4) with median survival times of 7.5 months *versus* 4.1 months respectively (p=0.003), log rank; Figure 8.4). However in multivariate analysis the interaction between HER2 and c-MET was not an independent prognostic factor.

Table 8.5 This table demonstrates the correlations between HER2/c-MET expressions.

Fisher's exact test (2-sided) = 0.037		c-MET		
		positive	negative	Total
HER2 expression	positive	50	6	56
	negative	6	4	10
Total		56	10	66

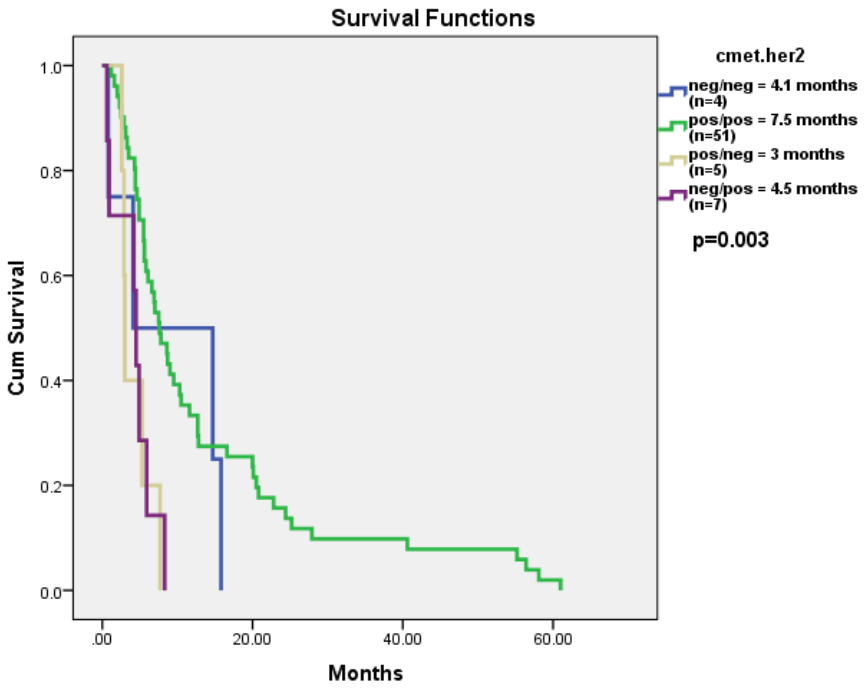


Figure 8.4 Survival analysis for combined c-MET/HER2 protein expression. Here the Kaplan Meier plot shows univariate analysis of combined c-MET/HER2 expression status (p=0.003). The median survival was 7.5 months in MET positive/HER2 positive cases (green line), 4.1 months in MET negative/HER2 negative cases (blue line), 3 months in MET positive/HER2 negative cases (yellow line) and 4.5 months in MET negative/HER2 positive cases (purple line).

8.2.6.2 Western blot

Immunoblot analysis performed as described in section 4.9 demonstrated the expression of c-MET in both mesothelioma and lung cancer cell lines. A 145-KDa protein band corresponding to the biologically active form of c-MET was clearly detected. Increased expression of c-MET was observed in the NCI-H2452 cells while A549 expression was relatively low. The loading control α-tubulin showed that proteins were equally loaded onto each well (Figure 8.5). Densitometric analysis also showed that NCI-H2052 cells had the least c-MET expression when compared to NCI-H2452 and MSTO-211H cells. A549 cells had low c-MET expression when compared to the mesothelioma cells. The expression of c-MET in the epithelial cell line NCI-H2452 was twice that of NCI-H2052 (Figure 8.6).

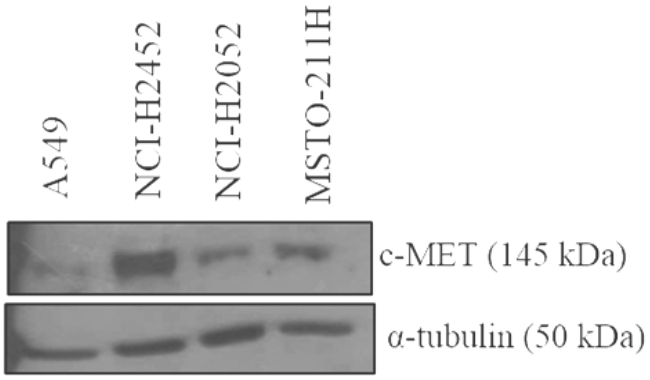


Figure 8.5: Representative western blot analysis of c-MET in MPM cell lines (NCI-H2452, NCI-H2052 and MSTO-211H. The biologically active form of c-MET was observed in all cell lines. For each lane 50 µg of protein was loaded. Different expression levels were displayed in each cell line. The bands were quantified against A549 cells and normalized to the loading control. Alpha tubulin served as the loading control to ensure equal amount of protein was loaded onto each well.

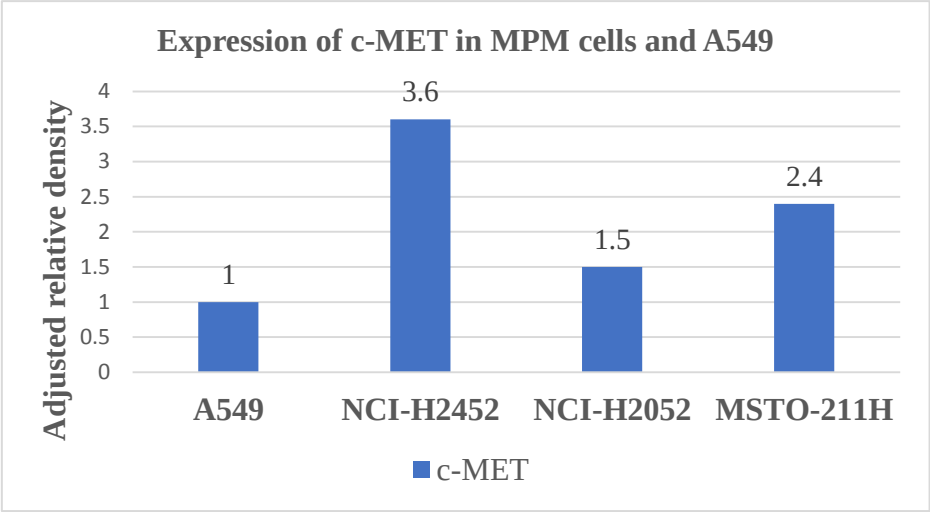


Figure 8.6 Graphical representation of the relative density of the c-MET protein in MPM cell lines. A549 cells were used as control to calculate relative density before normalizing against the control using Image J software. Alpha-tubulin was used as a loading control showing that difference in expression of c-MET is not as a result of unequal loading of proteins onto the gel (Figure 8.5).

8.2.6.3 MTS assay

Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H and A549 NSLC cancer cell line were treated with increasing concentrations (0 – 10 µM) of onartuzumab for up to 72 hours. Cell viability was assessed at 72 hours after treatment. Onartuzumab showed no significant growth inhibition in A549, NCI-H2452, NCI-H2052 and MSTO-211H (Figure 8.7). We were unable assess growth inhibition at higher concentrations than 10 µM due to insufficient amount of the antibody supplied.

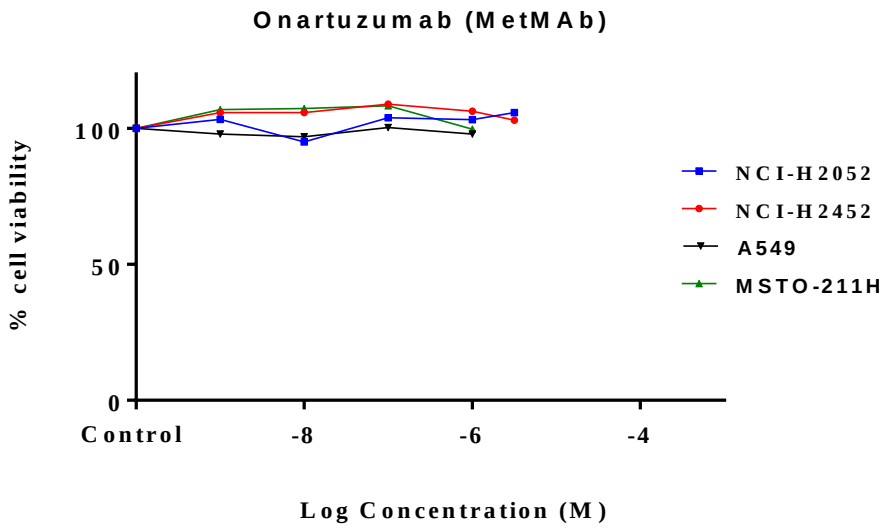


Figure 8.7 Antiproliferative effect of onartuzumab. The data presented represents the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. The cmax for onartuzumab is yet to be published.

The cell lines were also treated with increasing concentrations (0- 300 µM) of Tivantinib, Crizotinib and SU11274 for up to 72 hours. Cell viability was assessed at 72 hours after treatment. The results showed a dose-dependent decrease in cell viability in all analysed cell lines (Figure 8.8). MSTO-211H cells were the most sensitive to all three inhibitors with an IC50 of 0.3 µM (Tivantinib), 0.5 µM (Crizotinib) and 2.2 µM (SU11274) (Figure 8.8A-C). Tivantinib was the most potent inhibitor in all cell lines and reduced cell viability by 50% at concentrations lower than its clinically relevant dose of 4.5 µM. Only the MSTO-211H cells were seen to have an IC50 lower than the clinically relevant dose of crizotinib. A549 had the least c-MET expression it was also the least sensitive to the three c-MET inhibitors (Table 8.6).

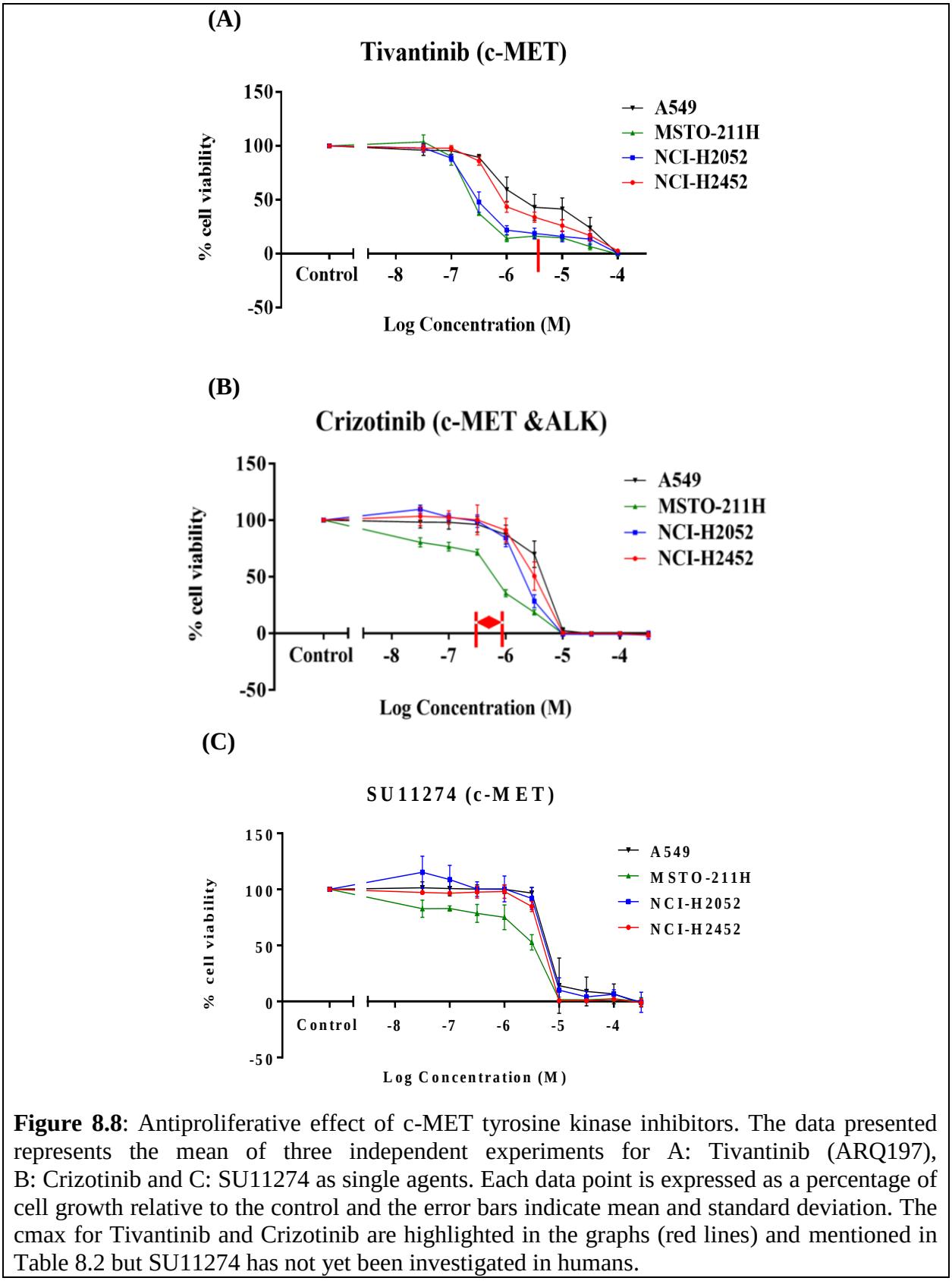


Figure 8.8: Antiproliferative effect of c-MET tyrosine kinase inhibitors. The data presented represents the mean of three independent experiments for A: Tivantinib (ARQ197), B: Crizotinib and C: SU11274 as single agents. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate mean and standard deviation. The cmax for Tivantinib and Crizotinib are highlighted in the graphs (red lines) and mentioned in Table 8.2 but SU11274 has not yet been investigated in humans.

Table 8.6: IC50 values of Tivantinib, Crizotinib and SU11274 in MPM and A549 cells. Values in bold are the IC50s within the clinical relevant dose of the inhibitor

	Protein expression	**Tivantinib cmax = 4.5 µM**	**Crizotinib cmax = 0.3-0.57 µM**	**SU11274 cmax = N/A**
Cell line	**c-MET**	**c-MET inhibitor**	**c-MET, ALK inhibitor**	**c-MET inhibitor**
A549	Expressed	**3.4 µM**	4.04 µM	6.7 µM
NCI-H2452	Expressed	**1.6 µM**	3.1 µM	4.3 µM
NCI-H2052	Expressed	**0.4 µM**	2.1 µM	5.8 µM
MSTO-211H	Expressed	**0.27 µM**	**0.54** µM	2.2 µM

8.2.7 Discussion

Immunohistochemistry

In this study, immunohistochemistry demonstrated nuclear and cytoplasmic c-MET expression in reactive and non-reactive mesothelial cells in benign pleura samples. Similar staining pattern was observed in 82% (58/70) of MPM tissue specimens. Immunoreactivity to c-MET was dominant in the epithelial and biphasic subtypes, (89% and 81% respectively) but reduced in the sarcomatoid subtype (64%). Cytoplasmic staining was observed in majority of the immunoreactive tissue specimens and a very few number of cases had nuclear localization. No plasma membrane staining was observed. Nuclear expression of c-MET has not been previously reported in MPM however in hepatic adenocarcinomas, c-MET translocates to the nucleus to interact with adaptor proteins that subsequently activates phospholipase C thus increasing calcium signalling. The mechanism by which c-MET translocates the nucleus and its topology within the nucleus is yet to be understood (Gomes et al., 2008). Our study further emphasizes the expression and activation of c-MET in a significantly large proportion of MPM patients. Tolnay et al (1998) reported cytoplasmic c-MET expression in 74% (29/39) of pleural mesotheliomas and the biphasic and epithelial subtypes had a strong positive reaction against c-MET. In the biphasic subtypes, the epithelial differentiated cells were intensely stained by the c-MET antibody than the sarcomatoid parts (Tolnay et al., 1998). Jagadeeswaran et al (2006) also reported c-MET expression in 82% (54/66) of MPM tissue samples displaying a spectrum of intensities but the localization of c-

MET protein was not reported. Kawaguchi et al (2009) further demonstrated positive c-MET expression in 80% (28/35) of malignant mesotheliomas but weak staining was observed in normal mesothelial cells. Our study and that of Tolnay et al (1996) and Jagadeeswaran et al (2009) utilized the same polyclonal anti-c-MET antibody from SantaCruz but neither of both studies evaluated the association of c-MET expression with survival. Recently, c-MET upregulation was found to be predominant in pleura mesotheliomas where the protein was localized in the plasma membrane in addition, its expression was associated with improved prognosis (p=0.043; multivariate analysis). Of the 157 pleura mesothelioma cases examined, 76% (119/157) were positive for c-MET expression and majority of the c-MET positive tissue samples were of the epithelial subtype (87%) (Levallet et al., 2012). Although in their study, cytoplasmic expression was observed in tumours with low c-MET expression, this present study demonstrated that cytoplasmic expression of c-MET is significantly associated with improved survival (p=0.019; log rank; Figure 8.2). Interestingly, we also observed a significant association between c-MET and HER2 expression in the cohort of 66 samples that were analysed (p=0.037; Fisher's exact). This is in keeping with the results of Thirkettle et al (2000) which demonstrated the presence of HER2 protein and c-MET protein in 97% and 100% of 29 mesothelioma tissue samples respectively showing that c-MET and HER2 co-express in mesothelioma cells. There was no report on the association of c-MET protein or HER2 protein with survival in their study (Thirkettle et al., 2000). The significant co-expression of c-MET and HER2 provides further evidence of a crosstalk between both pathways. Studies have shown that HGF has the ability to transactivate EGFR which can form a dimer with HER2 and the phosphorylation of EGFR can induce c-MET activation leading to synergistic effects on tumour growth (Xu & Yu, 2007; Spix et al., 2007; Reznik et al., 2008). c-MET and HER2 have been shown to synergize in promoting cellular invasion and the co-expression of both receptors yields aggressive tumours (Khoury et al., 2005). In breast cancer cells, c-MET was frequently expressed in HER2 upregulated cells and it contributed to the resistance of the cells to anti-HER2 monoclonal antibody (trastuzumab) treatment. Cell treatment with trastuzumab and c-MET specific RNA interference resulted in increased growth inhibition (Shattuck et al., 2008)

Western blot

The density of c-MET expression in two MPM cell lines MSTO-211H and NCI-H2452 was about 2 to 4-folds higher than that of the A549 cells. The expression of the c-MET protein in the three mesothelioma cell lines was very similar to those reported by Jagadeeswaran et al (2006) and the mRNA data of Kawaguchi et al (2009) in the same cell lines. In the two

previous studies, NCI-H2452 demonstrated high c-MET expression, MSTO-211H showed moderate expression and NCI-H2052 had the lowest c-MET expression density of the three cell lines. The expression pattern observed in the cell lines seems to reflect those observed in the tissue samples such that mesotheliomas of epithelial and biphasic origin displayed higher frequency of c-MET expression. Phospho-RTK array analysis further demonstrated the coactivation of c-MET and HER2 in the MSTO-211H cell line (Kawaguchi et al., 2009). Mesothelioma cell lines were observed to have a higher c-MET expression density than the A549 NSCLC cell line in this study.

MTS assay

Onartuzumab did not demonstrate significant growth inhibition in any of the cell lines at the concentrations applied. In A549 and MSTO-211H cells, 0–1 µM of onartuzumab was applied and in NCI-H2452 and NCI-H2052 cells, 0-10 µM was applied. Due to insufficient quantity and difficulty in acquiring additional volume of antibody, we could not determine its IC50 in each cell line. Further studies with increasing concentrations are warranted since there is no published study on its effect in mesothelioma. We also investigated and compared the antiproliferative effect of SU11274, Crizotinib and Tivantinib in NCI-H2452, NCI-H2052, MSTO-211H and A549 cell lines. SU11274 showed significant growth inhibition in all cell lines at concentrations between 2.2 µM and 6.7 µM. In a study by Jagadeeswaran et al (2006), SU11274 reduced cell proliferation in MSTO-211H, H513, H2596 and H28 cells in a dose-dependent manner with an IC50 between 2 and 3 µM but no significant effect was observed at 10 µM in NCI-H2452, NCI-H2052 cells and non-malignant MET-5A. The cells were cultured for 48 hours in the presence of increasing concentrations of SU11274 and cell growth assessed by MTT assay. In addition, the cell lines (H513 and H2596) that displayed increased sensitivity to SU11274 were observed to harbour T1010I mutation (Jagadeeswaran et al., 2006). This present study also showed MSTO-211H is more sensitive to SU11274 than the NCI-H2452 and NCI-H2052 cells. In contrast, a study by Kawaguchi et al (2009) investigated the effect of SU11274 on 13 MPM cells lines using the TetraColor one cell proliferation assay after 72 hours of exposing the cells to the inhibitor. None of the mesothelioma cells including MSTO-211H cells was sensitive to SU11274 at doses 0-10 µM were reported to be resistant to SU11274 (Kawaguchi et al., 2009). No MET mutation has been reported in NCI-H2452, NCI-H2052 and MSTO-211H therefore the difference in the antiproliferative effect across studies might be as a result of difference in methodology and exposure time. Crizotinib reduced 50% of cell growth at concentrations between 0.54 µM and 4.04 µM. The IC50 for crizotinib in MSTO-211H cells (0.54 µM) fell within the maximum

clinically relevant dose (0.6 µM) of the inhibitor. Crizotinib is known to be effective in anaplastic lymphoma kinase (ALK) positive NSCLC but mesotheliomas are not know to express the ALK protein. A study by Varesano et al (2014) reported the absence of ALK protein or translocation in 63 MPM tissue specimens (Varesano et al., 2014). Since the ALK gene might not be functional in mesotheliomas, the antitumour effect of crizotinib on MSTO-211H might be independent of ALK. Crizotinib demonstrated antiproliferative effect in NCI-H2052 cells (72 hour incubation) at an IC50 of 1.16 µM when evaluated using the MTS assay (Kanteti et al., 2014). There remains a paucity of data on the biological activity of crizotinib in mesothelioma cells. Tivantinib, the non-ATP competitive and selective c-MET inhibitor was the most potent c-MET inhibitor of the three inhibitor evaluated. In our study, the IC50s of tivantinib in all the cell lines ranged from 0.27 µM to 3.4 µM which is lower than its mean maximum plasma concentration (4.5 µM) reported by Goldman et al (2012). The study published by Kanteti et al (2014), demonstrated the effect of Tivantinib in MPM cell lines (H2596, H513, NCI-H2052, H2461) and the non-malignant mesothelial cell line Met-5A using the Alamar blue cell viability assay. Growth inhibition was observed in all cell lines and the dose required for 50% growth inhibition in the NCI-H2052 was 0.43 µM. The required dose was similar to the IC50 of tivantinib in NCI-H2052 cells (0.4 µM) observed in our study and also lower than the clinically relevant dose (Kanteti et al., 2014). Although tivantinib is a selective c-MET inhibitor, three recent studies have also shown that its antitumour properties might result from other off target effects. Katayama et al (2013) and Basilico et al (2013) both reported that tivantinib suppressed the growth of c-MET addicted and non-addicted cancer cell lines by inhibiting microtubule polymerization and inducing G2/M arrest in addition to inhibiting c-MET. Similarly, Calles et al (2014) subsequently compared tivantinib with other MET tyrosine kinase inhibitors (crizotinib and PHA-665752) in a series of NSCLC cell lines with different MET dependencies. Crizotinib and PHA-665752 inhibited the growth of c-MET dependent cell lines via the inhibition of downstream signalling proteins, initiation of G0/G1 cell cycle arrest and apoptosis. On the contrary, the increased potency of tivantinib was not restricted to c-MET dependent NSCLC cell lines and unlike the other two inhibitors; tivantinib induced G2/M cell cycle arrest and inhibited microtubule polymerization (Basilico et al., 2013; Katayama et al., 2013; Calles et al., 2015). Interestingly, a study by Suraokar et al (2014) demonstrated from their microarray data of 53 surgically resected MPM tumours and paired normal tissue that the cytoskeleton/spindle microtubules network was the second most significantly altered pathway identified by network analysis (Suraokar et al., 2014). Their findings suggest a role for microtubule-

targeting therapy such as epothilone B for MPM patients. Their findings also led Leon et al (2014) to investigate the molecular and cellular characteristics involved in the interaction between pemetrexed and tivantinib in mesothelioma cells. The growth of four MPM cell lines (NCI-H2452, NCI-H2052, MSTO-211H and H28) was inhibited with tivantinib (IC50 ranged from 0.31 µM to 2.4 µM) and pemetrexed in a dose-dependent manner. The combination of 10 µM of tivantinib with pemetrexed produced a synergistic effect in the NCI-H2052 (CI=0.37) and MSTO-211H (CI=0.70) cells. PCR analysis also demonstrated that the combination of tivantinib and pemetrexed decreased thymidylate synthase mRNA expression. Tivantinib reduced the phosphorylation of c-MET but also acts as a mitotic inhibitor by inhibiting microtubule assembly. In addition, tivantinib significantly reduced cell migration as a single agent and in combination with pemetrexed (Leon et al., 2014). The added therapeutic/biological activity of tivantinib might explain the increase in potency of the inhibitor compared to other c-MET TKIs in our study. There is a need for further studies to understand the molecular mechanism of tivantinib in mesothelioma cells in order to identify its therapeutic benefits in mesothelioma patients.

In this study, we demonstrated that cytoplasmic expression of c-MET was associated with improved prognosis in univariate analysis. The data supports other published reports of the overexpression of c-MET in mesothelioma. We were also able to establish that tivantinib showed increased cytotoxic effect in mesothelioma cells when compared to crizotinib and SU11274. The effect of c-MET inhibitor in the mesothelioma cell lines was not associated with the expression levels of the c-MET protein. Tivantinib was further investigated in combination with other tyrosine kinase inhibitors in chapter 10.

CHAPTER NINE

INHIBITION OF THE PI3K/AKT/mTOR PATHWAY IN MESOTHELIOMA CELLS

Chapter 9 Inhibition of the PI3K/AKT/mTOR pathway in mesothelioma cells

9.1 PI3K/AKT/mTOR pathway in MPM

The PI3K/AKT/mTOR/PTEN is an important intracellular signalling pathway in the hallmarks of cancer (section 2.3). It is a common intracellular signalling pathway for the tyrosine kinase receptors discussed in preceding chapters. The EGF, VEGF and HGF receptors activate the PI3K/AKT/MTOR pathway following the recruitment of the adaptor proteins (Section 2.3; Figure 2.10). In tumour cells AKT can be activated via a variety of mechanisms such as loss or downregulation of PTEN, activation of PI3K as a result of autocrine and paracrine stimulation of RTKs, Ras activation and/or mutation of the PI3K catalytic or regulator subunits PI3K3CA or PI3KR (Liu, 1998; Philp et al., 2001; Eng, 2003; Samuels et al., 2004). In mesothelioma, immunohistochemical studies have demonstrated the upregulation or loss of biomarkers within the PI3K/AKT/mTOR pathway (Table 9.1). Altomare et al (2005) reported expression of proteins in PI3K/AKT pathway in mesothelioma tissues via immunohistochemical analysis. PhosphoAKT and phosphoMTOR was upregulated in 65.4% and 92.3% of 26 MPM tissue specimen respectively but PTEN protein was absent in 8% although the histological subtypes of the tissue samples were not reported. Western blot analysis showed that the M43 mesothelioma cell line had an elevated AKT expression but PTEN was lost. Single-strand conformation polymorphism (SSCP) assay further confirmed the absence of PTEN exons 2-8 in the cells (Altomare et al., 2005). Garland et al (2007) also demonstrated PTEN loss in 16% of 19 MPM patient tumour samples but pAKT and pMTOR were upregulated in 84% and 74% of the tumour specimens respectively using immunohistochemistry. Surrounding non-neoplastic stroma cells or normal tissues served as internal negative controls (Garland et al., 2007). PTEN loss demonstrated by immunohistochemistry was further reported in 62% of 341 MPM cases and the absence of the protein was associated with poor median overall survival (9.7 months in PTEN negative versus 15.5 months in PTEN positive; log rank, p=0.0001) (Opitz et al., 2008). PTEN loss was also demonstrated by immunohistochemical analysis in 26.7% of the 86 MPM tissue samples evaluated in our laboratory but there was no significant association with survival (Agarwal et al., 2013). An interesting study by Bitanihirwe et al (2014) revealed dynamic changes in PTEN and pMTOR protein expression during induction chemotherapy and its effect on overall survival. Their study assessed two cohorts (cohort 1 = 107; cohort 2 = 46) of MPM patients uniformly treated with platinum-based induction chemotherapy followed by radical surgery at two different institutions. Low expression of pS6K in chemonaive patients

was associated with longer overall survival (p=0.02), in addition, a paired comparison of the biomarkers showed that reduced cytoplasmic PTEN and increased pMTOR was associated with worse overall survival (p=0.04 and p=0.03, respectively). However, reduced PTEN protein expression post chemotherapy was not as a result of homozygous gene deletion when analysed by fluorescence in situ hybridization (Bitanihirwe et al., 2014). Subsequent immunohistochemical studies have demonstrated the activation of the PI3K/AKT pathway in MPM tissue specimens and are listed in Table 9.1. Similarly, mesothelioma cell lines have also demonstrated the frequent activation of the PI3K/AKT/MTOR pathway. A study by Suzuki et al (2009) demonstrated loss of PTEN protein in 9.5% of the cell lines (ACC-MESO-1 and Y-MESO-25) and pAKT was upregulated in 62%. pAKT protein was absent in the NCI-H2452 and MSTO-211H cells but weakly expressed in the NCI-H2052 cells. PTEN expression was relatively low in the NCI-H2452, NCI-H2052 and MSTO-211H cells compared to the normal mesothelial cell line Met-5A (Suzuki et al., 2009). These findings support a possible role for activation of the PI3K pathway through the loss of PTEN and via autocrine and paracrine stimulation of receptor tyrosine kinases in some MPM, hence targeting the PI3K/AKT/MTOR pathway in MPM requires further exploration. Unlike other cancers mutations is the PI3K/AKT pathway are not common in mesothelioma. A previous study using the SSCP assay reported the absence of PTEN mutation in 18 MPM specimens another study demonstrated the presence of a homozygous deletion of PTEN in 1 of 9 MPM cell lines (Papp et al., 2001; Altomare et al., 2005). Suzuki et al (2009) did not observe any activating mutation in the 20 exons of the PIK3CA coding region and in the 9 exons of LKB1 genes of all 21 cell lines including NCI-H2452, NCI-H2052 and MSTO-211H cells. (Suzuki et al., 2009).

Table 9.1 Immunohistochemical analysis of biomarkers in the PI3K/ AKT/mTOR pathway in MPM

Study	Protein	No of tissue samples	Histological subtypes	Expression	Prognostic value
(Altomare et al., 2005)	pAKT	26	Not indicated	17 (65%)	N/A
	pMTOR			24 (92%)	
	PTEN			2 (8%)	
(Garland et al., 2007)	PTEN	19	Not indicated	Lost in 3 (16%)	N/A
	pAKT			16 (84%)	
	pMTOR			12 (74%)	
	P4EBP1			18 (95%)	
	pFOXO1			12 (74%)	
(Opitz et al., 2008)	PTEN	341	Epithelial – 112 Biphasic – 183 Sarcomatoid – 46	Lost in 211 (62%)	Poor prognosis (p=0.003; n=126)
(Watzka et al., 2011)	pAKT	74	Not indicated	65 (88%)	Not prognostic
(Cedrés et al., 2012)	PTEN	30	Epithelial – 21 Biphasic – 8 Sarcomatoid - 1	27 (90%)	N/A
	pMTOR			28 (93.3%)	
	pAKT			24 (80%)	
(Agarwal et al., 2012)	PTEN	86	Epithelial – 46 Biphasic – 24 Sarcomatoid - 16	Loss in 23 (26.7%)	No
(Bitanihirwe et al., 2014)	PTEN	107	Epithelial – 57 Biphasic – 45 Sarcomatoid - 5	75 (70%)	
	pMTOR	107		66 (62%)	Poor prognosis (p=0.03; n=66)
	pS6K	153		75 (49%)	
(Cedrés et al., 2016)	PTEN	26	Epithelial – 19 Biphasic – 5 Sarcomatoid – 1 Not specified -1	23 (88.5%)	No
	pMTOR	26		24 (92.3%)	
	pAKT	23		18 (78.3%)	
	P4EBP1	26		10 (38.5%)	
	pS6	23		23 (100%)	
	FOXo3a	24		24 (100%)	

9.2 Targeting the PI3K/AKT/MTOR pathway in mesothelioma

The targeting of the PI3K/AKT/MTOR pathway in mesothelioma is still in its early stages. The clinically approved inhibitors within this pathway are the first generation of MTOR inhibitors which are known as the analogs of rapamycin. Unfortunately this class of inhibitor have very limited activity as single agents and second line therapy in malignant pleura mesothelioma as evidenced by a phase II clinical trial. Ou et al (2015) demonstrated that Everolimus as a single agent in pre-treated MPM patients did not meet the primary endpoint of 4-month progression free survival (PFS) in 50% of the participants. The 4-months PFS rate was 29%, overall survival rate was 2% and the median overall survival was 6.3 months which is significantly lower than first line standard therapy. The authors concluded that further studies of this inhibitor as single agent in unselected MPM patients is not justified (Ou et al., 2015). A possible explanation for the failed attempt of this trial could be as a result of AKT activation via the mTOR C2 complex which is insensitive to rapamycin. Another phase II clinical investigated the effect of everolimus in selected MPM patients. Inactivation of Merlin/NF2 can lead to the activation of the MTOR pathway therefore loss of Merlin/NF2 was used as a sensitivity biomarker. The investigators had to terminate the study and could not report findings due to small numbers of participants (n=6) and technical setbacks which could lead to unreliable data (NCT01024946).

In vitro studies investigating other classes of PI3K/AKT/MTOR inhibitors have been explored as single agents and in combination with other inhibitors. The effect of LY294002, a broad and less specific PI3K inhibitor has been studied *in vitro* in mesothelioma cell lines and MPM tumour fragment spheroids. Treatment of M43 cells with LY294002 for 48 hours decreased AKT phosphorylation, MTOR activity and significantly increased apoptosis than MTORC1 inhibition using rapamycin. Furthermore, LY294002 enhanced the growth inhibitory effect of cisplatin and this was associated with increased constitutive AKT activity in the cell line (Altomare et al., 2005). In mesothelioma tumour spheroids, MTOR was a major mediator for acquired multicellular resistance. LY294002 reduced AKT phosphorylation but did not completely block p70S6K suggesting an active MTOR complex. On the other hand, rapamycin completely blocked p70S6K and reduced acquired apoptotic resistance but increased phosphorylated AKT via a positive feedback mechanism. PI-103, specific a PI3K and MTOR (both complexes mTOR1&mTOR2) inhibitor completely blocked p70S6K and p-AKT (Wilson et al., 2008). Although only rapamycin was observed to reduce apoptotic resistance by 40% and the concentration used was approximately two-fold less than its clinically relevant dose (13 nM), however this effect could not be replicated in MPM

patients (Jimeno et al., 2008; Wilson et al., 2008; Ou et al., 2015). A study by Mikami et al (2010) demonstrated that the PI3K/AKT pathway regulates cell proliferation and cell cycle progression using LY294002. Following a 24 hour treatment of mesothelioma cells with 20 µM LY294002, G1 cell cycle arrest was observed in treated cells compared to vehicle control cells in all cell lines (MS-1, REN, MSTO-211H, H28 and NCI-H2052). AKT phosphorylation and cyclin D1 protein was significantly downregulated and the CDK inhibitor p27 was up-regulated suggesting that the PI3K/AKT pathway might regulate cell cycle progression via the modulation of key regulators like p27 and cyclin D1. Significant inhibition of cell proliferation was also observed after treating the cells for an additional 48 hours (Mikami et al., 2010). Another study by Miyoshi et al (2012) further demonstrated synergistic and additive effects *in vitro* and in severe combined immunodeficient (SCID) mice respectively when LY294002 is combined with a MEK inhibitor (U0126). The combination inhibited mesothelioma cell growth via cell cycle arrest, apoptosis and inhibition of angiogenesis. In addition, the survival times was prolonged in the SCID mice when treated with the combination rather than with individual drugs (Miyoshi et al., 2012).

So far, the first PI3K/mTOR inhibitor that has made it into clinical trials in mesothelioma patients is VS-5584. VS-5584 is currently in two Phase I dose escalation studies in patients with advanced non-hemaologic malignancies or lymphoma and in combination with Defactinib (VS-6063) in patients with relapsed malignant mesothelioma (NCT01991938; NCT02372227). To the best of our knowledge, dual mTOR kinase inhibitors that selectively targets MTOR1 and MTOR2 complexes have not been evaluated in mesothelioma and no comparison has been made on the antiproliferative effects of dual mTOR inhibitors and dual PI3K/mTOR inhibitors (targeting PI3K and both mTOR complexes) *in vitro*. This study aimed to evaluate the antiproliferative effect of inhibitors that target both mTOR complexes (KU0006974 and XL388) and inhibitors that target PI3K in addition to both mTOR complexes (NVPBEZ235 and VS-5584) in mesothelioma cells (Figure 2.10). The concentration of NVPBEZ235 calculated based on the mean steady state plasma concentrations of 655.6 ng/mL at the maximum tolerated of 300 mg twice a day was 1.4 µM (Bendell et al., 2015) PTEN and p70S6K protein expression was also assessed in the cell lines.

Aims:

- To assess the expression of PTEN protein and p70S6K protein in mesothelioma cells (NCI-H2452, NCI-H2052 and MSTO-211H) and A549 by western blotting.
- To examine the antiproliferative effects of two dual mTOR1&2 inhibitors and two dual PI3K/AKT/mTOR 1&2 inhibitors on mesothelioma cells and A549.

9.3 Materials and Method

Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H were obtained from the ATCC and the lung cancer cell line A549 was obtained from the ECACC as detailed in section 4.2. All cell lines were maintained in RPMI 1640 cell culture medium as detailed in section 4.4.2. The dual mTOR 1&2 inhibitors (KU0063794 (#3725) and XL388 (#4893)) were purchased from Tocris Biosciences and the dual PI3K/mTOR inhibitors (NVPBEZ235 (#S1009) and VS-5584 (#S7016)) were purchased from Selleckchem. Stock solutions were prepared in 100% DMSO and stored at -80^{o}C as recommended by the manufacturer. The drug stock solution was diluted in fresh cell culture media prior to each experiment.

9.3.1 Western blot

Western blot analysis was carried in collaboration with Dr Agarwal. Cells were lysed in Laemmli buffer as detailed in section 4.9.1. Twenty micrograms of protein lysates was loaded onto a 12% precise protein gel (#25222, Thermo Scientific) and separated by one dimension gel electrophoresis. The separated proteins were transferred onto a nitrocellulose membrane and incubated with anti-PTEN primary antibody (ab32199, Abcam) and anti-p70S6K antibody (ab32359, Abcam) (detects both phosphylated and non-phosphorylated forms of p70S6K) diluted in 5% non-fat milk at 1:400 and 1:1000 respectively.

9.3.2 MTS assay

Each well in a 96 well plate was seeded with 1000 cells each and grown for 24 hours in fresh cell culture media. The cells were viewed under the microscope to ensure they had adhered to the bottom of the wells and no contamination was observed. Each cell line was then treated with increasing concentrations of freshly diluted dual mTOR1&2 inhibitors (XL388 and KU0063974) and dual PI3K/MTOR1&2 inhibitors (NVPBEZ235 and VS5584) ranging from 0-100 µM but up to 300 µM for XL388. The control wells received DMSO vehicle at a concentration equal to that of drug treated cells. Each inhibitor was used as a single agent on

each cell line. After 72 hours, 20 µl of MTS assay was added to each well and absorbance was read after a three hour incubation period. In each experiment six replicate wells were used for each drug concentration and the experiment was repeated three times. The IC50s were generated using a sigmoidal dose-response curve estimated by nonlinear regression analysis and comparison between cells was carried out using the one-way ANOVA with multiple comparisons. All statistical analysis was carried out on Graphpad prism 6.0.

9.4 Results

9.4.1 Western blot

PTEN protein was strongly expressed in the NCI-H2452 and NCI-H2052 cell lines but weak expression was observed in the A549 cell lines. In the MSTO-211H cell lines PTEN was absent. p70S6K protein was expressed in MSTO-211H and A549 cells, very weak expression in the NCI-H2452 cells but none in the NCI-H2052 cells (Figure 9.1; Figure 9.2).

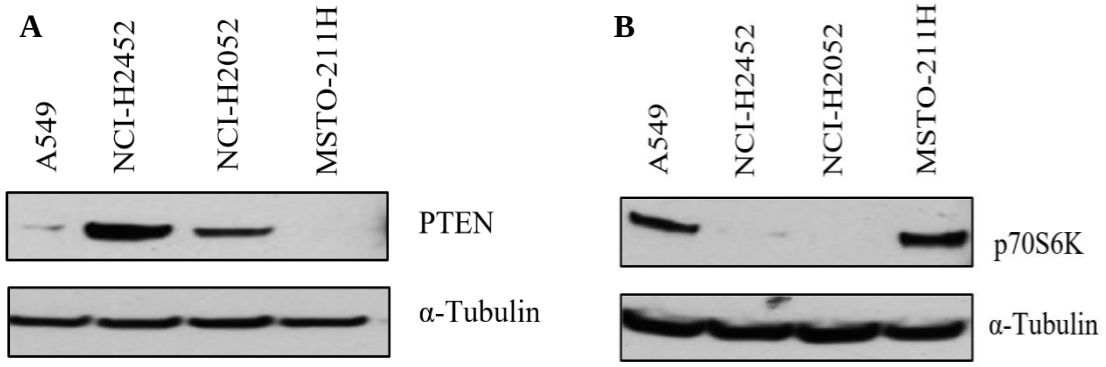


Figure 9.1 Expression of PTEN and p70S6K in mesothelioma cell lines. (A) and (B) show a representative immunoblot of mesothelioma cell lines for PTEN and p70S6K respectively. The image shows results with anti-PTEN antibody (ab32199, Abcam) at 47 kDa and anti-p70S6K antibody (ab32359, Abcam) at 70 kDa as expected. Alpha tubulin (ab7291, Abcam) was used as loading control at 50 kDa. A549 was used as positive control since it has been previously shown to express wild type PTEN (Janmaat et al., 2006)

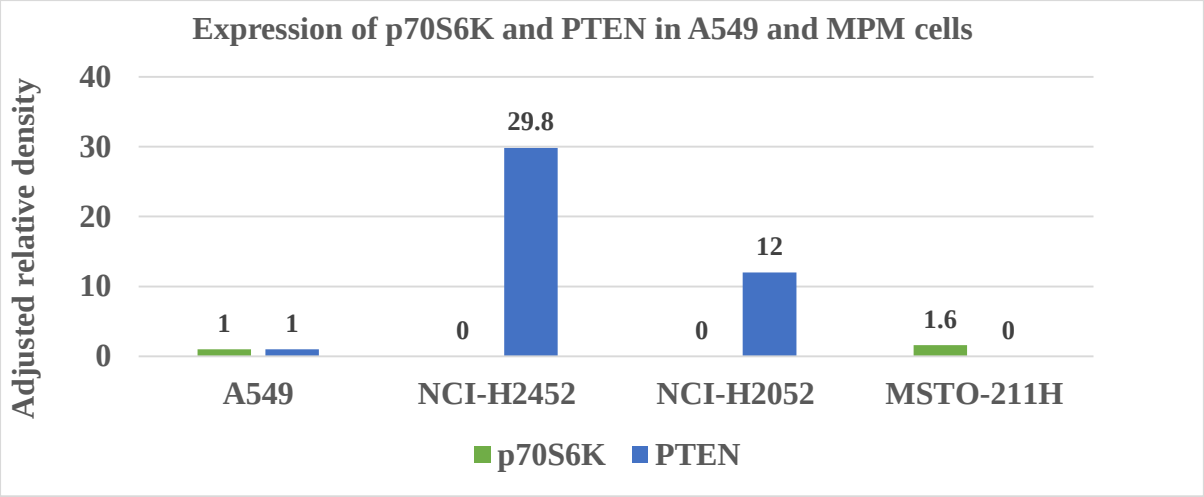


Figure 9.2 Quantitative analysis of p70S6K and PTEN expression in mesothelioma cell lines. There was expression of the PTEN protein in the NCI-H2452 and NCI-H2052 cell lines. MSTO-211H expressed p70S6K but not PTEN. Each band was normalized to the loading control (α-tubulin). A549 was used as positive control. Densitometry was carried out by Image J.

9.4.2 MTS assay

NCI-H2452, NCI-H2052, MSTO-211H and A549 in the exponential growth phases were exposed to increasing concentrations of KU006794 and XL388 and the effect on cell viability was examined after 72 hours of culture (Figure 9.3). Both inhibitors decreased call viability in all three mesothelioma cell lines in a dose-dependent manner. The MSTO-211H cell line was the most sensitive to KU0063794 and XL388 with an IC50 of 204 nM and 700 nM respectively (Table 9.2). However, the A549 cell line was the most resistant to XL388 with an IC50 of 5.3 µM. KU006794 was observed to have an increased cytotoxic effect as evidenced by the lower IC50 values in all cell lines when compared to XL388.

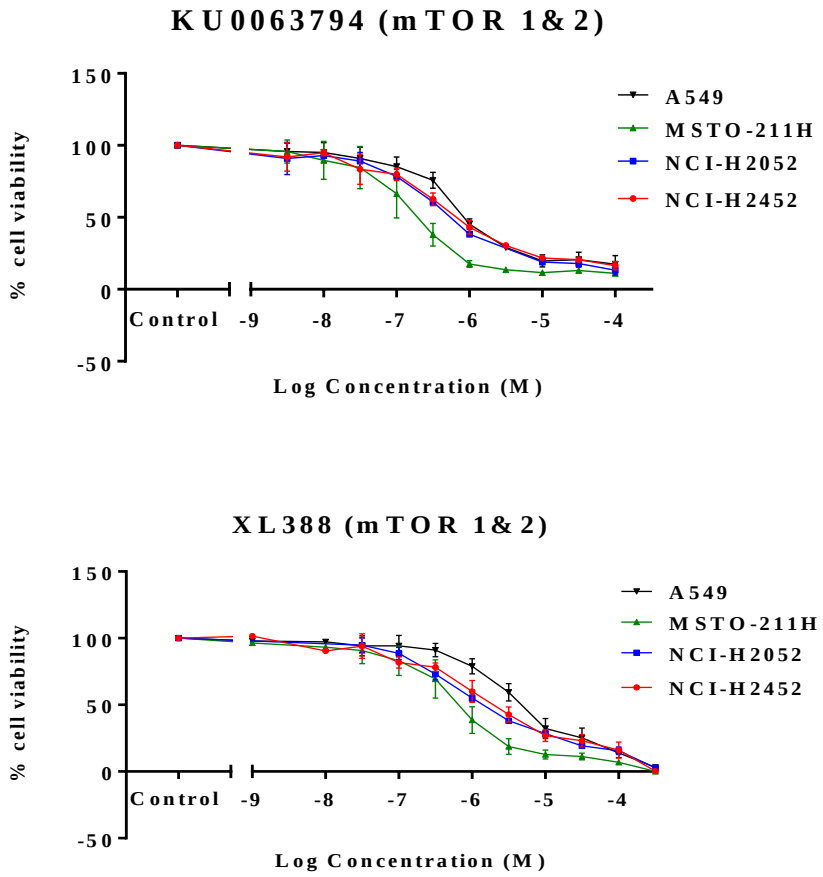


Figure 9.3: Antiproliferative effect of dual mTOR inhibitors**.** The data presented represents the mean of three independent experiments for A: KU0063794 and B: XL388 as single agents. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate mean and standard deviation. Both inhibitors are still in preclinical stage of development and their cmax values have not been determined.

Cell proliferation was determined in MPM and A549 cells following treatment with increasing concentrations of NVP-BEZ235 and VS-5544 for 72 hours. As shown in Figure 10.4, cell viability was significantly decreased in all cell lines in response to both inhibitors (Figure 9.4). The IC50s of NVPBEZ235 ranged from 4.8 nM to 51 nM and the IC50 of VS-5584 ranged from 90 nM to 390 nM (Table 9.2).

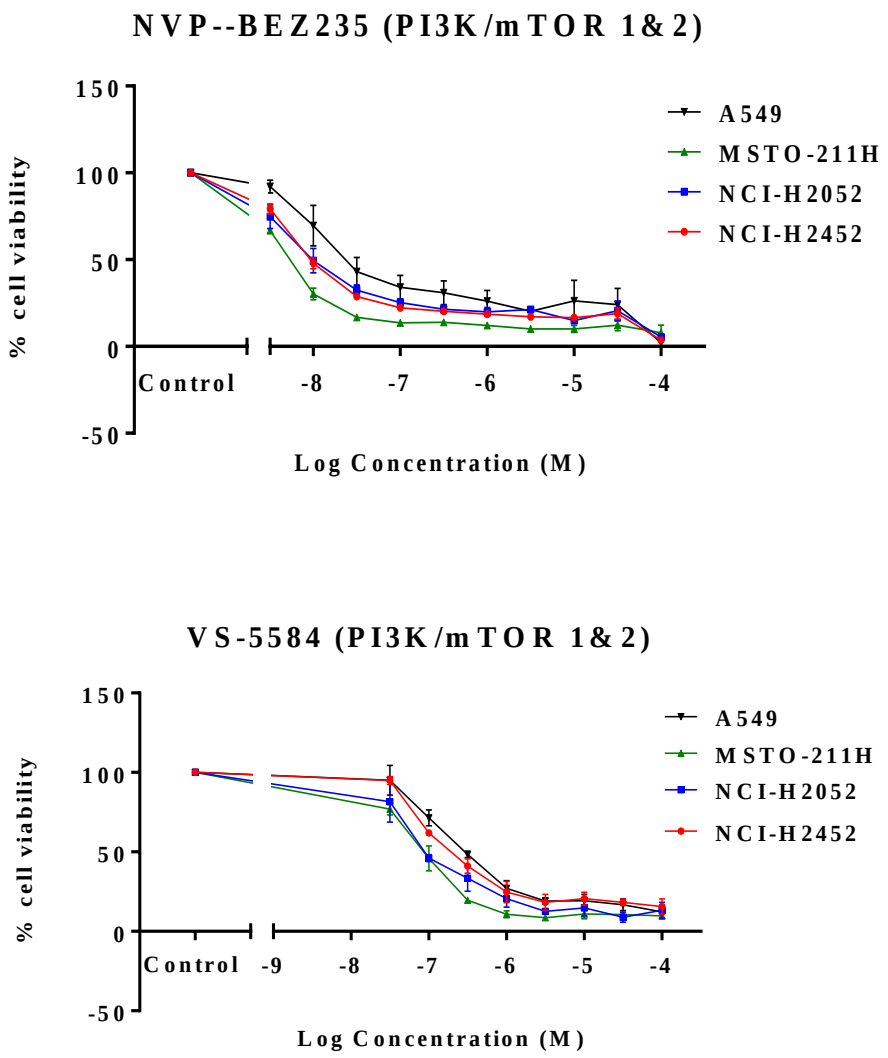


Figure 9.4 Antiproliferative effect of PI3K/mTOR inhibitors. The data presented represents the mean of three independent experiments for A: NVPBEZ235 and B: VS-5584 as single agents. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate mean and standard deviation. The cmax for NVPBEZ235 is 1.4 µM and VS-5584 has only recently entered a phase I clinical trial.

Table 9.2: IC50 values of PI3K/mTOR inhibitors in MPM and A549 cells. IC50s within the clinically relevant range are in bold

	KU0063794 cmax = N/A	**XL388 c-max = N/A**	**NVPBEZ235 cmax = 1.4 µM** (Bendell et al., 2015)	**VS-5584 cmax = N/A**
Cell line	**mTOR1&2**	**mTOR1&2**	**PI3K,mTOR, p110α,γ,δ,β**	**PI3K α/γ/δ/β mTOR 1&2**
A549	1.3 µM	5.3 µM	**0.051 µM**	0.39 µM
NCI-H2452	0.92 µM	2.2 µM	**0.012 µM**	0.29 µM
NCI-H2052	0.76 µM	1.8 µM	**0.013 µM**	0.14 µM
MSTO-211H	0.204 µM	0.7 µM	**0.0048 µM**	0.09 µM

Table 9.3 PTEN protein and p70S6K protein expression status in MPM and A549 cells

Cell lines	**PTEN protein**	**P70S6K protein**
A549	Weakly expressed	Expressed
NCI-H2452	Expressed	Not expressed
NCI-H2052	Expressed	Not expressed
MSTO-211H	Not expressed	Expressed

9.5 Discussion

Western blots

This study demonstrates the activation of the PI3K/AKT/mTOR pathway in mesothelioma. Western blot analysis showed that in the MSTO-211H cells, PTEN protein was absent but a weak expression was observed in the A549 cells (Figure 9.1; Table 9.3). PTEN protein expression in NCI-H2452 cells was nearly 3-fold higher than those observed in the NCI-H2052 cell line (Figure 9.2). Our western blot study for PTEN expression was consistent with those of Suzuki et al (2009) who also demonstrated the expression of PTEN protein in NCI-

H2052 and NCI-H2452 cell lines but very weak expression in the MSTO-211H cell line (Suzuki et al., 2009). The absence of PTEN could imply the activation of MTORC1 which is evidenced by the presence of p70S6K in the A549 and MSTO-211H cell lines. Since PTEN protein was absent in MSTO-211H cells we deduced that the PTEN/AKT/mTOR pathway may be active in these cells therefore mTOR inhibitors would be effective.

MTS assays

Since rapamycin was not very effective in mesothelioma patients based on the Phase II studies we hypothesized that the use of dual mTOR inhibitors and PI3K/mTOR inhibitors might limit negative feedback mechanisms and produce better clinical results. There was no significant difference in the effect of KU0063794 or XL388 across all cell lines as demonstrated by one-way ANOVA ($p=0.85$) and ($p=0.77$) respectively (Figure 9.3). Although MSTO-211H were the most sensitive cells with low IC50s for both inhibitors, this cannot be attributed to the absence of PTEN alone because the A549 cells had weak expression of both PTEN and p70S6K but were the least sensitive to both KU0063794 and XL388.

Dual inhibition of MTORC1 and MTORC2 in mesothelioma has not yet been reported. In this study, KU0063794 reduced cell viability in mesothelioma cells at IC50s ranging from 204 nM to 920 nM and XL388 was effective at 700 nM – 2.2 µM (Table 9.2). KU0063794 was therefore slightly more potent on cell viability than XL388. Previous studies have shown that KU0063794 and XL388 inhibits MTOR activity at less than 10 nM and both inhibitors have no cross reactivity with members of the PI3K lipid kinase superfamily at low nanomolar concentrations. The effect of KU0063794 on MTOR activity was assessed in human embryonic kidney cells (HEK-293), XL388 was assessed in prostate and breast xenograft tumours (García-Martínez et al., 2009; Takeuchi et al., 2013). However, their chemical structures are slightly different with XL388 possessing additional fluorine and sulfonyl groups (Figure 4.4). XL388 has been reported to inhibit MTORC1 and MTORC2 activity in the MCF-7 breast cancer cell line at IC50 values of 94 nM and 350 nM respectively. It was effective as a single agent and displayed a synergistic effect when combined with chemotherapeutic agents. In an MCF-7 xenograft model, XL388 also exhibited enhanced tumour growth inhibition (Miller, 2009). A study by Zhang et al (2013) compared KU0063794 and temsirolimus (a rapamycin analog) in preclinical renal cell cancer models. KU0063794 inhibited MTORC1 and MTORC2 activity and significantly reduced cell viability and growth by inducing cell cycle arrest and autophagy *in vitro*. Neither of the two

inhibitors induced apoptosis. However in mouse models temsirolimus significantly reduced tumour microvessel density therefore stimulating less angiogenesis (Zhang et al., 2013). This important additional effect of temsirolimus on the tumour microenvironment might suggest the inability of dual mTOR1&2 inhibitors to modulate host factors therefore they may require combinations with anti-angiogenic inhibitors since angiogenesis is an important hallmark in the pathogenesis of mesothelioma. Since there are currently no pharmacokinetic studies for KU0063794 and XL388 we are unable to compare the IC50s generated in our *in vitro* studies to a pharmaceutically relevant dose however, we observed that KU0063794 was more effective in all mesothelioma cell lines and the absence of PTEN in MSTO-211H cells could be a plausible reason for the increased sensitivity observed in the MSTO-211H cell line.

PI3K is upstream of AKT and MTOR with the ability to activate negative feedback mechanisms and induce other signalling pathways and proteins such as AKT in order to confer resistance to MTOR inhibitors. Simultaneous inhibition of PI3K and MTOR might therefore be a better approach than targeting MTOR alone. We investigated two dual PI3K/MTOR inhibitors in our cell-based assays. Both inhibitors were very effective on all cell lines in a dose-dependent manner. VS-5584 reduced cell viability in mesothelioma cells at IC50s ranging from 90 nM to 290 nM. A549 cells were the least sensitive to VS-5584 with an IC50 of 390 nM. VS-5584 was reported to be a potent inhibitor of multiple cancer cells including breast, ovarian, lung, melanoma, and was more effective on cells harbouring mutations in the PI3KCA gene (Hart et al., 2013). VS-5584 was shown to induce tumour growth inhibition in rapalog sensitive and resistant human xenograft models. More importantly, VS-5584 was reported to inhibit the proliferation and survival of cancer stem cells and remarkably reduced tumour-initiating capacity in multiple mouse xenograft models than chemotherapeutics such as paclitaxel and cisplatin (Hart et al., 2013; Kolev et al., 2015; Z. Shao et al., 2015). Cancer stem cells have been reported to confer chemoresistance to cisplatin and pemetrexed in mesothelioma cell lines NCI-H2052 and MSTO-211H (Cortes-Dericks et al., 2010). NVPBEZ235 has shown promising results against variety of cancers including lung, colorectal and glioma (Liu et al., 2009; Herrera et al., 2011; Roper et al., 2011). It induced growth arrest *in vitro* and *in vivo* was more effective than rapamycin in renal cell carcinoma cell lines (Cho et al., 2010). In glioma cells, NVPBEZ235 induced cell arrest at G0/G1 phase and suppressed the activity of AKT1 and S6K1 (p70S6K) resulting in reduced PI3K/MTOR signalling (Liu et al., 2009). In mesothelioma cells ZL34 and ZL55, NVPBEZ235 was reported to regulate ABCG2-mediated chemoresistance and increased sensitivity to pemetrexed (Fischer et al., 2012). Cell viability was strongly reduced by

NVPBEZ235 in mesothelioma cell lines (MESO924, MESO257, MESO296, MESO428) at IC50s ranging from 10.8 nM to 12 nM irrespective of histological subtype (S. Zhou et al., 2014). The concentration of NVPBEZ235 calculated based on the mean steady state plasma concentration of 655.6 ng/mL at the maximum tolerated dose of 300mg twice a day was 1.4 µM (Bendell et al., 2015). In our study, cell growth inhibition by NVPBEZ235 was observed at very low nanomolar range in all the cell lines. IC50 values for A549, NCI-H2452, NCI-H2052 and MSTO-211H were 51 nM, 12 nM, 13 nM and 4.8 nM respectively (Table 9.2). In keeping with our findings, Echeverry et al (2015) demonstrated that MSTO-211H, NCI-H2452 and NCI-H2052 were sensitive to NVPBEZ235 at concentrations below 200 nM. Similarly, Kanteti et al (2014) also reported that NVPBEZ235 inhibited the viability of NCI-H2052 cells at 13.86 nM after a 72 hour treatment (Kanteti et al., 2014; Echeverry et al., 2015). NVPBEZ235 has been investigated in several clinical trials however preliminary results from a phase II trial showed that progression free survival was better in the everolimus arm of patients with advanced pancreatic neuroendocrine tumours (NCT01628913). Reports from another Phase II study of BEZ235 in patients with advanced pancreatic neuroendocrine tumours that failed mTOR inhibition therapy using everolimus showed that NVPBEZ235 was not well tolerated by patients therefore could not progress into other stages of the trial (NCT01658436) (Fazio et al., 2016)

In summary, our findings show that dual PI3K/mTOR inhibitors are more potent than dual MTOR inhibitors on NCI-H2452, NCI-H2052 and MSTO-211H mesothelioma cells supporting the need to target multiple nodes in key survival pathways. NVPBEZ235 has been investigated *in vitro* as combination therapy by other authors for example the combination of NVPBEZ235 with selumetinib in metastatic colorectal tumour xenografts enhanced antitumor effects and induced antiangiogenic effects (E et al., 2015) VS-5584 is yet to be explored *in vitro* in combination with other inhibitor in mesothelioma. In Chapter 10, we therefore aimed to combine VS-5584 with either EGFR/HER2 inhibitor (Afatinib) or c-MET inhibitor (Tivantinib) to evaluate the effects of combined therapy. The ability of VS-5584 to also target cancer stem cells make it a preferred inhibitor to investigate.

CHAPTER TEN

Co-targeting receptor tyrosine kinase in MPM cells

Chapter 10 Co-targeting receptor tyrosine kinases in MPM cells

10.1 Introduction

Immunohistochemical studies and immunoblot studies have implicated several receptor tyrosine kinase in the pathogenesis of malignant pleural mesothelioma. This includes c-MET and EGFR/HER2 (Brevet et al., 2011). HER2 and c-MET proteins may be co-expressed in mesothelioma tumours as demonstrated by immunohistochemistry in section 8.2.6.1.2. In addition to previous studies, we have also demonstrated that mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H express both EGFR and c-MET proteins (Figure 7.7 and Figure 8.5). Crosstalk between MET and EGFR or HER2 has emerged as a major mechanism for cancer progression and resistance to tyrosine kinase inhibitors. Upregulation and activation of c-MET, EGFR and HER2 in mesothelioma was reported to result in the activation of major downstream intracellular PI3K/mTOR and MAPK signalling pathways resulting in cell growth and metastasis (Figure 10.1) (S. Zhou et al., 2014). Co-expression of c-MET and its ligand HGF have been shown to be frequent and contribute to the invasion and proliferation of mesothelioma cells (Klominek et al., 1998; Harvey et al., 2000). Although there are indications for tyrosine kinase activation in mesothelioma pathogenesis, anti-tyrosine kinase therapies have not been successful in MPM patients. The heterogeneous nature of mesothelioma and the activation of multiple pathways provides a rationale to target c-MET along with targets that can potentially synergize to inhibit cancer cells and prevent resistance. Tivantinib is the only selective non-ATP competitive inhibitor in advanced clinical development amongst MET inhibitors. Studies have suggested that tivantinib not only inhibits c-MET but also inhibits microtubule polymerization therefore suppressing the growth of c-MET dependent and independent cancers (Basilico et al., 2013; Katayama et al., 2013). Leon et al (2014) further investigated the effect of tivantinib with pemetrexed in MPM cells. Their results showed that tivantinib was able to inhibit c-MET in addition to being a mitotic inhibitor. There was increase in the percentages of cells in the G2/M phase and reduction in tubulin content leading to the inhibition of the microtubule assembly. Synergistic interaction was observed when tivantinib was combined with pemetrexed and this was mediated by the reduction of thymidylate synthase expression by tivantinib therefore increasing the sensitivity of mesothelioma cells to pemetrexed (Leon et al., 2014). Previous studies have also shown that combined targeting of kinase signalling pathways as well as combinations between targeted and chemotherapeutic agents are more effective than single agents in mesothelioma cells. Brevet et al (2011) demonstrated the co-activation of MET and EGFR in 43% (6/14) of MPM cell lines including NCI-H2452, NCI-H2052 and MSTO-211H. Significant crosstalk

between EGFR and MET was also demonstrated by decrease in phospho-EGFR when cells were treated with a MET inhibitor or MET siRNA. There was an increase in phospho-EGFR on HGF stimulation and an increase in phospho-MET on EGF stimulation. Co-targeting both RTKs with PHA-665752 (MET inhibitor) and erlotinib (EGFR inhibitor) supressed cell proliferation that with inhibitors as single agents in the Meso10, Meso9 and JMN cell lines. In addition, the MSTO-211H cell line was sensitive to the combination of rapamycin and PHA-665752 (Brevet et al., 2011).

AIMS:

- To evaluate the antiproliferative effect of tivantinib in combination with targeted agents (VS-5584 or Afatinib) and chemotherapy agents (cisplatin or pemetrexed).
- To assess the effect of combined targeted therapy on the migration and intracellular signalling pathways of mesothelioma cells NCI-H2452, NCI-H2052 and MSTO-211H.

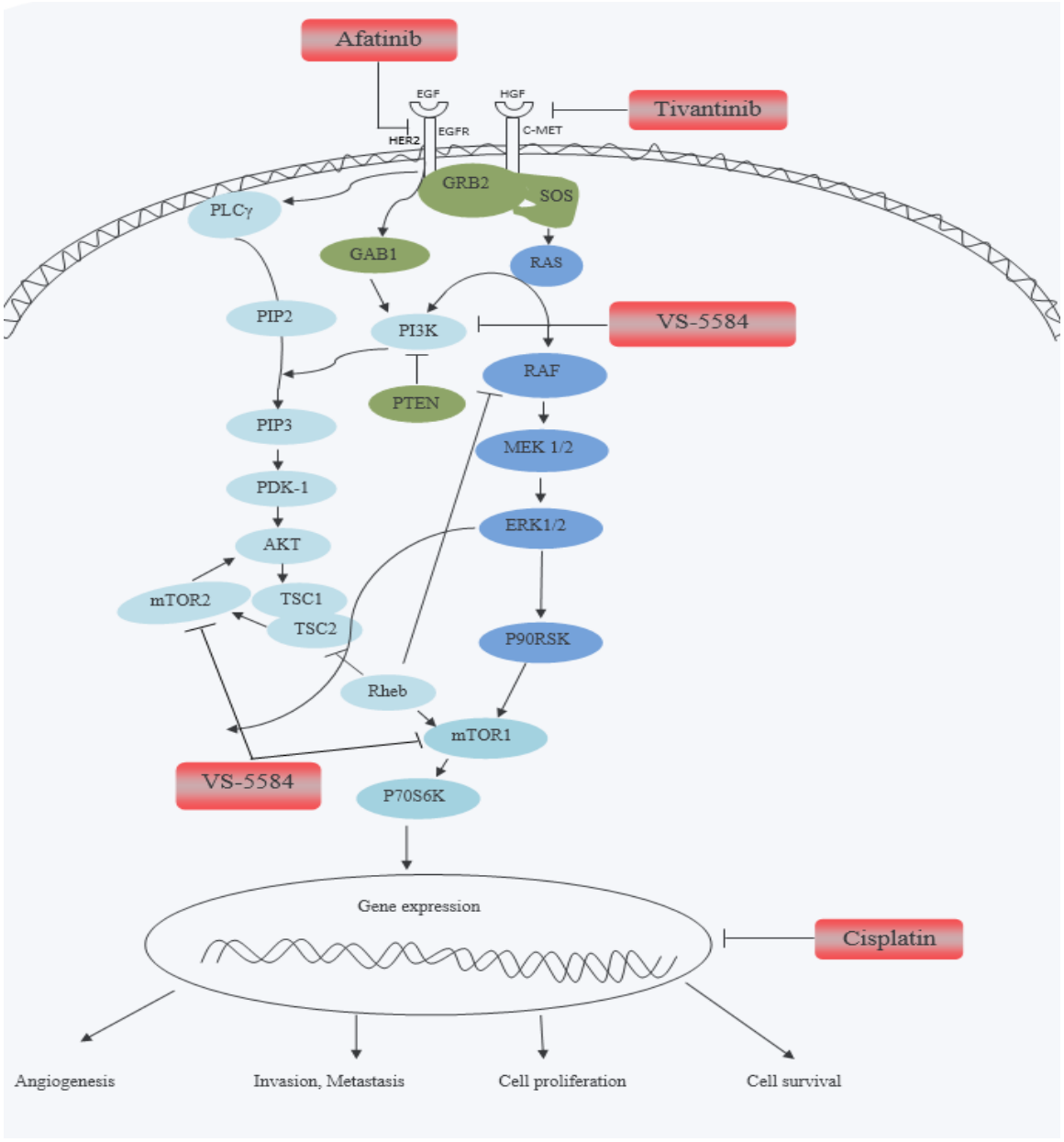


Figure 10.1 EGFR/MET pathway interactions. This figure demonstrates the interaction between c-MET, EGFR, MAPK and the PI3K/mTOR pathway. The target proteins investigated in this chapter by Tivantinib, Afatinib and VS-5584 are shown.

10.2 Materials and Method

Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H were obtained from the ATCC and the lung cancer cell line A549 was obtained from the ECACC as detailed in section 4.2. All cell lines were maintained in RPMI 1640 cell culture medium as detailed in section 4.4.2. Tivantinib (#S2753), Afatinib (#S11011) and VS-5584 (#S7016) were purchased from Selleckchem. Cisplatin (#P4394) was purchased from Sigma-Aldrich in a lyophilised form and reconstituted in distilled water. Pemetrexed was obtain by material transfer agreement from EliLilly in a lyophilised form. Another batch of pemetrexed was purchased from Selleckchem (#S1135). Stock solution for the tyrosine kinase inhibitors was prepared in 100% DMSO and stored at -80^{o}C as recommended by the manufacturer. Pemetrexed purchased from Selleckchem was reconstituted in PBS and the pemetrexed obtained from EliLilly was prepared in 100% or PBS as recommended by the manufacturers. Each drug was diluted in fresh cell culture media prior to each experiment.

Polyclonal c-MET, ERK2 and p-ERK1/2 antibodies were obtained from Santa Cruz Biotechnology. Antibodies against p-AKT, PTEN and ALDHA1 were obtained from R&D Systems. Caspase 3 antibody was obtained from Biolegend and EGFR polyclonal antibody was obtained from Abcam.

10.2.1 MTS assay

A 96 well plate was seeded with 1000 cells each and grown for 24 hours in fresh cell culture media. The cells were viewed under the microscope to ensure they had adhered to the bottom of the wells and no contamination was observed. Each cell line was treated with increasing concentrations of Cisplatin ranging from 0-100 µM. The mean plasma concentration of cisplatin following a single short-term infusion with 80 mg/m2 was 3.31±0.29 µg/ml (Ikeda et al., 1998). The IC50 values of cisplatin in A549, NCI-H2452, NCI-H2052 and MSTO-211H has been previously reported as 10.9 µM, 91.5 µM, 95.6 µM, and 20.4 µM respectively (Wellcome Trust Sanger Institute, 2016). Lennon et al (2016) have also reported the IC50 of cisplatin in NCI-H2052 and NCI-H2452 as 7.08 µM and 80.13 µM respectively (Lennon et al., 2016). Pemetrexed failed to show any effect on the cell lines at very high concentrations therefore it was exempted from all experiments. The control wells received media only. The IC50s of Afatinib, Tivantinib and VS-5584 were obtained from previous experiments in Chapters 7, 8 & 9 (Table 10.1). The IC25s of each inhibitor was calculated from the IC50 values (Table 10.2). For single agent treatment, each cell line was treated with the IC50 of

cisplatin or the IC25 of Tivantinib, Afatinib and VS-5584 for 72 hours. The IC25s of tyrosine kinase inhibitors were used in order to limit cytotoxicity.

For combinations, each cell line was treated with IC25s of Tivantinib and Afatinib or Tivantinib and VS-5584 or Afatinib and VS-5584 for 72 hours. The combination of cisplatin with each inhibitor and combinations was also evaluated. After 72 hours, 20µl of MTS assay was added to each well and absorbance was read after a three hour incubation period. In each experiment, six replicate wells were used for each drug concentration and the experiment was repeated three times. Cell viability was calculated based on the percentage of viable cells as discussed in Section 4.7.1.2. Comparison between treatments was carried out using the one-way ANOVA with multiple comparisons. All statistical analysis was carried out on Graphpad prism 6.0. Statistically significant differences between control, single agents and combinations are defined as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Table 10.1: IC50 values of Tivantinib, Afatinib and VS-5584 in MPM and A549 cells as demonstrated in Chapters 7, 8 & 9.

	Cisplatin	**Tivantinib**	**Afatinib**	**VS-5584**
Cell line	**DNA**	**c-MET**	**EGFR/HER2**	**PI3K/mTOR**
A549	9.0 µM	3.4 µM	3.1 µM	0.39 µM
NCI-H2452	9.9 µM	1.6 µM	1 µM	0.29 µM
NCI-H2052	2.1 µM	0.4 µM	1.4 µM	0.14 µM
MSTO-211H	0.86 µM	0.27 µM	1 µM	0.09 µM

Table 10.2 Calculated IC25 values for Tivantinib, Afatinib and VS-5584 in MPM and A549 cells

	Tivantinib Cmax = 4.5 µM	**Afatinib Cmax= 131.7 nM**	**VS-5584 Cmax= N/A**
Cell line	**c-MET**	**EGFR/HER2**	**PI3K/mTOR**
A549	1.13 µM	1.03 µM	0.13 µM
NCI-H2452	0.53 µM	0.33 µM	0.097 µM
NCI-H2052	0.13 µM	0.47 µM	0.047 µM
MSTO-211H	0.09 µM	0.33 µM	0.03 µM

10.2.2 Western blot

Cells were cultured in T75 flasks to reach 70% confluency. Inhibitors diluted in fresh media were added to each cell line over a period of 3 hour or 48 hour treatment. Each cell line was treated with the IC25 of Tivantinib, Afatinib and VS-5584 as single agents. Combinations included the addition of the IC25 of Tivantinib and Afatinib or Tivantinib and VS-5584 to each cell line. Whole cell lysates were prepared by scraping the cells in ice cold Laemmli buffer (Appendix B). Lysates were cleared by centrifugation at 16,000xg for 15 minutes at 4^{o}C, and lysates protein concentrations were determined using a Bio-Rad protein assay described in Section 4.9.2. Electrophoresis and western blotting were performed as previously described in Section 4.9.3 and Section 4.9.6 respectively. Briefly, 40 µg micrograms of protein lysates was loaded onto a 4-20% Tris-HEPES precise gradient gel (#25224, Thermo Scientific) and separated by one dimension gel electrophoresis. The separated proteins were transferred onto a nitrocellulose membrane and incubated with antibodies listed in Table 10.3.

Table 10.3 List of antibodies attempted and used for western blotting

Antibody (company)	Catalogue number	Molecular weight (kDa)	Host species	Blocking agent	Dilution	Application
Anti-alpha tubulin (Abcam) (monoclonal)	ab7291	50	Mouse	5% non-fat milk	1:1000	WB (loading control)
EGFR (Abcam) (polyclonal)	ab2340	185	Rabbit	5% non-fat milk	1:250	WB
p-AKT (S473) (R&D) (polyclonal)	AF887	60	Rabbit	5% non-fat milk	1:250	WB
PTEN (R&D) (monoclonal)	MAB847	50	Mouse	5% non-fat milk	Unable to optimise	WB
ALDH1A1 (R&D) (monoclonal)	MAB5869	56	Mouse	5% non-fat milk	1:1000	WB
ERK 2 (SantaCruz Biotechnology) (polyclonal)	sc 154	44	Mouse	5% non-fat milk	1:500	WB
p-ERK1/2 (Tyr 204) (SantaCruz Biotechnology) (monoclonal)	sc 7383	42/44	Mouse	5% non-fat milk	1:500	WB
p-MTOR (S2448) (Abcam) (polyclonal)	ab51044	289	Rabbit	5% non-fat milk	Unable to optimise	WB
Caspase 3 (clone 4-1-18) (Biolegend) (monoclonal)	622701	32 cleaved - 17	Mouse	5% non-fat milk	Unable to optimise	WB

10.2.3 *In vitro* Scratch assay

Cell migration plays an important role in normal physiological processes as well as disease processes such as cancer metastasis. In vitro scratch assay is an easy and economical method that involves the response of confluent monolayer of cells to mechanical wound created by using a pipette tip to remove a portion of the confluent cells grown in tissue culture flasks, 96-well plates or coverslips (Kramer et al., 2013). The HGF/c-MET pathway and the MAPK and PI3K intracellular signalling cascades have been reported to promote cell motility leading

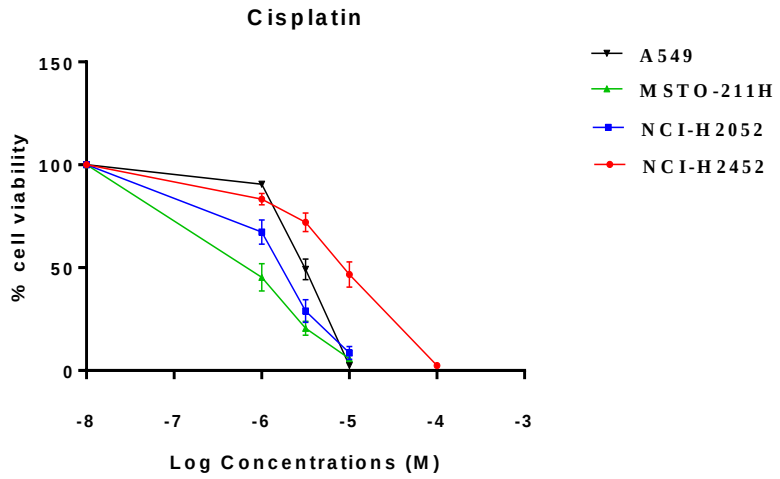
to tumour invasion (Kanteti et al., 2014; Spina et al., 2015). We hypothesized that inhibition of c-MET and downstream PI3K proteins might reduce the migration of mesothelioma cells. The protocol was adapted and modified from (Yue et al., 2010) and (Kramer et al., 2013). Briefly, each cell line was grown to confluence in a T25 tissue culture flask and a single scrape was made in the confluent monolayer using a sterile pipette tip. The monolayer was rinsed twice with PBS, then complete medium containing inhibitors as single agents, combinations or DMSO alone (vehicle control) was added. Photographs of the scraped section were taken at 0h and 24 h. Three data points were measured for each photograph and the experiment was repeated three times. The percentage of wound closure was calculated as: (Average distance before treatment – Average distance after treatment) / Average distance before treatment x 100 (Yue et al., 2010). One way ANOVA was used to compare statistical differences between control and other treatments. Significance was set at $p<0.05$.

10.3 Results

10.3.1 MTS assay

10.3.1.1 Antiproliferative effect of Cisplatin

A dose-dependence inhibition of tumour cell growth was observed with cisplatin in all three MPM cell lines and A549 cells. The IC50 values of cisplatin ranged from 0.86 µM to 2.1 µM in MSTO-211H and NCI-H2052 cells respectively. In NCI-H2452 and A549 cells, IC50 values of cisplatin were 9.9 µM and 9.0 µM respectively (Figure 10.2). MSTO-211H cells were the most sensitive to cisplatin. The IC50 value of cisplatin for each cell line was later used in combination with the IC25s of receptor tyrosine kinase (RTK) inhibitors in subsequent experiments.



Cell line	IC50
A549	9.0 µM
NCI-H2452	9.9 µM
NCI-H2052	2.1 µM
MSTO-211H	0.86 µM

Figure 10.2 Antiproliferative effect of cisplatin in mesothelioma cell lines and A549. The data presented represents the mean of three independent experiments. Each data point (n=6) is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. The above table is a list of the IC50 of cisplatin each cell line.

10.3.1.2 Effect of combined use of c-MET and EGFR/HER2 inhibitors on Cell Viability

In previous Chapters, all cell lines demonstrated positive expression of the EGFR protein (Figure 7.7) and MET protein (Figure 8.5). Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H showed increased EGFR and c-MET expression compared to the A549 positive control cells. We further evaluated the influence of the IC25 values of tivantinib and afatinib on cell viability as single agent and combinations by exposing the cells to the inhibitors for 72 hours. In NCI-H2452 cells, combination of tivantinib and afatinib reduced cell viability by 30% compared to either inhibitors alone (Figure 10.3A). Addition of cisplatin to the combination produced an additive effect and further reduced cell viability significantly ($p<0.01$) (Figure 10.3B). Similar effect was observed in the NCI-H2052 cells. The MSTO-211H cells were sensitive to the combination of tivantinib and afatinib but the addition of cisplatin to the combination did not show a significant difference when compared to the initial combination (Figure 10.4A & B). The combination of tivantinib and afatinib reduced cell proliferation in the A549 cells by 60% when compared to the control cells. Addition of cisplatin further reduced cell proliferation by 30% (Figure 10.4C & D). The combination of cisplatin with the tivantinib and afatinib cocktail had an additive effect in all cell lines except the MSTO-211H cells. The non-significant effect observed in the MSTO-

211H cells could be a reflection of the heterogeneous nature of mesothelioma cells. Addition of cisplatin to either tivantinib or afatinib reduced cell proliferation by approximately 50% similar to the combination of afatinib and tivantinib in all cell lines.

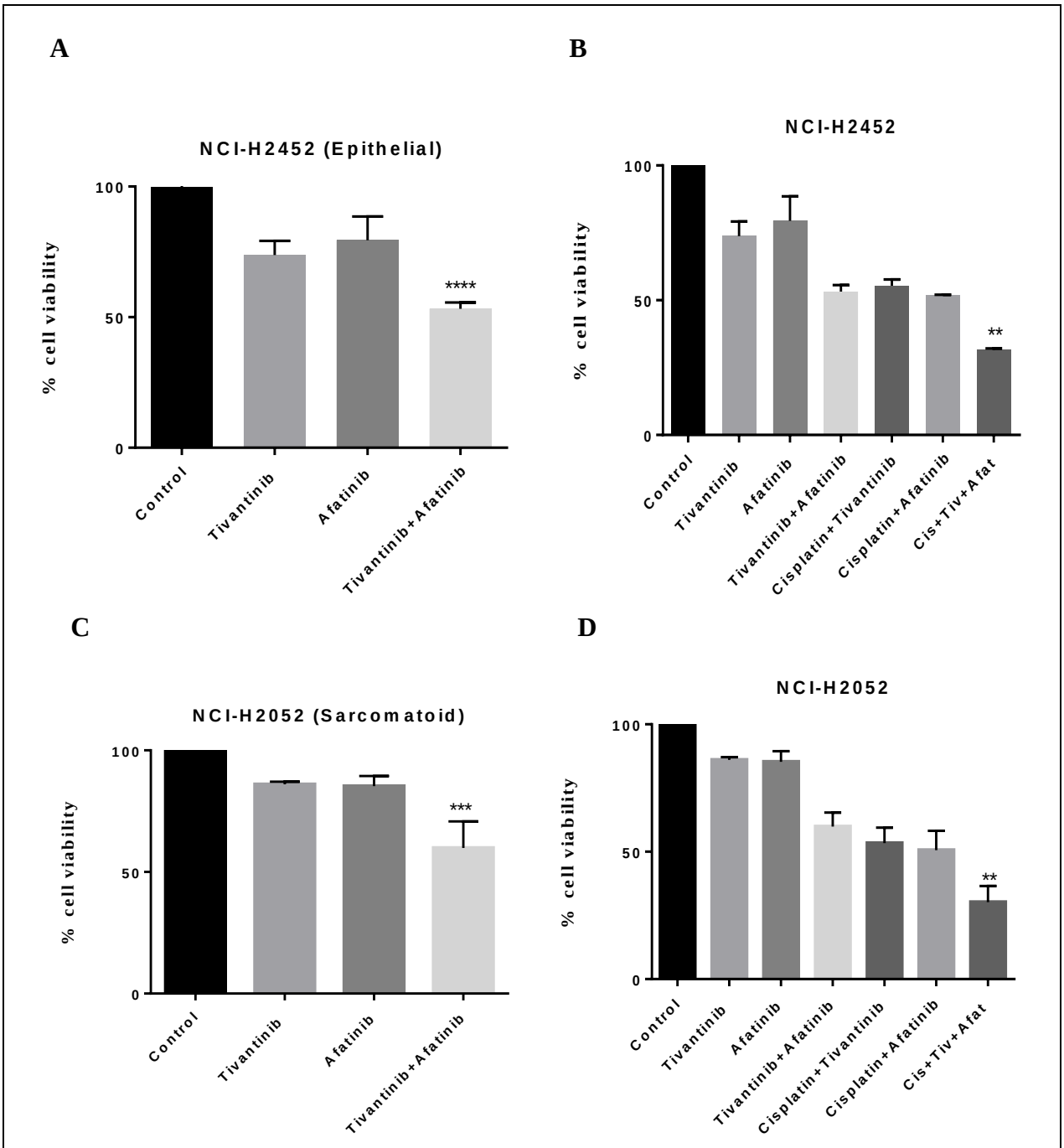


Figure 10.3 Reduction of cell viability in NCI-H2452 and NCI-H2052 cells with treatment by Tivantinib, Afatinib and Cisplatin. Cell proliferation assay of (A&B) NCI-H2452 cells and (C&D) NCI-H2052 cells with a single agent or combination treatment with the IC25 values (**Table 10.2**) of Tivantinib, Afatinib, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

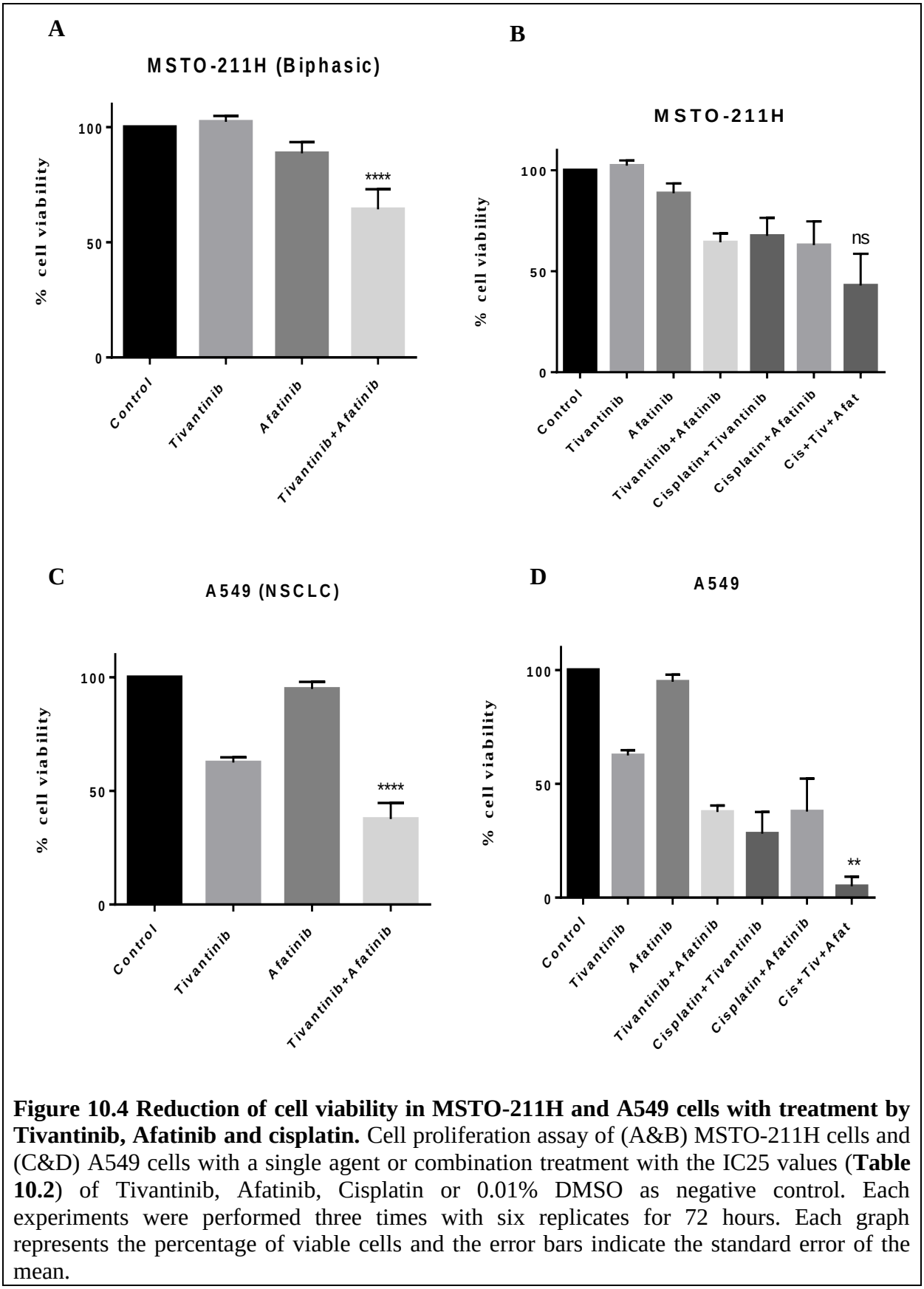


Figure 10.4 Reduction of cell viability in MSTO-211H and A549 cells with treatment by Tivantinib, Afatinib and cisplatin. Cell proliferation assay of (A&B) MSTO-211H cells and (C&D) A549 cells with a single agent or combination treatment with the IC25 values (**Table 10.2**) of Tivantinib, Afatinib, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

10.3.1.3 Effect of combined use of c-MET and PI3K/mTOR dual inhibitors on Cell Viability

Tivantinib was combined with the dual PI3K/mTOR inhibitor VS-5584 in all cell lines. We observed that all the cell lines had increased sensitivity to the IC25 of VS-5584 resulting in further reduction (20 - 30%) in cell proliferation (Figure 10.5 & Figure 10.6). Combination of tivantinib and VS-5584 produced an additive effect when compared to each inhibitor as single agents. The addition of cisplatin to the combination further reduced cell proliferation in all cell lines (Figure 10.5A-D and Figure 10.6A-D). The percentages of cell inhibition after 72-hour exposure to a combination of cisplatin with tivantinib and afatinib were approximately 85%, 78%, 79% and 98% in NCI-H2452, NCI-H2052, MSTO-211H and A549 cells respectively, when compared to control cells (Figure 10.5B&D; Figure 10.6B&D). VS-5584 was also very effective when combined with cisplatin alone.

In Figure 10.7 and Figure 10.8, afatinib and VS-5584 were assessed as single agents and combination therapy in all cell lines. Although the combination of both inhibitors was significantly more effective than afatinib alone, VS-5584 as a single agent reduced cell proliferation by 50% at low nanomolar range (30-130 nM) (Table 10.2). The addition of afatinib to VS-5584 did not produce an additive effect in all mesothelioma cell lines and A549 cells when compared to VS-5584 alone. Subsequent addition of cisplatin to the combination significantly reduced cell proliferation by approximately 30% when compared to the combination of afatinib and VS-5584. However, the addition of cisplatin to the combination of afatinib and VS-5584 was not significantly different from when cisplatin was added to VS-5584 in all cell lines.

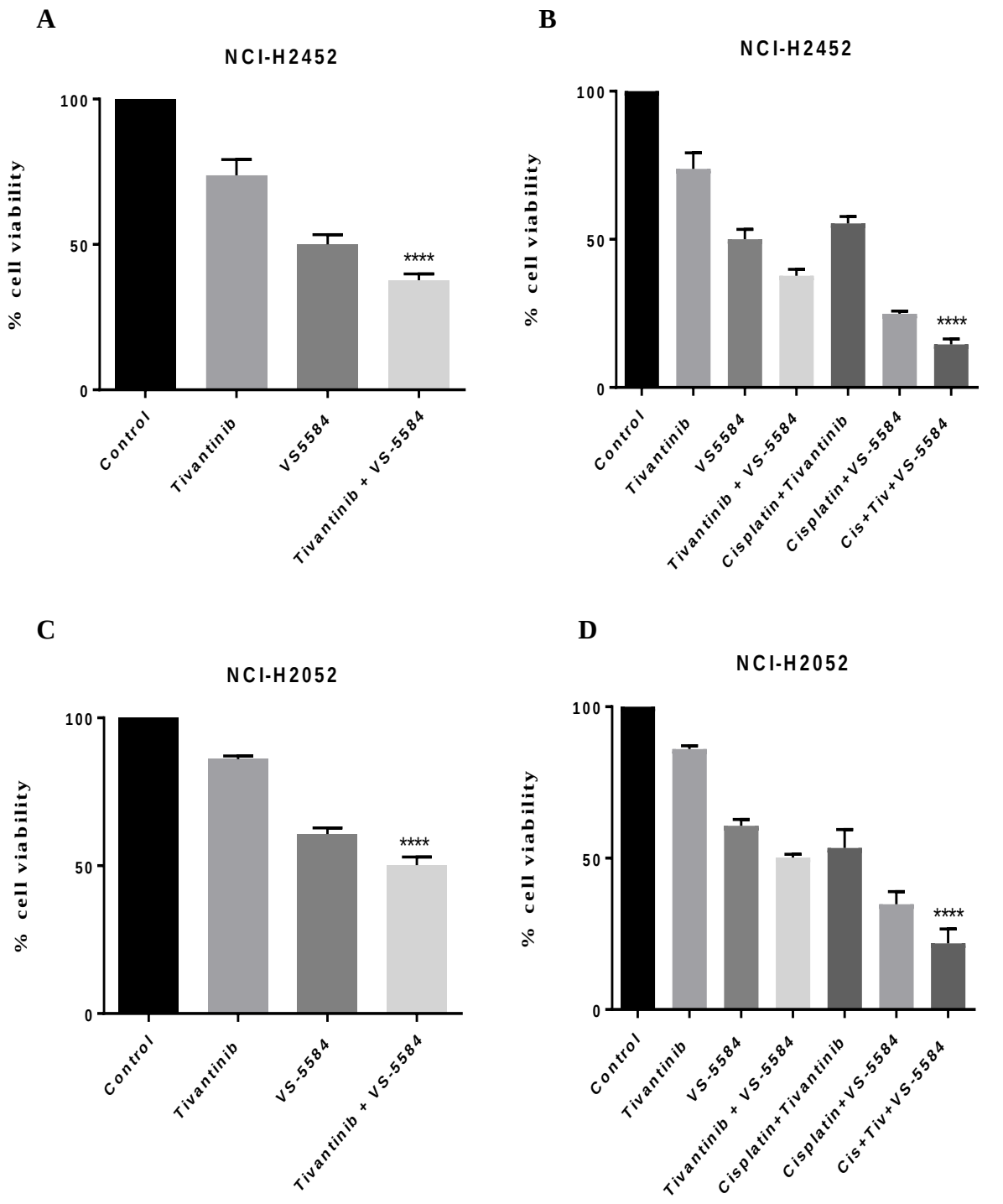


Figure 10.5 Reduction of cell viability in NCI-H2452 and NCI-H2052 cells with treatment by Tivantinib, VS-5584 and cisplatin. Cell proliferation assay of (A&B) NCI-H2452 cells and (C&D) NCI-H2052 with a single agent or combination treatment with the IC25 values of Tivantinib, VS-5584, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

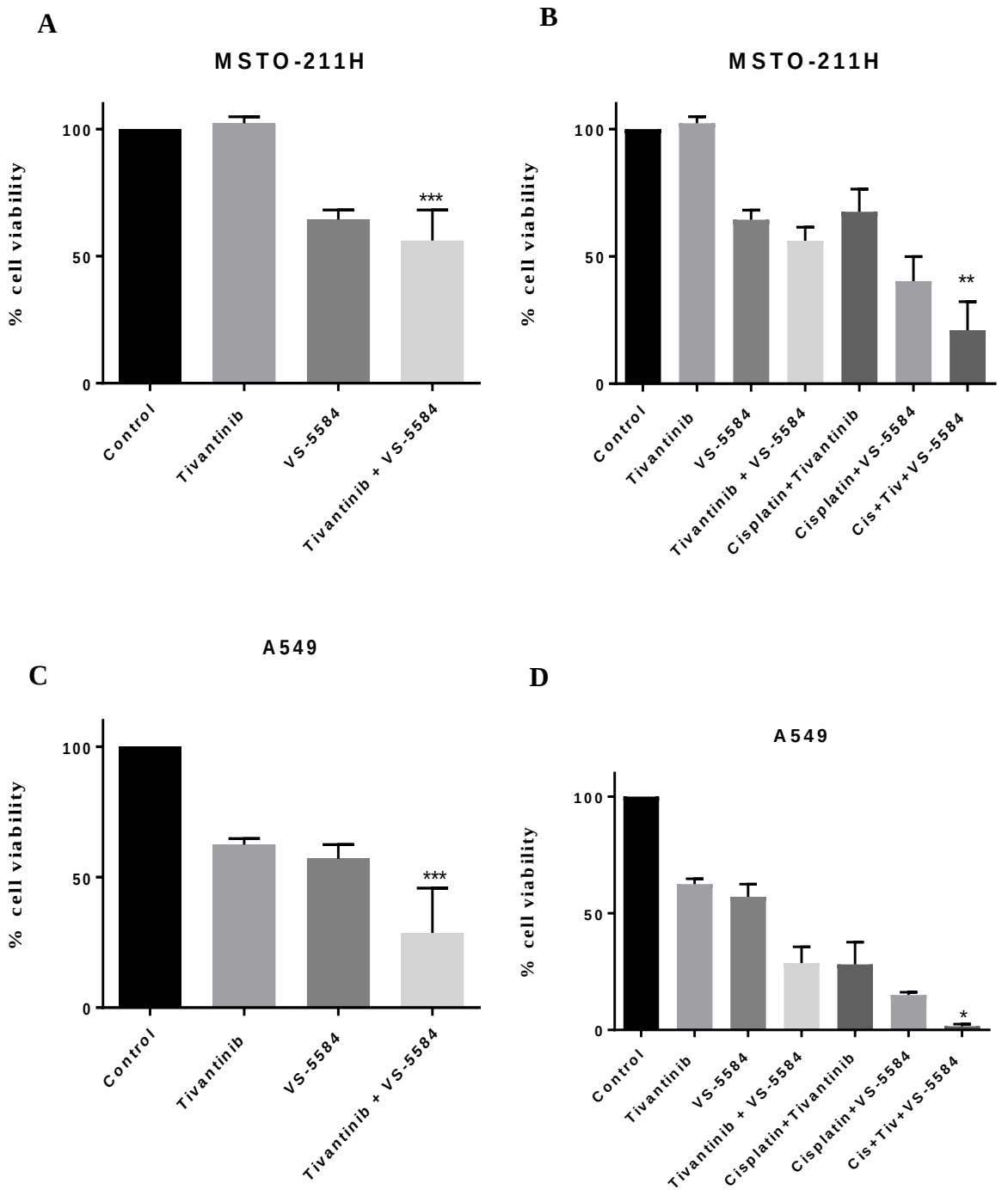


Figure 10.6 Reduction of cell viability in MSTO-211H and A549 cells with treatment by Tivantinib, VS-5548 and cisplatin. Cell proliferation assay of (A&B) MSTO-211H cells and (C&D) A549 cells with a single agent or combination treatment with the IC25 values of Tivantinib, VS-5584, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

10.3.1.4 Effect of combined use of EGFR/HER2 and PI3K/mTOR dual inhibitors on Cell Viability

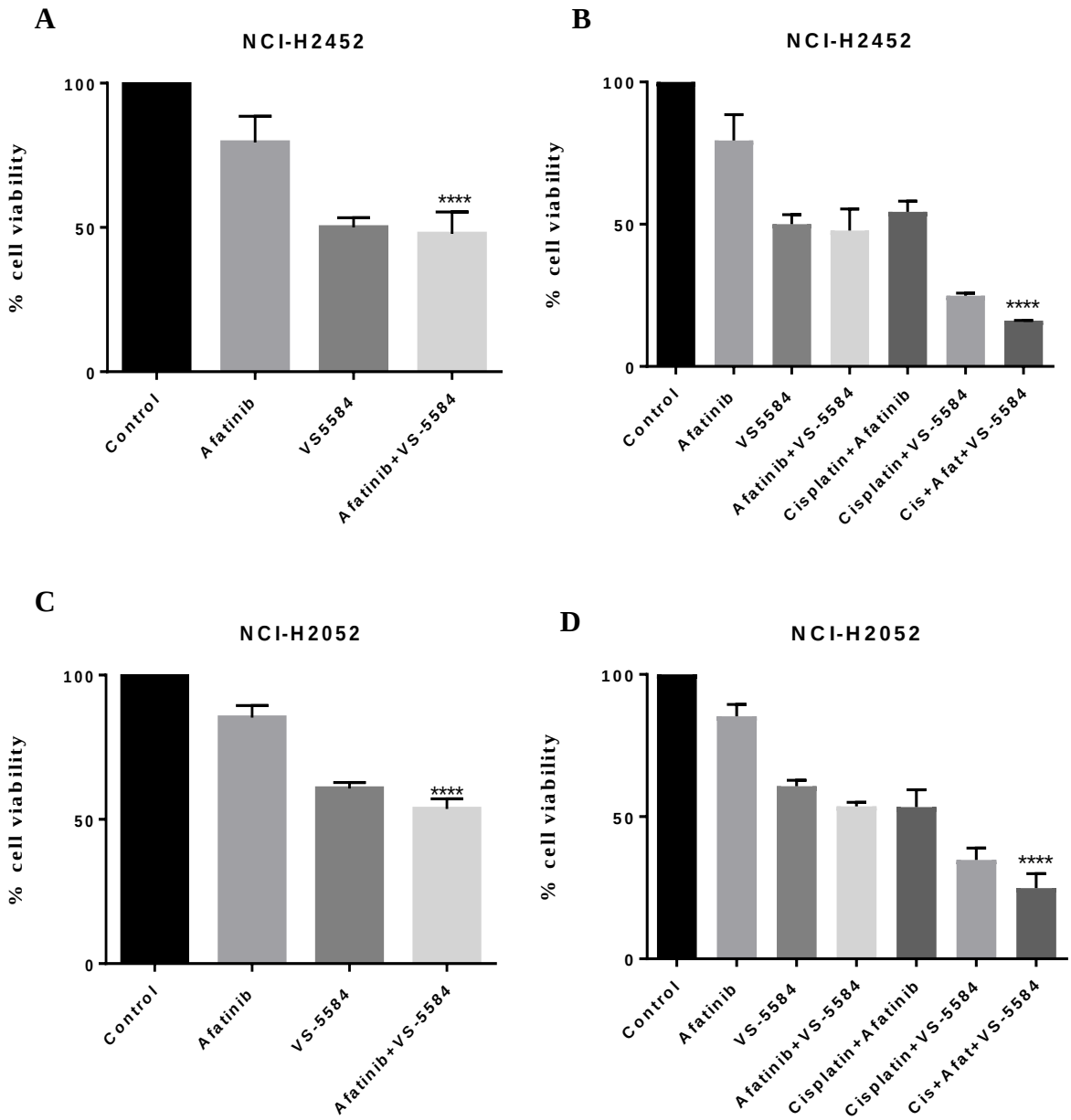


Figure 10.7 Reduction of cell viability in NCI-H2452 and NCI-H2052 cells with treatment by Afatinib, VS-5584 and cisplatin. Cell proliferation assay of (A&B) NCI-H2452 cells and (C&D) NCI-H2052 with a single agent or combination treatment with the IC25 values of Afatinib, VS-5584, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

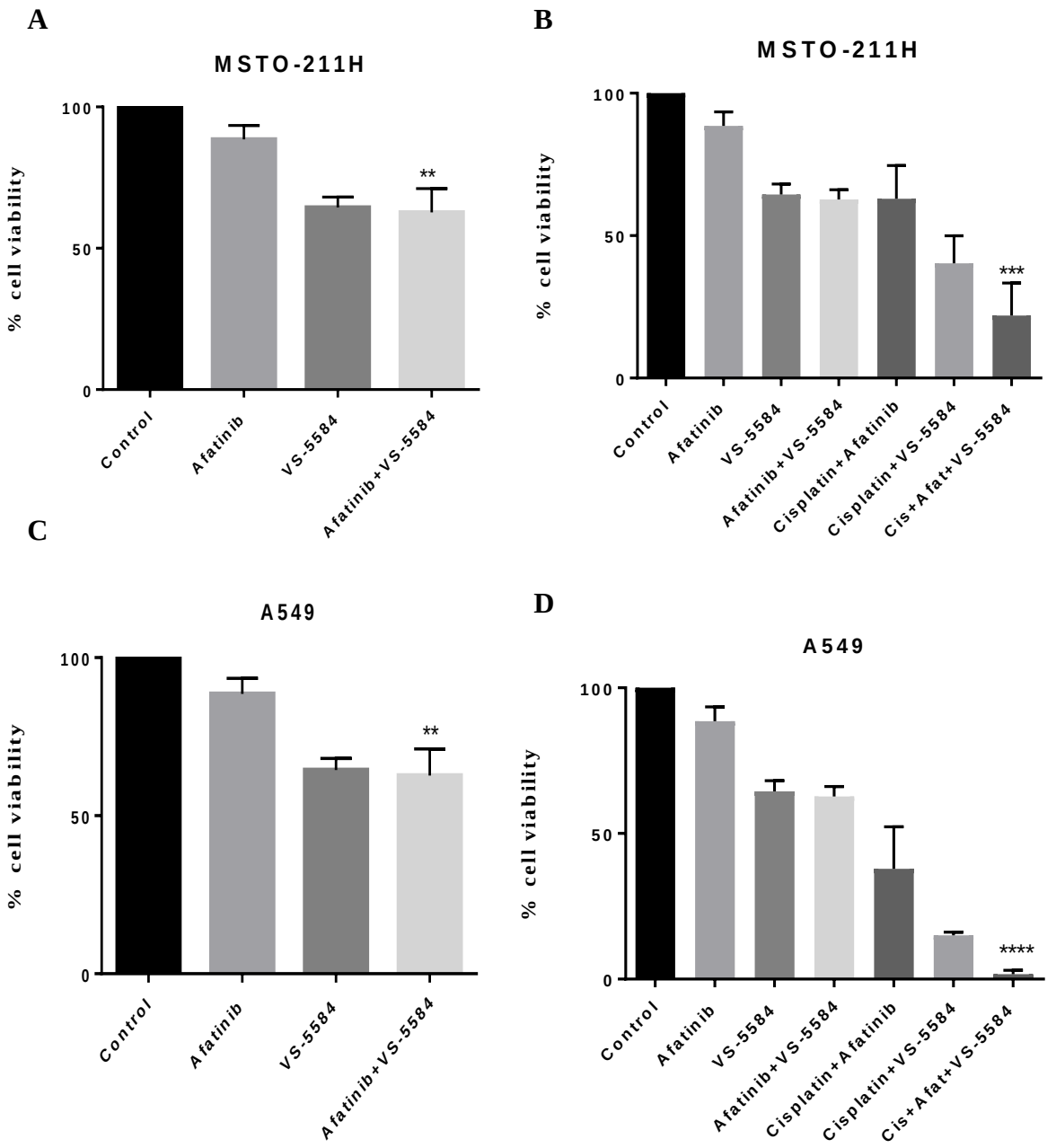


Figure 10.8 Reduction of cell viability in MSTO-211H and A549 cells with treatment by Afatinib, VS-5548 and cisplatin. Cell proliferation assay of (A&B) MSTO-211H cells and (C&D) A549 cells with a single agent or combination treatment with the IC25 values of Afatinib, VS-5584, Cisplatin or 0.01% DMSO as negative control. Each experiments were performed three times with six replicates for 72 hours. Each graph represents the percentage of viable cells and the error bars indicate the standard error of the mean.

10.3.2 Western blot

10.3.2.1 Effect of combined use of c-MET and EGFR/HER2 inhibitor or PI3K/mTOR inhibitor on downstream signalling pathways

Mesothelioma cell lines NCI-H2452, NCI-H2052 and MSTO-211H and the NSCLC cell line A549 were treated with the IC25s of tivantinib, or Afatinib or VS-5584 alone or in combination (tivantinib/afatinib and tivantinib/VS-5584) for 3 hours to investigate the effect of these inhibitors on downstream signalling of the MAPK and AKT pathways. Since VS-5584 inhibits cancer stem cells, ALDH1 expression was also investigated. ALDH1 has been previously described as a marker for cells with stem-cell like properties (Ginestier et al., 2007; Nishikawa et al., 2013). However, only A549 cells expressed the ALDH1A1 protein. Forty micrograms of whole cell lysates were subjected to western blotting and representative blots are shown in Figure 10.9A-D. The expression status of each protein in each cell line is described in Table 10.4. Tivantinib alone slightly inhibited p-ERK in H2052 cells but strongly reduced the expression of p-AKT in MSTO-211H cells (Figure 10.9C&D). Afatinib alone significantly reduced p-ERK expression in A549 and NCI-H2052 cells. EGFR expression was reduced by afatinib in A549 cells and also inhibited by tivantinib and afatinib combination in MSTO-211H cells (A&D). VS-5584 had no effect on ALDH1A1 although VS-5584 alone inhibited p-AKT in MSTO-211H and slightly reduced the expression of p-ERK in A549 cells. The combination of tivantinib and VS-5584 reduced the expression of p-AKT in A549 and MSTO-211H cells. The combination also reduced the expression of p-ERK in NCI-H2052 cells. Combination of tivantinib with afatinib reduced p-ERK expression in A549 cells, EGFR expression in NCI-H2452 cells, EGFR and p-ERK expression in NCI-H2052 cells and EGFR and p-AKT in MSTO-211H cells.

Table 10.4 Tabular description of protein expression in A549 and mesothelioma treated cell lines

		Protein status				
Cell lines	**Treatment (targets)**	**EGFR**	**p-AKT**	**ALDH1**	**ERK**	**p-ERK**
A549	Untreated	+++	+++	+++	+++	+++
	Tivantinib (c-MET)	+++	+++	+++	+++	+++
	Afatinib (EGFR/HER2)	++	++	+++	+++	+
	VS-5584 (PI3K/mTOR)	++	++	+++	+++	++
	Tivantinib +VS-5584	+++	+	+++	+++	+++
	Tivantinib +Afatinib	+++	++	+++	+++	++
NCI-H2452	Untreated	+++	N/A	0	+++	++
	Tivantinib (c-MET)	+++	N/A	0	+++	+++
	Afatinib (EGFR/HER2)	+++	N/A	0	+++	+++
	VS-5584 (PI3K/MTOR)	+++	N/A	0	+++	+++
	Tivantinib +VS-5584	+++	N/A	0	+++	+++
	Tivantinib +Afatinib	+	N/A	0	+++	++
NCI-H2052	Untreated	+++	N/A	0	N/A	+++
	Tivantinib (c-MET)	+++	N/A	0	N/A	++
	Afatinib (EGFR/HER2)	+++	N/A	0	N/A	+
	VS-5584 (PI3K/MTOR)	+++	N/A	0	N/A	++
	Tivantinib +VS-5584	+++	N/A	0	N/A	++
	Tivantinib +Afatinib	++	N/A	0	N/A	+
MSTO-211H	Untreated	+++	+++	0	N/A	+++
	Tivantinib (c-MET)	++	+	0	N/A	+++
	Afatinib (EGFR/HER2)	++	+	0	N/A	+++
	VS-5584 (PI3K/MTOR)	++	+	0	N/A	+++
	Tivantinib +VS-5584	+++	++	0	N/A	+++
	Tivantinib +Afatinib	++	+	0	N/A	+++

Key	
Strong expression	+++
Moderate	++
Weak expression	+
Absent	0
Not optimized	N/A

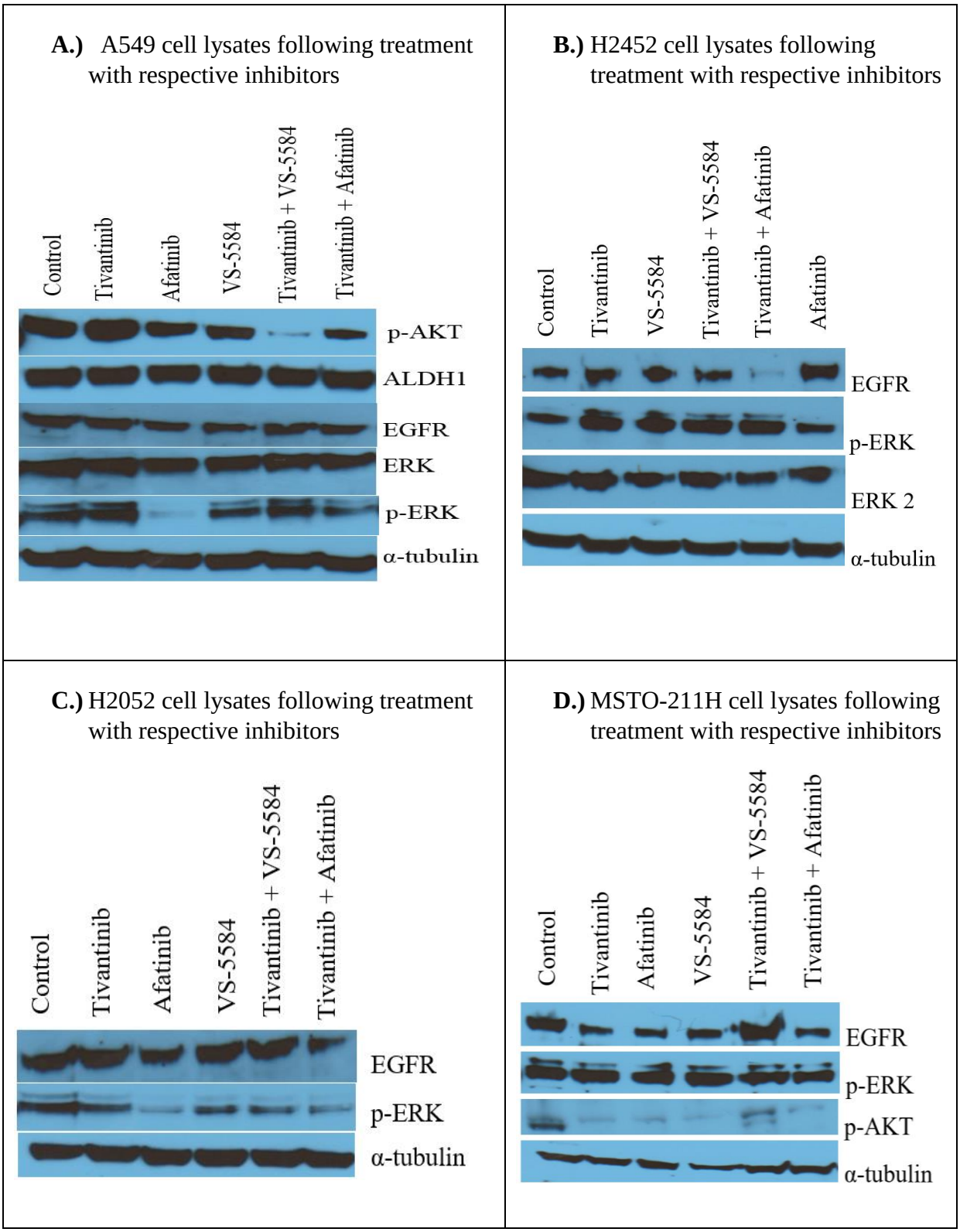


Figure 10.9 Effects of inhibitors of c-MET (Tivantinib), EGFR/HER2 (Afatinib) and PI3K/mTOR (VS-5584) on AKT and MAPK activation. Western blotting was carried out with 40 µg total cell lysates after 3-hour treatment with the IC25s of tivantinib, afatinib or VS-5584 as single agents or as combinations in supplemented cell culture media. α-tubulin was used as loading control.

10.3.3 Effect of combined use of c-MET and EGFR/HER2 inhibitor or PI3K/mTOR inhibitor on Cell Motility

We hypothesize that targeting proteins that promote mobility might reduce the migration of treated cells. The effect of tivantinib, afatinib, VS-5584 and their combination on migratory behaviour was investigated in NCI-H2452, NCI-H2052 and A549 cells using *in vitro* scratch assay. All three cell lines were assessed but MSTO-211H cells did not adhere firmly to the surface of the flask after the scratch was made therefore it was difficult to measure accurately. Poly-lysine was used to coat the flask but there was no success achieved. This cell line was therefore exempted from this experiment.

In the NCI-H2052 cells, afatinib and tivantinib/VS-5584 combination reduced cell migration by approximately 35% and 36% respectively. There was no statistical difference between the treated cells and untreated (control) cells (Figure 10.10A-B). As shown in Figure 10.11, afatinib, tivantinib and VS-5584 reduced cell migration in NCI-H2452 cells when compared to the control cells following a 24-hour exposure to the inhibitors. Although the difference between the tivantinib and VS-5584 single agent treated cells and control cells was not statistically significant. Afatinib significantly reduced cell migration with a percentage wound healing area of 14%. The percentages of the wound healing area were approximately 15% and 16% in the tivantinib/afatinib and tivantinib/VS-5584 combination treated cells respectively. The combination of tivantinib with either afatinib or VS-5584 significantly reduced cell migration in the H2452 cells (Figure 10.11A-B).

Similarly in the A549 cells, afatinib, tivantinib and VS-5584 reduced cell migration when compared to control cells. The percentages of the wound healing area were approximately 15% and 19% in the tivantinib/afatinib and tivantinib/VS-5584 combination treated cells respectively. The combination of tivantinib with either afatinib ($p=0.007$) or VS-5584 ($p=0.0123$) significantly reduced cell migration in the A549 cells when compared to the untreated cells (Figure 10.12A-B).

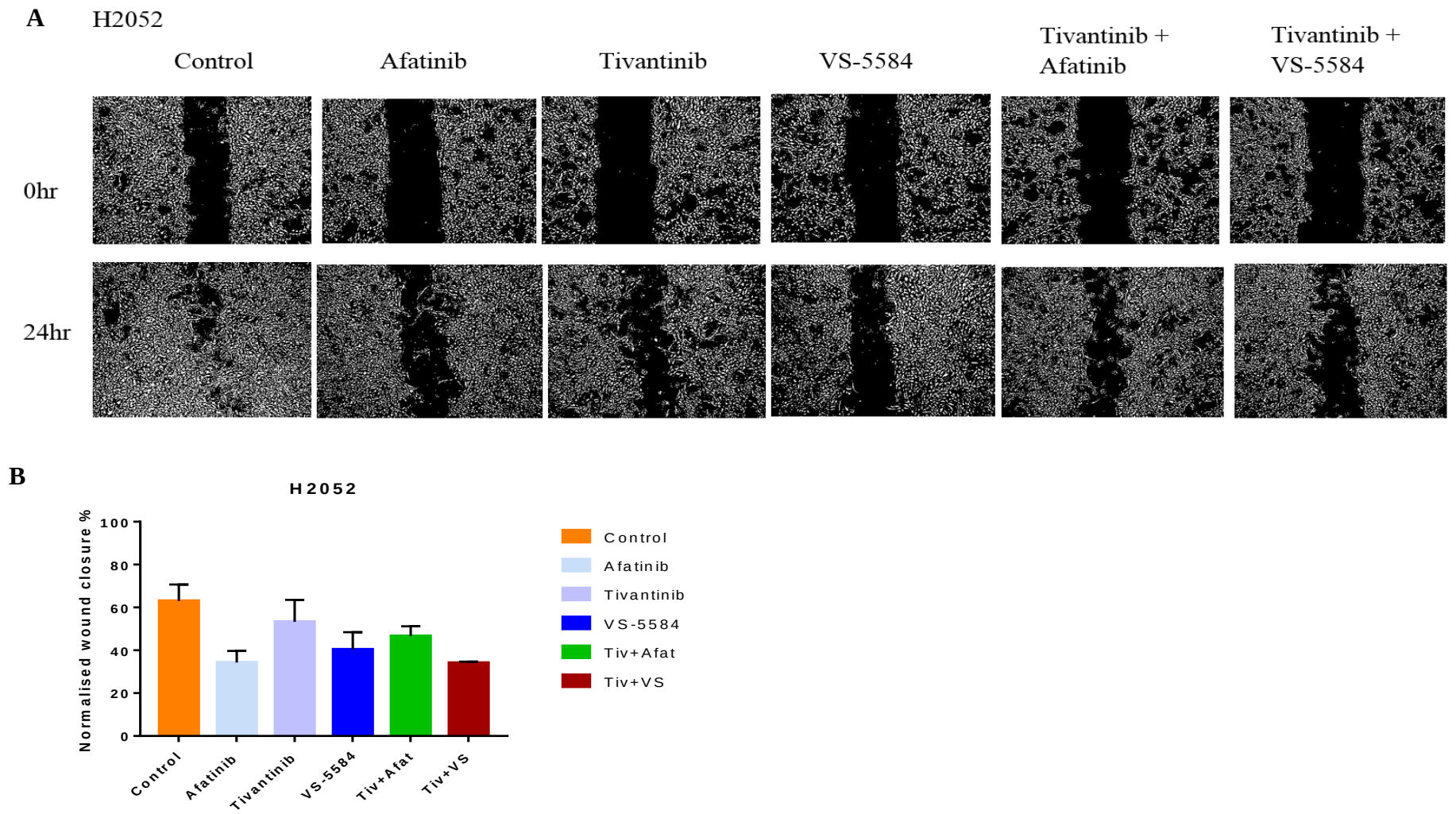


Figure 10.10 Inhibition of cell migration of H2052 cells by tyrosine kinase inhibitors. Wound healing assay was performed in H2052 cells treated with Afatinib (470 nM), Tivantinib (130 nM), VS-5584 (47 nM), or combinations for 24 hours. (A) shows representative pictures of the degree of wound closure in control and treated (x100). (B) cell migration at each point was quantified and normalised to 0 h in the H2052 cells. Experiments were repeated three times and three points were measured. The error bars indicate the standard error of the mean.

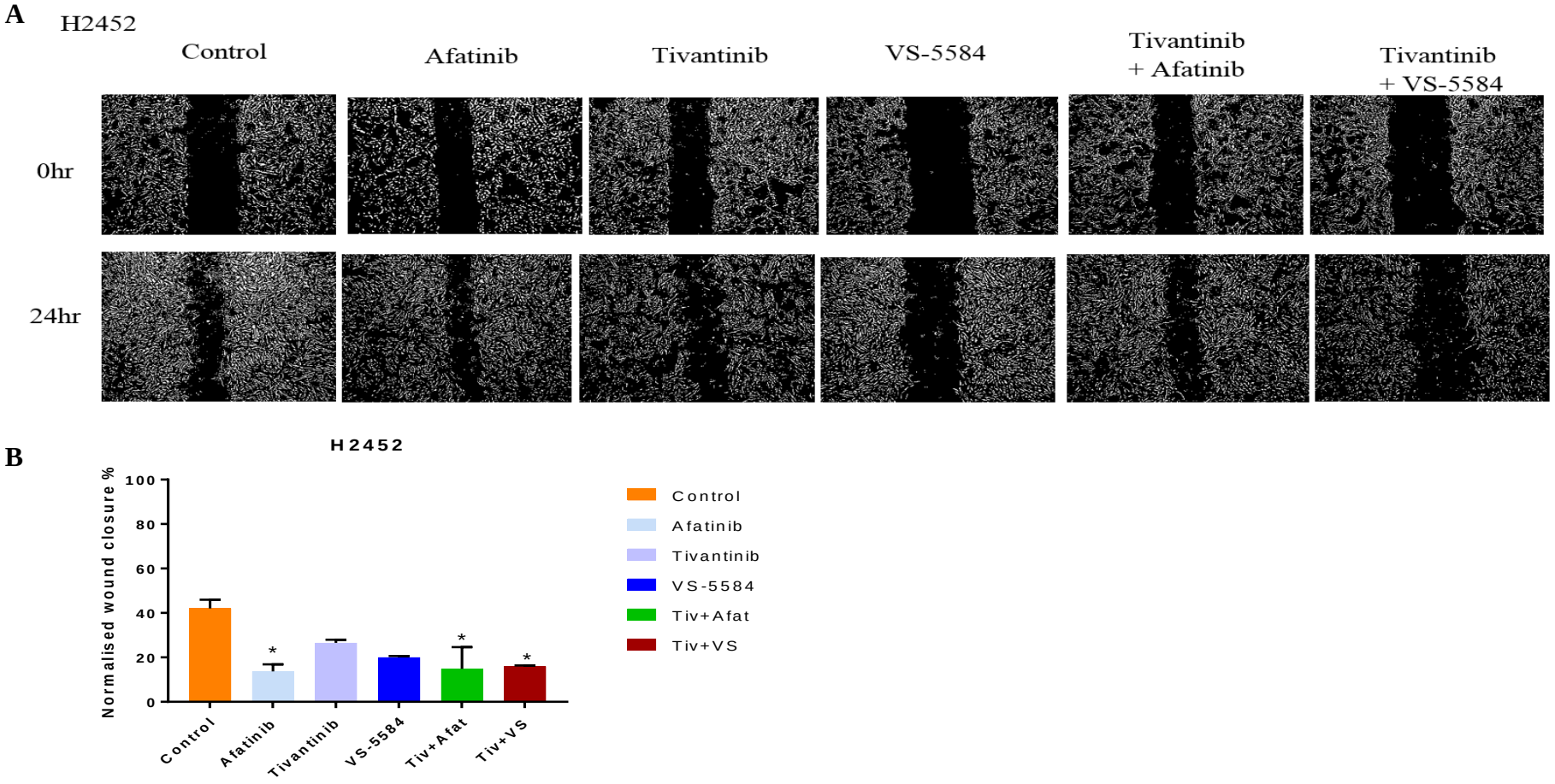


Figure 10.11 Inhibition of cell migration of H2452 cells by tyrosine kinase inhibitors. Wound healing assay was performed in H2452 cells treated with Afatinib (330 nM), Tivantinib (530 nM), VS-5584 (97 nM), or combinations for 24 hours. (A) shows representative pictures of the degree of wound closure in control and treated (x100). (B) cell migration at each point was quantified and normalised to 0 h in the H2452 cells. Experiments were repeated three times and three points were measured. The error bars indicate the standard error of the mean.

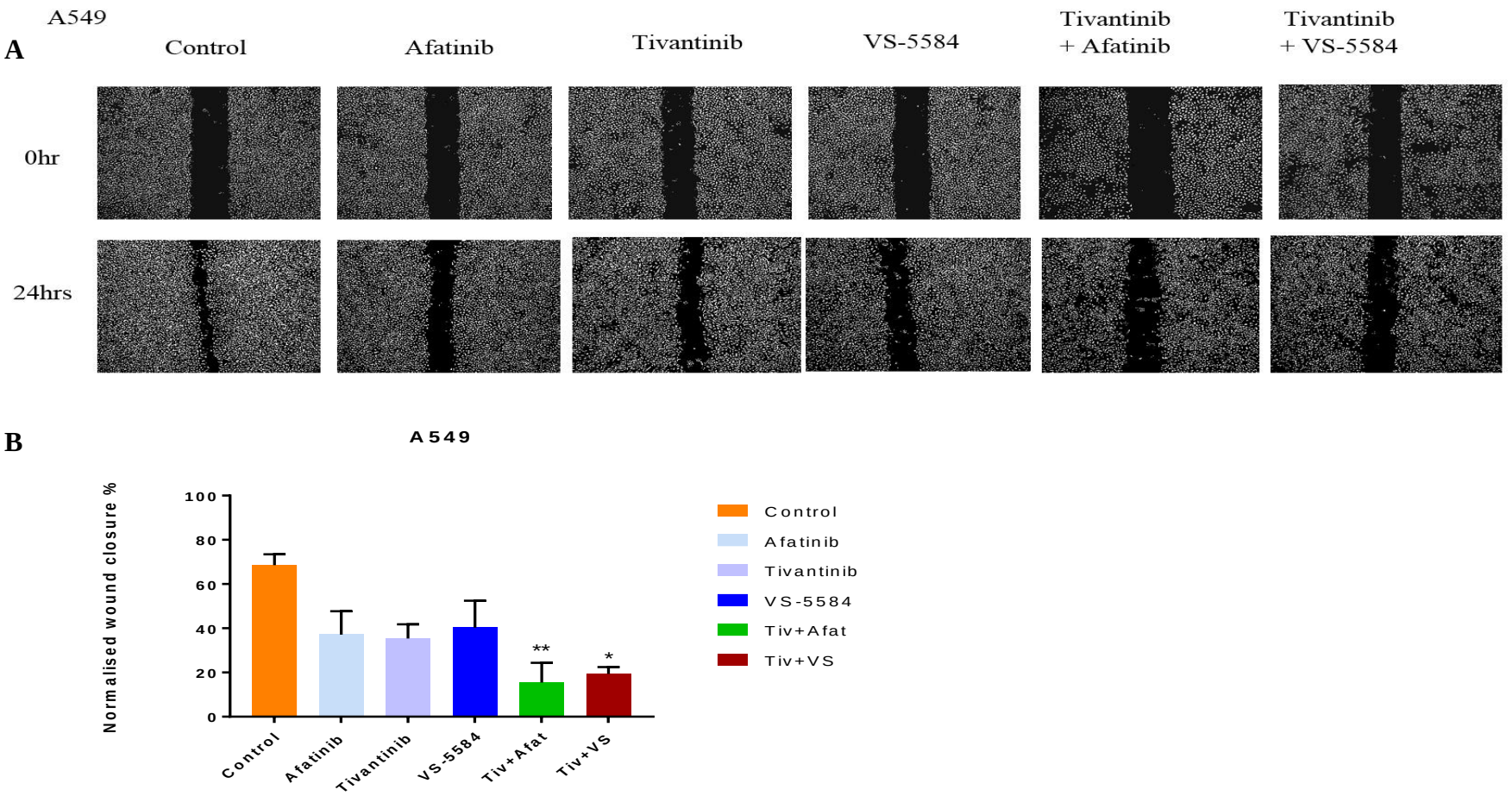


Figure 10.12 Inhibition of cell migration of A549 cells by tyrosine kinase inhibitors. Wound healing assay was performed in A549 cells treated with Afatinib (1.03 µM), Tivantinib (1.13 µM), VS-5584 (130 nM), or combinations for 24 hours. (A) shows representative pictures of the degree of wound closure in control and treated (x100). (B) cell migration at each point was quantified and normalised to 0 h in the A549 cells. Experiments were repeated three times and three points were measured. The error bars indicate the standard error of the mean.

Table 10.5 c-MET, EGFR, PTEN and p70S6K protein expression status in A549 and mesothelioma cell lines as demonstrated in Chapters 7, 8 & 9.

Cell lines	c-MET protein	EGFR protein	PTEN protein	p70S6K protein
A549	Expressed	Expressed	Weakly expressed	Expressed
NCI-H2452	Expressed	Expressed	Expressed	Not expressed
NCI-H2052	Expressed	Expressed	Expressed	Not expressed
MSTO-211H	Expressed	Expressed	Not expressed	Expressed

10.4 Discussion

In this study we demonstrated the benefit of combined targeting of receptor tyrosine kinase c-MET and EGFR or major intracellular signal transducer PI3K *in vitro*. We had previously shown in Chapters 7, 8 & 9 that the c-MET inhibitor tivantinib, EGFR/HER2 inhibitor and PI3K/mTOR inhibitor VS-5584 significantly reduced cell viability in a dose-dependent manner when used alone. EGFR protein and c-MET protein were expressed in all cells lines. PTEN protein was expressed in the NCI-H2452 and NCI-H2052 cell lines but p70S6K protein was absent suggesting the inactivation of the PI3K/AKT/MTOR pathway in both cell lines. Weak expression and loss of PTEN in the A549 and MSTO-211H cells may suggest that PTEN/AKT/MTOR pathway may be activated in these cells therefore enhance their sensitivity to PI3K/MTOR inhibitors. The presence of c-MET and EGFR protein also provides a rationale to use tyrosine kinase inhibitors that would inhibit the proteins. We hypothesized that co-targeting different nodes in the EGFR, c-MET and PI3K/MTOR pathway might reduce cell viability which might also be enhanced by the addition of a chemotherapeutic agent. The IC50 values of cisplatin in A549, NCI-H2452, NCI-H2052 and MSTO-211H cells were 9 µM, 9.9 µM, 2.1 µM and 0.86 µM respectively. O'Kane et al (2010) using MTT assay, have previously reported the IC50 values for cisplatin in this same cell lines as 24.6 µM (A549), 4.0 µM (NCI-H2452), 2.2 µM (NCI-H2052) and 3.4 µM (MSTO-211H). The IC50 values for pemetrexed was also reported as 5.28 µM (A549), 1.08 µM (NCI-H2452), 0.204 µM (NCI-H2052) and 1.61 µM (MSTO-211H). (O'Kane et al., 2010). Due to technical challenges we were unable to obtain IC50 values for pemetrexed for each cell line in this study.

In all cell lines targeting both EGFR/HER2 and c-MET was more effective than using a single inhibitor (tivantinib or afatinib). Addition of cisplatin to the combination of tivantinib

with afatinib further reduced cell viability significantly in all cell lines except the MSTO-211H cells (Figure 10.3 and Figure 10.4). Next we investigated the effect of targeting c-MET and PI3K/mTOR together with a chemotherapeutic agent. We demonstrated that the combination of tivantinib and VS-5584 was significantly more effective than using either agents alone in the NCI-H2452, NCI-H2052 and A549 cells (Figure 10.5A&C; Figure 10.6C). Although in the MSTO-211H cells we did not observe significant difference in the VS-5584 only treated cells and the combination (Figure 10.6A). We concluded that it might be as a result of variation between experiments because of the rise in the error bar. Extra care was taken to ensure that every experiment was carried out in the same manner. Addition of cisplatin to the combination was also significantly more effective than the combination in all cell lines. Combination of VS-5584 with cisplatin had a strong synergistic effect that was more effective than the tivantinib and VS-5584 combination in all cells lines. This effect seemed to be independent of the status of the PI3K/AKT/MTOR pathway in this cells thus suggesting that VS-5584 inhibits cell growth through other unknown mechanisms. This interaction also suggests that VS-5584 might be an excellent candidate for *in vivo* testing in addition to cisplatin. We further examined co-targeting EGFR/HER2 and PI3K/mTOR in all cell lines by combining afatinib and VS-5584 (Figure 10.7; Figure 10.8). In comparison to afatinib alone the combination seemed effective but when compared to VS-5584 only treated cells, there was no significant difference observed. The cytotoxic effect of VS-5584 seemed to be stronger than that of afatinib hence no additive effect was observed in all the cell lines. Although the addition of cisplatin drastically reduced cell viability by approximately 35% in mesothelioma cells and 45% in the A549 cells when compared to the combination of afatinib and VS-5584. Afatinib alone or in combination with tivantinib suppressed p-ERK expression in A549, NCI-H2452 and NCI-H2052 cells (Figure 10.9A-C). The combination of tivantinib with afatinib also reduced the expression of phosphorylated AKT in the MSTO-211H cells. p-AKT is a downstream target of the PI3K which is a key downstream signal transducer for RTKs including c-MET and EGFR. p-AKT was also supressed by tivantinib, afatinib and VS-5584 as single agents in MSTO-211H cells (Figure 10.9D). The underlying reason for the reduced AKT phosphorylation could be as a result of the complete loss of PTEN protein and the low expression of ERK in the MSTO-211H cells, suggesting that PI3K/MTOR pathway might be a major player in the proliferation of these cells. In the NCI-H2452 and NCI-H2052 cells, Afatinib and VS-5584 adversely affected cell motility independent of the HGF/c-MET pathway which is popularly known to promote cell motility. This might imply that afatinib and VS-5584 supress the expression of other proteins that promote cell motility but this was

not explored in this study. The combination therapy (tivantinib/afatinib or tivantinib/VS-5584) significantly reduced cell motility in the NCI-H2452 and A549 cells than single agents (Figure 10.11; Figure 10.12). It is noteworthy to mention that the highest concentration of tivantinib used within these experiments was approximately 4-fold lower than its maximum clinically relevant dose of 4.5 µM.

The study of Kanteti et al (2014) in MPM cell lines (H2596 and H513) analysed the cytotoxic effect of combining tivantinib with NVP-BEZ25 or GDC-0980 (two PI3K/mTOR inhibitors) for 72 hours using the Alamar blue assay. There was a synergistic effect in MPM cells when tivantinib was combined with either NVP-BEZ235 or GDC-0980 (Kanteti et al., 2014). This was in keeping with our findings when tivantinib was combined with VS-5584. To the best of our knowledge there is no published report on the effect of combining VS-5584 with other inhibitors in advanced cancers especially mesothelioma. Our study is the second to evaluate the combination of MET and PI3K inhibitors in mesothelioma. Recently, a study reported the significant synergistic effect of combining a MEK inhibitor U0126 and the PI3K inhibitor LY294002 in *in vitro* and *in vivo* MPM experimental models. The combination therapy was more effective than individual drugs and prolonged the survival time of EHMES-10 cell bearing mice. In addition, the combination therapy induced apoptosis and strongly reduced tumour angiogenesis by suppressing the expression of the VEGF (vascular endothelial growth factor) and HIF1α (hypoxia-induced factor 1-alpha) proteins (Miyoshi et al., 2012). Tivantinib has been shown to have a synergistic effect when combined with pemetrexed in mesothelioma cells (Leon et al., 2014). Our study demonstrates that tivantinib also exhibits enhanced antiproliferative effects when combined with cisplatin. A study by Kawaguchi et al (2009) reported the co-activation of receptor tyrosine kinases including c-MET, EGFR and HER2 in a panel of MPM cell lines using phospho-RTK array analysis. Combined inhibition with c-MET and EGFR inhibitors effectively supressed cell growth in H2373 and H290 cells than either inhibitors alone (Kawaguchi et al., 2009). Similarly, a combination of afatinib and tivantinib was reported to be effective against erlotinib-resistant lung cancer cell line H1975 by inducing apoptosis and cell cycle arrest in addition to significantly inhibiting cell growth compared to either inhibitors alone or a combination of erlotinib and tivantinib (Qu et al., 2014). A preliminary report on a clinical trial investigating the effect of tivantinib alone in MPM patients as second line therapy failed to reach its primary endpoint of complete or partial radiologic response in 12 months. No correlation was observed between c-MET expression or mutation and progression free survival or overall survival in their preliminary analysis. Of the 18 patients enrolled, 56% had high p-AKT expression (Maron et al., 2015).

Our study and others have reported the activation of multiple pathways for the survival of mesothelioma cells and the likelihood of resistance via shunting of pathways. This could be a reason for the failed response in the above trial since there was functional high expression of p-AKT indicating a functional PI3K/AKT pathway which needed to be inhibited. While we await the results from the ongoing open-phase 1b trials of tivantinib in combination with pemetrexed and carboplatin as first line therapy in MPM patients (NCT02049060) and the phase 1 dose escalation study of VS-5584 in combination with Defactinib in relapsed mesothelioma (NCT0237227), there is a need for further studies to identify predictive biomarkers for tivantinib and VS-5584. The response of each cell line to combination therapy seemed to be independent of the status of the protein in the cell. The combination of Tivantinib with Afatinib or VS-5584 in A549 cells produced additive effect but the combination of Afatinib with VS-5584 did not significantly reduce cell growth. We expected that A549 cells should respond to the combination of Afatinib and VS-5584 due to the increased expression of MAPK proteins, KRAS mutation status and the expression of p70S6K proteins. Synergistic effect was only seen when cisplatin was added to the combination. Our results suggests that there might be other mechanisms that are involved in driving growth inhibition in each cell line in response to the inhibitors.

In summary, our results indicates that multiple receptor tyrosine kinase are frequently activated in mesothelioma cells therefore, simultaneous inhibition of multiple tyrosine kinases might be potential therapeutic strategies for the treatment of MPM. Combination of multiple inhibitors is beneficial such that it may delay or circumvent mechanisms of drug resistance by interfering cell survival pathways and the crosstalk established between them. However, this comes at increased financial cost and the likelihood of side effects arising from individual drugs or interactions between two drugs. Importantly, mesothelioma cells are heterogeneous and we have observed from our findings that cell lines do not respond to therapy in the same manner. It is therefore important that further studies evaluate the molecular mechanism of action of tivantinib, afatinib and VS-5584 using comparative proteomic tools in order to identify predictive markers for the inhibitors and provide accurate clinical inferences for their use as combination therapy. Our study provides as rationale for the evaluation of ATP non-competitive c-MET inhibitors and PI3K/AKT/MTOR inhibitors as combinations in preclinical MPM models. Future studies will evaluate the effect of combining VS-5584 with Tivantinib or Afatinib in 2D and 3D cell culture systems. We will determine the dose of VS-5584 required to achieve significant growth inhibition when combined with the clinically achievable dose of afatinib in each cell line. The interaction of

individual inhibitors with pemetrexed will also be evaluated since the combination of cisplatin and pemetrexed is the first line therapy in mesothelioma. In order to identify biomarkers that can predict response or of prognostic relevance, we will look at the difference in the proteomic landscape of each cell line using the electrospray ionization mass spectrometry (ESI/MS) platform.

CHAPTER ELEVEN

CONCLUSIONS

Chapter 11 Conclusion

In summary, this study has demonstrated that

- 5-LOX and 12LOX proteins are expressed in 73% (56/77) and 83% (69/83) of archival MPM tissue samples respectively. Our study is the first to evaluate and show that the combination of a COX-2 inhibitor (Celecoxib) with a LOX inhibitor (Baicalein) produced an additive effect in MSTO-211H cells.
- VEGFR-2 protein is expressed in 93.8% (75/80) of archival MPM tissue samples. The VEGFR1, 2, 3 inhibitor (Cediranib) did demonstrate cytotoxicity but at doses significantly higher than those that can be achieved therapeutically. MGCD265 also reduced cell growth by 50% but it is not known if the dose used can be clinically achievable. Our study is the first to evaluate the cytotoxic effect of MGCD265 a c-MET, RON &TIE-2 inhibitor in MPM.
- HER-2 protein is expressed in 86.2% (69/80) of archival tissue samples. Our study is the first to evaluate the cytotoxic effect of Selumetinib and Afatinib in MPM. Afatinib did demonstrate cytotoxicity but at doses significantly higher than the clinically achievable dose.
- c-MET protein is expressed in 82% (58/71) of archival tissue samples. Tivantinib did demonstrate cytotoxicity at doses significantly lower than the clinically achievable dose in the NCI-H2452, NCI-H2052, MSTO-211H and A549 cell lines. Crizotinib demonstrated cytotoxicity within a clinically achievable dose range in only the MSTO-211H cells.
- MTOR kinase inhibitors (XL388 and Ku0063794) and PI3K/AKT/MTOR inhibitors (NVPBEZ235 and VS-5584) demonstrated significant cytotoxicity in NCI-H2452, NCI-H2052, MSTO-211H and A549 cells. It is not known if the dose used can be clinically achievable. Our study is the first to evaluate MTOR kinase inhibitors in MPM.
- Combined inhibition of Tivantinib and Afatinib demonstrated enhanced inhibition in all cell lines when compared to either inhibitors alone. The combination also reduced cell migration in NCI-H2452 and A549 cells.
- Combined inhibition of VS-5584 with Tivantinib or Afatinib demonstrated enhanced inhibition when combined with cisplatin in all cell lines.
- Combination of Tivantinib and VS-5588 reduced cell migration in NCI-H2452 and A549 cells.

- Afatinib alone significantly reduced cell migration in NCI-H2452 cell when compared to untreated cells.

11.1 *In vitro* cell line models and normal pleura samples

Most preclinical cytotoxicity studies are performed using cell lines grown in 2D monolayer which is a convenient and simple method for evaluating the effect of inhibitors in cancer cells. Cell lines offer several advantages over clinical tissue samples such as ease of use, cost effectiveness, limitless replicative ability and provide a homogenous population of tumour cells. Nevertheless, evidence suggests that cells undergo changes when placed in cultures resulting in doubts about the originality of the cell lines in comparison to the original tissue (Čunderlíková, 2013). Due to the continuous culture of cell lines, it is important to acquire cells from reputable sources for provenance, keep to low passage numbers and ensure routine mycoplasma tests. The tumour microenvironment consists of cancer cells, inflammatory cells, cytokines, blood vessels, endothelial cells and extracellular matrix. Significant interaction occur in the tumour microenvironment via cell-cell crosstalk which plays a vital role in the morphology and phenotype of cancer initiation and progression (Bissell & Radisky, 2001; Balkwill et al., 2013). Lack of a heterogeneous cell population and tissue architecture in cell cultures often abolishes cell-cell interaction and other cell functions. Unlike cell lines, human primary cells closely mimic the physiological state of cells *in vivo* and are likely to generate more relevant data representing living systems. However, they are extremely sensitive, difficult to maintain in culture and studies requiring several passages cannot be formed with primary cells. A bioinformatic analysis of proteomic phenotypes by Pan et al (2009) demonstrated that the Hepa 1-6 cell lines were deficient in mitochondria, reflecting rearrangement of metabolic pathways when compared to primary hepatocytes (Pan et al., 2009). Three dimensional cell cultures have also been developed to create cellular models that mimic the functions of living tissues and bridge the gap between 2D cell culture and live tissue (Pampaloni et al., 2007). Mesothelioma cells grown in 3D spheroids have been shown to acquire multicellular resistance mechanisms to a variety of apoptotic stimuli including TRAIL, histone deacetylase and proteasome inhibitors that were highly effective in mesothelioma cells grown in monolayers (Barbone et al., 2008; Barbone et al., 2011). It is therefore essential that complementary pre-clinical experiments using 3D cultures, primary cells or mouse xenografts are carried out to validate our MTS assay findings. In the search for biomarkers using proteomics, there is a need to promote the use of matched benign (normal) pleura tissue samples for robust interpretations and reproducible results in the understanding

of mesothelioma. An analysis of The Cancer Genome Atlas (TCGA) data on different cancer types showed that paired normal samples provided more information on patient survival than tumours because of the involvement of the tumour microenvironment (Huang et al., 2016). Proteomics studies in mesothelioma involving tissue samples should seek to incorporate appropriate matched control samples in all experiments for comparing proteomic aberrations.

11.2 Targeted therapy in mesothelioma

The Arachidonic acid pathway

The expression of arachidonic acid metabolising enzymes COX-2, 5-LOX and 12-LOX in MPM archival tissue samples and cell lines suggest that their metabolic pathways may be active in mesothelioma. Studies have shown crosstalk between COX-2 and 5-LOX or 12-LOX pathways demonstrating a shunt in a metabolic pathway when another is blocked. In colorectal cancer cell lines (HCA7, HT-29 & LoVo) expressing 5-LOX and different levels of COX-2 expression, COX inhibitors reduced prostaglandin E_2 production but enhanced leukotriene B_4 secretion indicating a shunt in the arachidonic acid metabolism (Ganesh et al., 2012). COX-2 inhibitors and LOX inhibitors have also demonstrated cytotoxic effects in MPM cell lines as single agents (Marrogi et al., 2000; Romano et al., 2001; DeLong et al., 2003; Catalano et al., 2004; O'Kane et al., 2010). In our study, Licofelone, a dual COX/5-LOX inhibitor did not demonstrate cytotoxicity in A549, NCI-H2452, NCI-H2052 and MSTO-211H cell lines at therapeutically relevant dose. This could be as result of negative feedback mechanism via other signalling pathways. We also used MTS assay to determine the cytotoxic effect of combining baicalein with a therapeutically relevant dose of celecoxib but an additive effect was only observed in MSTO-211H cells. To further clarify the mechanisms of the COX-2, 5-LOX & 12-LOX pathway in mesothelioma, expression of several downstream proteins and metabolites such as PGE2, leukotrienes and VEGF should be assayed. Agarwal et al 2013 demonstrated that DuP-697 the parent compound of celecoxib inhibits mesothelioma cells by inducing apoptosis (Agarwal et al., 2013). Other authors have also suggested that celecoxib may act independent of COX-2/PGE2 inhibition to inhibit cancer cell growth (Lai et al., 2003; Kim et al., 2010). A similar proteomic study might be able to identify the exact molecular mechanism of action of celecoxib and baicalein in mesothelioma cells.

The VEGFR pathway

Preclinical studies including ours have shown that VEGF and VEGF receptors (VEGFR) are highly expressed in MPM and circulating VEGF levels are raised in mesothelioma patients

compared with healthy individuals or patients with other malignancies (Yasumitsu et al., 2010). So far, antiangiogenic inhibitors have shown modest efficacy in mesothelioma. Small molecules that inhibit VEGFR such as vatalanib, cediranib, dovitinib, pazopanib, nintedanib and axitinib have been evaluated in clinical trials in mesothelioma patients. Our study showed that cediranib demonstrates cytotoxicity to mesothelioma cells but at doses higher than its maximum tolerated dose. To the best of our knowledge no other study has evaluated the *in vitro* effect of cediranib in mesothelioma cell lines. A Phase II trial reported that cediranib as monotherapy has limited or no activity in mesothelioma patients and is associated with grade 3 toxicities particularly hypertension. In pre-treated MPM patients, cediranib had modest activity with a disease control rate of 42%. The initial dose of 45 mg daily was not tolerated therefore majority of patients required dose reduction (Garland et al., 2011; Campbell et al., 2012). Cediranib is currently in Phase II randomised trial evaluating side effects and best tolerated dose when combined with present standard of care, cisplatin and pemetrexed (NCT01064648). Nintedanib is also being investigated in Phase II/III trials in combination with cisplatin and pemetrexed (LUME-Meso; NCT01907100). In a recent randomised controlled open-label phase 3 trial, bevacizumab in addition to pemetrexed and cisplatin was reported to improve overall survival (18.8 months) when compared to patient treated with cisplatin and pemetrexed only (16.1 months) but at the cost of manageable toxicity (Zalcman et al., 2016). The authors did not evaluate the effect to VEGF expression or any other biomarker in this study. Median progression free survival overall survival in this trial were longer than in those reported in the other two phase II studies with gemcitabine, cisplatin and bevacizumab or pemetrexed, carboplatin and bevacizumab (Kindler et al., 2012; Ceresoli et al., 2013). Differences in patient enrolment and study design as well as low efficacy of the gemcitabine-based therapy might explain the discrepancy. The major setbacks in the use of antiangiogenic therapy is the absence of biomarkers or polymorphisms to stratify patients that would respond to anti-VEGF based therapies, side effects because of its effect on the normal vasculature and tumour resistance due to compensations from parallel signalling pathways. A study is currently evaluating biomarkers of angiogenesis and disease in 47 patients with unresectable malignant mesothelioma treated with vatalanib in the completed CALGB-30107 trial (NCT00898547). Although, biomarker studies require analysis of larger sample sizes to improve the biological significance of the identified proteins. There is currently no proteomic study that has extensively compared the proteomic landscape of anti-VEGF inhibitor treated samples *vs* untreated to understand the molecular mechanism of the inhibitors and circumvent side effects by identifying vulnerable signalling pathways.

The EGFR pathway

The expression of EGFR has been well established in mesothelioma but there has been little or no progress in the use of EGFR inhibitors in MPM. Phase II studies in advanced or recurrent MPM patients have shown that first generation EGFR inhibitors (erlotinib and gefitinib) were not effective as single agents despite overexpression of EGFR in 50-95% of cases (Govindan et al., 2005; Garland et al., 2007). Although, the patients were not selected based on their EGFR status. Low prevalence of EGFR activating mutations might explain the lack of efficacy. Our study evaluated the cytotoxic effect of afatinib an irreversible EGFR/HER2 inhibitor and showed that afatinib was able to reduce the growth of A549, NCI-H2452, NCI-H2052 and MSTO-211H cells but this was at doses higher than the maximum tolerated dose. Okita et al (2015) reported that lapatinib (EGFR/HER2 inhibitor) was able to enhance trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC) in MPM cell lines (NCI-H28 and NCI-H2052) and in primary mesothelioma cells lapatinib upregulated the binding sites of both cetuximab and trastuzumab resulting in enhanced cetuximab or trastuzumab medicated ADCC. Afatinib did not enhance trastuzumab-mediated ADCC (Okita et al., 2015). The dose of lapatinib (1 µM) used in their study was lower than the mean plasma concentration of lapatinib (6 µM) when 1250 mg was administered daily (Iwata et al., 2015). Cetuximab is currently being evaluated in a Phase II trial in combination with cisplatin or carboplatin/pemetrexed as first line treatment in MPM patients (NCT00996567). Co-activation of multiple receptor tyrosine kinases and constitutive activation of downstream signalling cascades that activate other pathways such as COX-2/PGE pathway have been proposed as additional mechanisms of resistance to EGFR inhibition in mesothelioma. Activation of EGFR and subsequent activation of MAPK results in an enhanced transcription of COX-2 and production of prostaglandins including PGE2. COX-2 derived PGE2 can activate EGFR via activation of the Src protein thereby stimulating cell proliferation, migration and increased amphiregulin (EGFR ligand) expression (Pai et al., 2002; Buchanan, 2003; Shao et al., 2003). Activation of EGFR via COX-2 can form a positive feedback leading to the production of COX-2/PGE which can continuously activate the EGFR pathway (Dannenberg et al., 2005; Lippman et al., 2005). Stoppoloni et al (2010) reported that combination of gefitinib and rofecoxib was synergistic in a mesothelioma cell line (1st-Mes-2) (Stoppoloni et al., 2010). Although it is uncertain whether the doses used in their study can be achieved clinically. Combination of afatinib with tivantinib in our study significantly reduced growth when compared to either inhibitors as single agents but this will

require further evaluation in *in vivo* models. The incidence of EGFR, KRAS and BRAF mutations also needs further evaluation in a large cohort of MPM tissue samples.

The c-MET pathway

The hepatocyte growth factor (HGF) and its receptor c-Met are activated in MPM. Activation of c-MET contributes to tumour pathogenesis by promoting cell proliferation, migration, invasiveness and adhesion (Klominek et al., 1998). Our study and those of other groups have evaluated the effect of c-MET small molecule inhibitors such as SU11274, Crizotinib and Tivantinib (ARG197) in mesothelioma cell lines. Kanteti et al (2014) demonstrated that the combination of tivantinib with GDC-0980 or NVPBEZ235 was strongly synergic in inhibiting MPM cell proliferation and tumour growth in MPM cell lines and mouse xenograft models (Kanteti et al., 2014). The group also reported that crizotinib in combination with BKM120 (pan-class I PI3K inhibitor) was highly synergistic in inhibiting MPM tumour growth in *in vitro* and *in vivo* models (Kanteti et al., 2016). Tivantinib inhibited c-MET activity and microtubule polymerization leading to reduced growth of MSTO-211H and NCI-H2052 cells. It also synergistically enhanced the cytotoxic and apoptotic activity of pemetrexed by reducing the expression of thymidylate synthase expression and cell migration (Leon et al., 2014). In line with previous studies, our study also demonstrated that combination of tivantinib with VS-5584 (PI3K/mTOR inhibitor) further reduced MPM cell growth when compared with either inhibitor alone and the addition of cisplatin to the combination was synergistic. This would require further evaluation in *in vitro* models. There is still a need to identify predictive biomarkers of response to c-MET inhibitors. Tivantinib is currently being evaluated in a Phase I/II study in combination with carboplatin and pemetrexed as first line therapy in NSCLC and MPM patients (NCT02049060). Preliminary results have shown that the addition of tivantinib to chemotherapy is safe with preliminary evidence of antitumour activity (Zucali et al., 2015). There is currently no *in vitro* or *in vivo* study evaluating the effect of anti-HGF monoclonal antibodies (e.g. rilotumumab, ficlatuzumab, TAK701) or anti-c-MET monoclonal antibodies (e.g. onartuzumab, LY2875358) in mesothelioma.

The PI3K/AKT/MTOR pathway

Constitutive activation of receptor tyrosine kinases in mesothelioma is associated with the upregulation of downstream signalling cascades such as the PI3K/AKT/MTOR pathways which plays a significant role in cell growth, proliferation and survival (Didier et al., 2012). Loss of PTEN expression also account for PI3K/AKT signalling activation in MPM (Opitz et

al., 2008; Agarwal et al., 2013). The MTOR inhibitor everolimus was evaluated in a phase II trial as second line therapy but showed no efficacy in unselected MPM patients (Ou et al., 2015). Another phase II trial investigating the role of everolimus in MPM patients with merlin/NF2 loss has been completed and the results are awaited (NCT01024946). MTOR1 inhibition alone may result in the compensatory upregulation of PI3KCA resulting in the restoration of PI3K and AKT signalling. Dual MTOR kinase (MTOR1 and MTOR 2) inhibitors and PI3K/mTOR inhibitors might address this resistance mechanism by inhibiting MTOR2 which is required for the full activation of AKT. Our study demonstrated that the MTOR kinase inhibitors KU0063794 and XL388 exerted cytotoxic effects in NCI-H2452, NCI-H2052, MSTO-211H and A549 cells. NVPBEZ235 and VS-5584 two PI3K/MTOR inhibitors also demonstrated significant cytotoxic effects on the cell lines. We also evaluated the combination of VS-5584 with Tivantinib or Afatinib for the first time. The combination of tivantinib with VS-5584 further reduced cell proliferation than when compared to both inhibitors alone. Combination of afatinib with VS-5584 did not improve the single agent effect of VS-5584. The combination of tivantinib and VS-5584 needs to be evaluated in *in vivo* models. The inhibitory effect of MTOR kinase inhibitors also require further evaluation in mesothelioma at clinically relevant doses. VS-5584 (NCT02372227) and LY3023414 (NCT01655225) are currently being evaluated in phase I trials in mesothelioma patients.

11.3 Future research in mesothelioma

Due to the rarity of mesothelioma, there are so many research questions that needs to be answered. Lack of reliable pre-clinical models, lack of sufficient tissue samples and appropriate controls have hampered progress in this area of research making it difficult to validate significant findings. However, over the past decade there has been considerable progress in the basic understanding of the biology or mesothelioma. The development of next generation sequencing platforms and the advent of mesothelioma biobanks have paved the way for high throughput genomic studies with tissue samples and primary cell lines can be established from tissue. There is still a lot of collaboration to be done to effectively progress. The integration of genomics, transcriptomics and proteomic information might lead to the identification of predictive biomarkers in malignant pleural mesothelioma which will in turn facilitate the introduction of personalised therapeutic strategies. This project has demonstrated new therapeutic strategies in mesothelioma that require validating in pre-clinical and *in vivo* models. Future work in this area may consider investigating the proteomic landscape of well characterized malignant pleura mesothelioma in the different histological

subtypes for the identification of differential expressed proteins that might be of clinical relevance. Malignant pleural mesothelioma is characterised into three major different histological subtypes with uniquely different prognosis. Future trials might benefit from stratifying patients by subtype when analysing response data as there is an urgent need to fully understand the biology of each subtype.

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APPENDICES

APPENDIX A

Table 1 Evaluation of CDKN2A/ARF inactivation in malignant mesothelioma

Study/Author	Samples	Methodology	Results
(Cheng et al., 1994)	40 cell lines 23 primary MM tumour specimens	Southern blot and PCR	Homozygous deletion of $p16^{INK4a}$ in 85%(34) cell lines and in 22% (5) primary tumours
(Kratzke et al., 1995)	11 primary resections of thoracic mesothelioma 1 cell block of a malignant pleural effusion 15 mesothelioma cell lines 1 NSCLC cell line 1 non-malignant pleural biopsy	Immunohistochemistry and Immunoblot	All tumour specimens and cell lines showed expression of wild-type Rb protein Uniform absence of $p16^{INK4}$ in 10 of 12 specimens. No $p16^{INK4}$ expression in all mesothelioma cell lines
(Xio et al., 1995)	50 primary mesothelioma	FISH	Codeletion of $p15^{INK4B}$ and $p16^{INK4A}$ in 36 (72%) of mesothelioma including all cases with spindle-cell components
(Illei et al., 2003)	95 MPM cases; 71 Epithelial, 19 Biphasic, 5 Sarcomatoid	FISH	Homozygous deletion of $p16^{INK4a}$/$p14^{ARF}$ observed in 74% (70/95) cases. 70% (49/71) Epithelial 89% (16/19) Biphasic 100% (5/5) Sarcomatoid

(Usami et al., 2006)	4 MPM cell lines	PCR, SSCP analysis and Immunoblot	Homozygous deletion of $p16^{INK4a}$/$p14^{ARF}$ in all four MPM cell lines
(Fischer et al., 2006)	43 MM specimens	Nested methylation-specific PCR DNA	$p16^{INK4a}$ and $p14^{ARF}$ promoter region methylation in 28.2% and 44.2% respectively.
(Taniguchi et al., 2007)	17MPM biopsy 9 MPM cell lines 1 non-malignant mesothelial cell line	RT-PCR, Genome wide array-based comparative genomic hybridization analysis	Homozygous deletion of $p16^{INK4a}$/ $p14^{ARF}$ in 41% (7/17) MPM tissue samples and 100% (9/9) of MPM cell lines
(Chiosea et al., 2008)	52 MPM specimens 21 Peritoneal samples	FISH	Homozygous deletion of 9p21 in 67% (35/52) of MPM cases. 25% (5/20) of peritoneal mesothelioma
(Onofre et al., 2008)	33 MM specimens	FISH	9p21 deletions in 90.9% of MM case (homozygous deletion in 48.5% (16/33); heterozygous deletion in 36.4% (12/33) and both deletions in 6% (2/33).
(Takeda et al., 2010)	40 MM cases (37 pleural, 1 peritoneal and 2 pericardial)	FISH	Homozygous deletion in 88% (35/40) MM cases.
(Takeda et al., 2012)	42 MM specimens (35 pleural, 5 peritoneal and 2 pericardial)	FISH	Homozygous deletion in 83% (35/42) of MM cases. (Epithelioid 77% (23/30), Biphasic/sarcomatoid 100% (12/12).
(Matsumoto et al., 2013)	15 Epithelioid MPM cases	FISH	All 15 MPM cases were positive for 9p21 deletion. 12 were positive for homozygous deletion and 3 had

			both heterozygous and homozygous deletions
(Tochigi et al., 2013)	32 sarcomatoid mesotheliomas 15 sarcomatoid carcinomas 32 solitary fibrous tumours 13 high-grade sarcomas	FISH	81% (26/32) of sarcomatoid mesothelioma positive for deletions. Homozygous deletion in 96% (25/26) and 4% (1/26) combined homozygous and hemizygous deletion
(Wu et al., 2013)	50 MPM cases	FISH, PCR	Homozygous deletion observed in 56% (10/18) epithelioid, 100% (22/22) sarcomatoid and 88% (7/8) biphasic.

FISH= fluorescence in situ hybridization; SSCP= single-strand conformation polymorphism; PCR= polymerase chain reaction.

Table 2. Evaluation of NF2 gene expression in malignant mesothelioma.

Study/Author	Samples	Methodology	Results
(Deguen et al., 1998)	18 MPM cell lines	Northern blot, RT-PCR and PCR	NF2 alterations were observed at a genomic level in 39% (7/18) cell lines and were associated with marked decrease in the concentration of the NF2 transcript
(Cheng et al., 1999)	25 MPM cell lines	Immunoblot, SSCP and DNA sequence analyses	No NF2 expression was observed in 56% (14/25), 72% (18/25) showed losses at one or both loci tested
(Usami et al., 2006)	4 MPM cell lines	PCR, SSCP analysis and Immunoblot	Point mutation observed in 25% (1/4)
(Taniguchi et al., 2007)	17MPM biopsy 9 MPM cell lines 1 non-malignant mesothelial cell line	RT-PCR, Genome wide array-based comparative genomic hybridization analysis	Small deletions resulting in frameshift mutation were observed in 2 cell lines and 1 MPM specimen
(Thurneysen et al., 2009)	44 MPM specimen (46 epithelioid and 18 biphasic)	Nested PCR, Immunoblot	Truncated NF2 transcripts were observed in 50% (13/26) epithelioid and 22% (4/18) biphasic samples

(Murakami et al., 2011)	20 MPM cell lines	PCR, array-based comparative genomic hybridization	Mutations including homozygous deletions were found in 50% (10/20) of the cell lines
(Bott et al., 2011)	53 primary MPM tumour samples	Comparative genomic hybridization arrays, FISH	Frequent inactivating mutations were found in 21% (11/53) MPM samples
(Takeda et al., 2012)	42 MPM specimens (35 pleural, 5 peritoneal and 2 pericardial)	FISH	Homozygous deletion of NF2 was found in 38% of MPM specimens

FISH= fluorescence in situ hybridization; SSCP= single-strand conformation polymorphism; RT-PCR= reverse transcription polymerase chain reaction.

Table 3 EGFR and HER2 inhibitors in clinical practice

Drugs/Company	Year Approved	Target	Approved indication
Gefitinib (AstraZeneca)	2003	EGFR	First and second line treatment of NSCLC with EGFR mutation
Erlotinib (Genentech/OSI)	2003	EGFR	AS first-line treatment of NSCLC with EGFR mutation or second-line treatment following platinum based chemotherapy. In combination with gemcitabine as first line treatment of advanced pancreatic cancer.
Cetuximab (ImClone/EliLilly/Bristol Myers)	2004	EGFR	In combination with cytotoxic therapy for metastatic wild type KRAS colorectal cancer Combined with radiation or cytotoxic chemotherapy in head and neck cancers
Panitumumab (Genentech	2006	EGFR	Second-line treatment for metastatic colorectal after cytotoxic therapies.
Afatinib (Boehringer Ingelheim)	2013	EGFR HER2 HER4	First line treatment of NSCLC with EGFR mutation (exon-19 deletion or exon-21 L858R mutation)
Lapatinib (GlaxoSmithKline)	2007	EGFR HER2	Second-line treatment in combination with capecitabine in HER2 positive breast cancer
Trastuzumab (Genentech)	1998	HER2	HER2-positive breast, gastric and gastroesophageal junction cancer.
Pertuzumab (Abgenix)	2012	HER2	In combination with trastuzumab and docetaxel in HER2-positive metastatic breast cancer as neoadjuvant therapy
Ado-trastuzumab Emtansine (Genentech)	2013	HER2	Second-line treatment of HER2 positive metastatic breast cancer previously treated with trastuzumab and/or taxane.

NSCLC - Non-small cell Lung cancer

APPENDIX B- Buffers and Reagents

Cell culture Medium

Rosewell Park Memorial Institute (RPMI) Medium (#31870, Invitrogen)	500 mL
Fetal Bovine Serum (#31053, Invitrogen)	50 mL
L-glutamine (#25030, Invitrogen)	5 mL
Fungizone – Amphotericin B (#1590, Invitrogen)	5 mL
Penicillin/Streptomycin (PenStrep) (#15140, Invitrogen)	5 mL

Freezing medium

Dimethyl Sulphoxide (DMSO)	5 mL
RPMI cell culture medium (#31870, Invitrogen)	45 mL

Western blot (WB) extraction buffers

Laemmli buffer
4 ml dH_20
1 ml 0.5M Tris:HCL pH6.8
0.8 ml glycerol
1.6 ml 10% sodium dodecyl sulphate (SDS)
200 µl 0.05% Bromophenol Blue

RIPA buffer (R0278, Sigma)
50 mM Tris-HCl pH8.0
150 mM sodium chloride
1.0% Igepal CA-630 (NP-40)
0.5% sodium deoxycholate
0.1% SDS

TBS-Tween20

TBS stock (concentrated)
121 g Trizma Base (#93304, Fluka)
170 g Sodium Chloride ()
Made to 1 litre with dH_20
Adjusted to pH 7.6 with 37% HCl

Working Solution
250 ml TBS stock
4750 ml dH_20
2.5 ml Tween20 (#P5972, Sigma Aldrich)

Western Blot blocking solution (5% Non-Fat Milk)

Non-fat dried milk powder (Marvel)	2 g
TBS / TWEEN 0.05% (as above)	40 mL

APPENDIX C – Optimization of seeding density for cell growth

NCI-H2452, NCI-H2052, MSTO-211H and A549 cell lines were seeded in 96 well plates at cell density of 1000 cells per well, 2000 cell per well, 5000 cell per well and 10000 cell per well in triplicates with and without 0.01% DMSO (drug carrier) and incubated for 5 days. Cell growth was calculated at the end of day 5 by MTS assay read at 3 hrs. The aim was to achieve maximal growth without exceeding 80% confluence at the end of day 5 to avoid growth inhibition due to cell-cell interaction, competing for nutrients and space. Confluence was estimated by directly visualizing the total area occupied by cells in relation to the total area of the well by light microscopy (magnification 100x). Maximal growth was seen in 1000 cell per well for NCI-H2452, NCI-H2052, MSTO-211H and A549 cell lines. Confluence was seen at 70-80% in the cell lines at this seeding density. The concentration of DMSO used was well tolerated in all cell lines.

Table 4 Percentage cell growth and the effect of 0.0% DMSO in cell lines based on their seeding density

Cell lines	Cell seeding density	% Growth	% Growth with 0.01% DMSO
NCI-H2452	1000	241.86	248.33
	2000	85.65	82.29
	5000	46.98	41.21
	10000	25.40	24.95
NCI-H2052	1000	184.62	183.04
	2000	120.94	126.32
	5000	86.15	88.02
	10000	78.73	75.19
MSTO-211H	1000	150.84	148.50
	2000	128.37	125.30
	5000	96.38	100.80
	10000	57.53	54.34
A549	1000	346.11	349.67
	2000	118.93	109.21
	5000	64.32	60.07
	10000	10.89	10.56

APPENDIX D- Table of external approvals

Type of Document	Title	Approving body	Reference number
Ethics Approval	An Immuno staining study of prognosis and response to chemotherapy in patients with malignant mesothelioma	Hull and East Riding Local Research Ethics Committee	11/00/212
Material transfer agreement	Agreement classifying Licofelone free acid	c-a-i-r biosciences GmbH (Prof. Dr. Stefan Laufer)	0158(73)
Material transfer agreement	Combined effects of Pemetrexed, LOX, COX-2 and receptor tyrosine kinase inhibitors in Mesothelioma	EliLilly	0158(79)
Material transfer agreement	The *in vitro* study of the effect of MetMab in mesothelioma cell lines	Genentech	OR-214168

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