The significance of the EGFR pathway in Malignant Pleural Mesothelioma

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Abstract

Introduction

EGFR, MTOR and COX2 are up regulated in malignant pleural mesothelioma (MPM). In this study we aimed to determine the expression of Lipoxygenase enzymes (LOX), absence of PTEN protein expression and the cytotoxic effect of EGFR, MTOR and COX2 inhibitors in MPM.

Materials and Methods

Immunohistochemical analysis was performed in 93 archival MPM tissue samples to determine the expression of 5-LOX and 12-LOX and PTEN protein. The COX-2 positive cell lines MSTO-211H, NCI-H2052, NCI-H2452 (mesothelioma) and A549 (lung cancer) were utilised. All cell lines were tested for EGFR, KRAS and BRAF mutations. Cells were incubated with Cetuximab, Gefitinib, Rapamycin, Ku0063794 (MTOR kinase inhibitor) and Celecoxib as single agents and in combinations and analysed using the MTS assay.

Results

Positive 5-LOX expression was seen in 73% and positive 12-LOX expression was seen in 83% of MPM samples. PTEN protein expression was absent in 27% of the samples. A549 cells had a KRAS missense mutation at codon 12. No other EGFR, KRAS and BRAF mutations were identified in any of the cell lines. Cetuximab showed 50% cell growth inhibition in MSTO-211H cells at a concentration of 1.6 μM. All other cell lines were resistant to Cetuximab. All cell lines were resistant to Gefitinib. Rapamycin and Ku0063794 demonstrated 50% cell growth inhibition in NCI-H2052, NCI-H2452 and A549. Celecoxib demonstrated 50% cell growth inhibition in all cell lines. Cetuximab and Gefitinib were combined in turn with Rapamycin, Ku0063794 and Celecoxib. Cetuximab when combined with Celecoxib (NCI-H2052, NCI-H2452 and A549 cells) and Ku0063794 (MSTO-211H cells) demonstrated significant growth inhibition.

Conclusions

Our study suggests that 5LOX and 12LOX are expressed in the majority and PTEN protein expression is absent is a significant proportion of MPM tissue samples. Inhibition of MTOR pathway may be an important therapeutic strategy in patients with MPM.

Table of Contents

List of figures

List of tables

Abbreviations

AKT	Protein kinase B
ARMS	Amplification Refractory Mutation System
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 Leukemia Group B
CO2	Carbon dioxide
COX	Cyclooxygenase
CR	Complete response
DAB	3,30-Diaminobenzidine Tetrahydrochloride
DEP	Differentially expressed protein
DH2O	Distilled water
DMF	Dimethylformamide
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
EMEA	European Medicines Agency
EP	Prostaglandin E receptor
ERCC1	Excision repair cross-complementing group 1
FDA	Food and Drug Administration
FLAP	5LOX activating protein
FGF	Fibroblast Growth Factor
FSC	Forward scatter
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	Hydroxyeicosatetraenoic
HGF	Hepatocyte Growth Factor
HNSCC	head and neck squamous cell cancer
HR	Hazard Ratio
HRP	Horseradish peroxidise
HUVEC	Human umbilical vein endothelial cells

IC50	Half maximal inhibitory concentration
IDEAL	Iressa Dose Evaluation in Advanced Lung Cancer trial
IGF-1R	Insulin-like Growth Factor 1 Receptor
IHC	Immunohistochemistry
IMIG	International Mesothelioma Interest Group
IPA	Ingenuity Pathway Analysis
IPASS	Iressa Pan Asia Study
IPKB	Ingenuity pathways knowledge base
IRS1	Insulin receptor substrate 1
KRAS	Kirsten rat sarcoma viral oncogene homolog
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
MM	Matrix metalloproteinases
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
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Progressive disease
PDGFR	Platelet Derived Growth Factor Receptor
PFS	Progression free survival
PG	Prostaglandins
PI3K	phosphoinositide-3-kinase
PIK3CA	phosphoinositide-3-kinase catalytic alpha polypeptide
PIP	Phosphatidylinositol phosphate
PKA	Protein kinase A
PLAGF	Placental Growth Factor
PR	Partial response
PTEN	phosphatase and tensin homolog
RIDEP	Repeatedly identified differentially expressed protein
RNA	Ribonucleic acid
RR	Response rate
SD	Stable response
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SSC	Side scatter
SV40	Simian Virus 40
tBID	Truncated BID
TBS	Tris buffered saline

TGF-a	Transforming Growth Factor alpha
TKI	tyrosine kinase inhibitors
TNF-R1	Tumour necrosis factor alpha receptors
TNFα	Tumour necrosis factor alpha
TRAIL	TNF related apoptosis-inducing ligand
TSC	Tuberous sclerosis complex
VEGF	Vascular Endothelial Growth Factor
VEGF	Vascular Endothelial Growth Factor Receptor

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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.

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Chapter 1. Malignant pleural mesothelioma

1.1 **Incidence**

Mesothelium is a single layer of mesothelial cells that line the serosal cavities such as pleura, pericardium, peritoneum and tunica vaginalis of testes. Malignant pleural mesothelioma (MPM) is a rare, aggressive tumour of the mesothelial cells of the pleura. The incidence of MPM is on the rise in most parts of the world including Europe, Australia and Asia. In the United States of America the incidence of MPM reached its peak in the early to mid 1990's and is now on a downward trend (Weill, Hughes et al. 2004). Great Britain, Australia and Belgium have the highest annual crude incidence rates of 30 cases per million population with United States of America and Japan having annual crude incidence rates of approximately 9 and 7 cases per million respectively (**Figure 1.1**) (Bianchi and Bianchi 2007). Britain has one of the highest mortality rates from MPM and it is predicted that the mortality rates in Britain are likely to peak in 2016 with over 2000 deaths per year (Tan, Warren et al. 2010). It is estimated that there will have been be over 60000 predicted deaths from mesothelioma in UK alone from 2007 onwards (Tan, Warren et al. 2010). Similar predictions in other parts of Western Europe suggest that the incidence of MPM is on the rise (Peto, Decarli et al. 1999).

In emerging economies such as India and China, the use of asbestos is widespread with little or no government regulation. They do not report mesothelioma cases and hence it is very difficult to analyse the burden in these countries. A recently published study attempted to predict the number of cases of mesothelioma in countries that do not report such cases using mathematical models and extrapolating from those countries who do report (Park, Takahashi et al. 2011). The authors identified 56 countries who reported data on the use of asbestos and mesothelioma frequency and 33 countries who reported data only on the use of asbestos, but not on mesothelioma. These 89 countries represented 82.6% of the global

population in 2000. Among the countries which reported data for asbestos and mesothelioma, the authors found a statistically significant linear relationship between the cumulative use of asbestos from 1920 to 1970 and the cumulative deaths from mesothelioma from 1984 to 2008. In the 56 countries that recorded data on mesothelioma the cumulative asbestos use was 51.2 million metric tons and the total number of mesothelioma deaths in the 15 year period was 174,300. Using this data, the authors predicted that 14.2 million metric tons of asbestos was used in the 33 countries that did not report data on mesothelioma and estimated that 38,900 deaths due to mesothelioma may have occurred in this 15 year period. The leading countries in this group were Russia (21,300 deaths), Kazakhstan (6,500 deaths), China (5,100 deaths), India (2,200 deaths) and Thailand (500 deaths). Despite the ban on the use of asbestos in most developed countries the cumulative useage of asbestos has increased from 65 million metric tons in 1970 to 124 million metric tons currently. Most of the increase in the use of asbestos is likely from the developing countries with the 33 countries that did not report data on mesothelioma quadrupling its use in the same period.

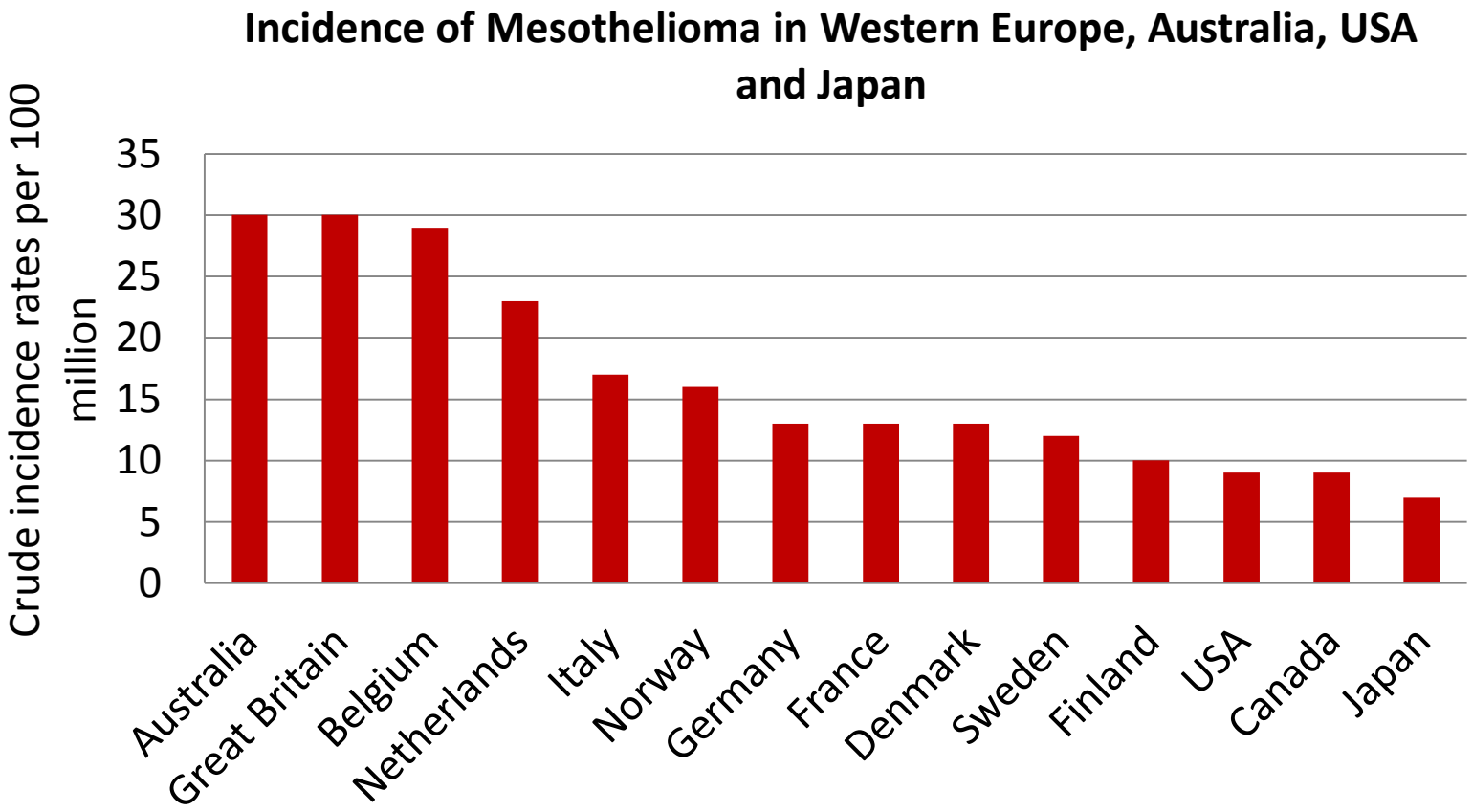

Figure 1.1 Incidence of Mesothelioma. Crude Incidence rates of Mesothelioma per 100 million population in Western Europe, Australia, USA and Japan. Graph drawn by incorporating data obtained from (Bianchi and Bianchi 2007).

1.2 **Causes and pathophysiology**

In 80% of cases, MPM can be attributed to asbestos fibre exposure (Carbone, Kratzke et al. 2002) with a median latency of at least 32 years (Lanphear and Buncher 1992). Simian virus 40 (SV40) has also been implicated as one of the other potential causes of MPM (De Luca, Baldi et al. 1997; Testa, Carbone et al. 1998; Carbone, Kratzke et al. 2002). Familial transmission in an autosomal dominant fashion has been reported in the Cappadocian region of Turkey (Roushdy-Hammady, Siegel et al. 2001; Dogan, Baris et al. 2006).

1.2.1 **Asbestos and mesothelioma**

Asbestos is a natural fibrous mineral that is fire, acid, corrosive and stress resistant, flexible and durable. These excellent properties of asbestos led to its widespread commercial use for more than a century in various industries such as construction, transportation and mining. There are two types of asbestos fibres; amphibole (crocidolite, amosite, anthrophyllite, tremolite and actinolite) and serpentine (chrysotile). The exposure of humans to asbestos fibres resulted in its harmful effects and was first reported in 1907 by Murray in his report to the Departmental Committee, London, among textile workers in Great Britain (Baldi 2008). Wenger *et al* (1960) described a case series of 33 patients with histologically proved MPM who were associated with the Cape asbestos field (South Africa) due to the probable exposure to crocidolite asbestos (Wagner, Sleggs et al. 1960) . Since then, there have been numerous reports of the detrimental effects of asbestos and its association with MPM, which led to the production and use of asbestos being 'controlled' in most parts of the world in the second half of 20th Century. Most of the exposure to asbestos is of occupational origin with workers handling raw asbestos. However, MPM is also observed in patients with non occupational exposure to asbestos, such as in people living near

asbestos factories or end users of asbestos products. Although all commercial asbestos has the capacity to induce mesothelioma, the amphibole fibres such as crocidolite, and amosite have a higher carcinogenic potential than chrysolite with exposure specific risk of mesothelioma being 1:100:500 for chrysotile, amosite and crocidolite respectively (Hodgson and Darnton 2000). Various studies have demonstrated a dose response relationship between asbestos exposure and mesothelioma with the risk being greatest among those heavily exposed to asbestos (Seidman, Selikoff et al. 1986; Hansen, de Klerk et al. 1998; Iwatsubo, Pairon et al. 1998). A population based case controlled French study demonstrated a significant dose-response relationship between cumulative asbestos exposure and occurrence of MPM with the odds ratio increasing from 1.2 (95% CI 0.8-1.8) for low exposure category to 8.7 (95% CI 4.1 – 18.5) in the highest exposure category (Iwatsubo, Pairon et al. 1998). Peto *et al* (1982) demonstrated a linear dose respone relationship using a mathematical model in which the risk of mesothelioma increased with the third and fourth power of time since first exposure in a cohort of North American insulators (Peto, Seidman et al. 1982). Newhouse *et al* (1985) studied the mortality rates in asbestos factory workers (3000 males, 1400 laggers and 700 women) of East London. They classified them into four categories depending upon the degree and duration of exposure and demonstrated that the mortality from mesothelioma increased according to the severity and the duration of exposure (Newhouse, Berry et al. 1985). An Italian study evaluated the latency period of 2544 patients diagnosed with mesothelioma and demonstrated a median latency period of 44.6 years with a shorter latency period of 43 years among those patients who were occupationally exposed to asbestos and a longer latency period of 48 years among those patients non occupational exposure to asbestos (Marinaccio, Binazzi et al. 2007). This may suggest that a high exposure to asbestos fibres (occupational) may have

less latency than low exposure to asbestos fibres (non occupational). Analysis of mesothelioma trends in the United States demonstrated a life time risk of mesothelioma among males as approximately 2.1×10^{-3} for the 1925 – 1929 birth cohort with a downward trend in the incidence of mesothelioma in subsequent cohorts (Price and Ware 2004). Among the female population the life time risk of mesothelioma has remained constant at approximately 3.6×10^{-4} . The high risk of mesothelioma in males is likely due to a high occupational exposure to asbestos in contrast to the low risk in females where the asbestos exposure has been mainly environmental or domestic (wives of men exposed to asbestos).

One of the possible mechanisms of asbestos induced oncogenesis is failure of apoptosis via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway. Inhalation of asbestos fibres such as crocidolite causes accumulation of macrophages in the lungs, which result in release of Tumour necrosis factor alpha (TNFα). Asbestos fibres also induce normal mesothelial cells to release TNFα in an autocrine and paracrine fashion and express TNFα receptors (TNF-R1). TNFα then acts on the NF-kB pathway resulting in failure of apoptosis. This allows the mesothelial cells that have undergone deoxyribonucleic acid (DNA) damage by the asbestos fibres to survive, resulting in increased chances of malignant transformation (**Figure 1.2**) (Yang, Bocchetta et al. 2006; Baldi 2008).

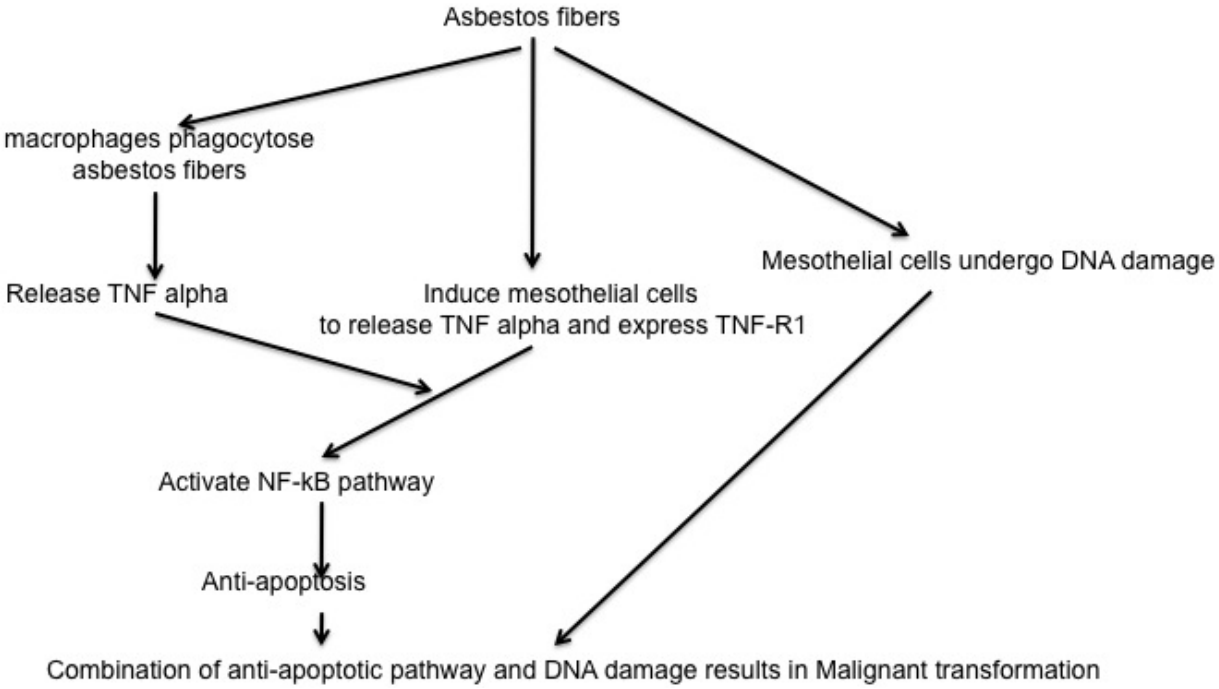

Figure 1.2 Pathophysiology of asbestos induced mesothelioma. Inhaled asbestos fibres are phagocytosed by the macrophages resulting in the release of TNFα. These fibres induce normal mesothelial cells to release TNFα in an autocrine and paracrine fashion and express TNF-R1. TNFα then acts on the NF-kB pathway resulting in failure of apoptosis. (Adapted from Baldi, A. (2008). Mesothelioma from bench side to clinic. New York, Nova Biomedical Books, page 107).

1.2.2 **SV40 and mesothelioma**

SV40 may act as a co-carcinogen along with asbestos fibres resulting in the development of malignant mesothelioma (Bocchetta, Di Resta et al. 2000). SV40 infection of the human mesothelial cells results in cell survival. SV40 virus integrates itself into the mesothelial cells resulting in expression of SV40 tumour antigens (large antigen Tag; small antigen tag) (Bocchetta, Di Resta et al. 2000; Cacciotti, Libener et al. 2001; Yu and Alwine 2002; Carbone, Pass et al. 2003) . Cells infected with SV40 virus, when exposed to asbestos fibres may result in production of toxic oxygen free radicals and chromosomal aberrations

causing malignant transformation (Wang, Jaurand et al. 1987; Broaddus, Yang et al. 1996). Cacciotti *et al* (2005) demonstrated that a mesothelioma cell line (MMP) expressing SV40 Tag was intrinsically more resistant to apoptosis when exposed to asbestos resulting in cell survival. (Cacciotti, Barbone et al. 2005). In the presence of Tag the cell survival was mediated through activation of PI3K/AKT pathway (Chapter 3) either independently or in response to growth factors such as hepatocyte growth factor in SV40 negative cell lines (Broaddus, Yang et al. 1996).

However, the role of SV40 in MPM remains controversial. Manfredi *et al* (2005) did a PCR based analysis in 69 MPM tissue samples to detect SV40 large T antigen DNA and did not identify the gene in any of them arguing against the role of SV40 in MPM (Manfredi, Dong et al. 2005). Another similar study failed to demonstrate the presence of SV40 large T antigen DNA in 71 MPM tissue samples (Lopez-Rios, Illei et al. 2004). A large population in the United States received SV40 contaminated live polio virus during 1955-163. Epidemiological studies conducted failed to demonstrate an increased risk of MPM in this cohort of population when compared to another similar group of different birth cohort (Strickler, Rosenberg et al. 1998).

1.2.3 **Other causes of MPM**

There are various other factors which are known to cause MPM such as radiation therapy, erionite exposure and genetic predisposition. MPM is known to occur as second malignancies in patients with primary cancers such as lymphomas and breast cancers who have received therapeutic radiation therapy and had a median latency of 4.3 years (Cavazza, Travis et al. 1996; Teta, Lau et al. 2007). MPM is found to be endemic in the Cappadocia, Turkey, resulting in 50% of the deaths in three small villages (Carbone, Emri et al. 2007). Pedigree studies have demonstrated an autosomal dominant pattern of

inheritance among the families suffering from MPM. The Cappadocian region is also dominated by volcanic rocks having a high concentration of the mineral erionite. The houses of the villages are made out of stone taken from the nearby mountains or rivers and have a high content of erionite. Exposure to erionite is strongly associated with the development of MPM in *in vivo* studies. MPM was seen in 100% (40/40) rats injected with erionite compared with 48% (19/40) of rats injected with asbestos (Zucali, Ceresoli et al. 2011). The MPM epidemic in the Cappadocian region is likely due to high erionite exposure in genetically predisposed individuals.

1.3 **Pathology**

Histologically, MPM can be categorised as epithelioid, sarcomatoid or mixed (biphasic) phenotype containing both epithelioid and sarcomatoid elements (**Figure 1.3**). Epithelioid phenotype is associated with better prognosis (Ceresoli, Locati et al. 2001; O'Kane, Cawkwell et al. 2005). Immunohistochemical staining of various biomarkers are required to distinguish MPM from adenocarcinoma of lung or metastasis from other sites. A consensus statement from International Mesothelioma Interest Group recommended that markers should have either specificity or sensitivity greater than 80% (Husain, Colby et al. 2009). Immunohistochemical staining for pancytokeratin is useful as virtually all mesothelioma are positive except some rare sarcomatoid mesothelioma. The International Mesothelioma Interest Group recommends that at least two positive markers and two negative markers be used in addition to pancytokeratin. Virtually all epithelioid tumours are positive for Calretinin and Mesothelin. Keratin 5/6 is expressed in 75% to 100% , Wilms Tumour-1 protein in 43% to 93% and Podoplanin in 86% to 100% of epithelioid mesothelioma. Thyroid transcription factor-1 is not expressed in mesothelioma. Similarly only 2% to 20%

of mesotheliomas express MOC-31, BG8 (Lewis-Y blood group), Ber-EP4, CD15 and Carcinoembryonic antigen.

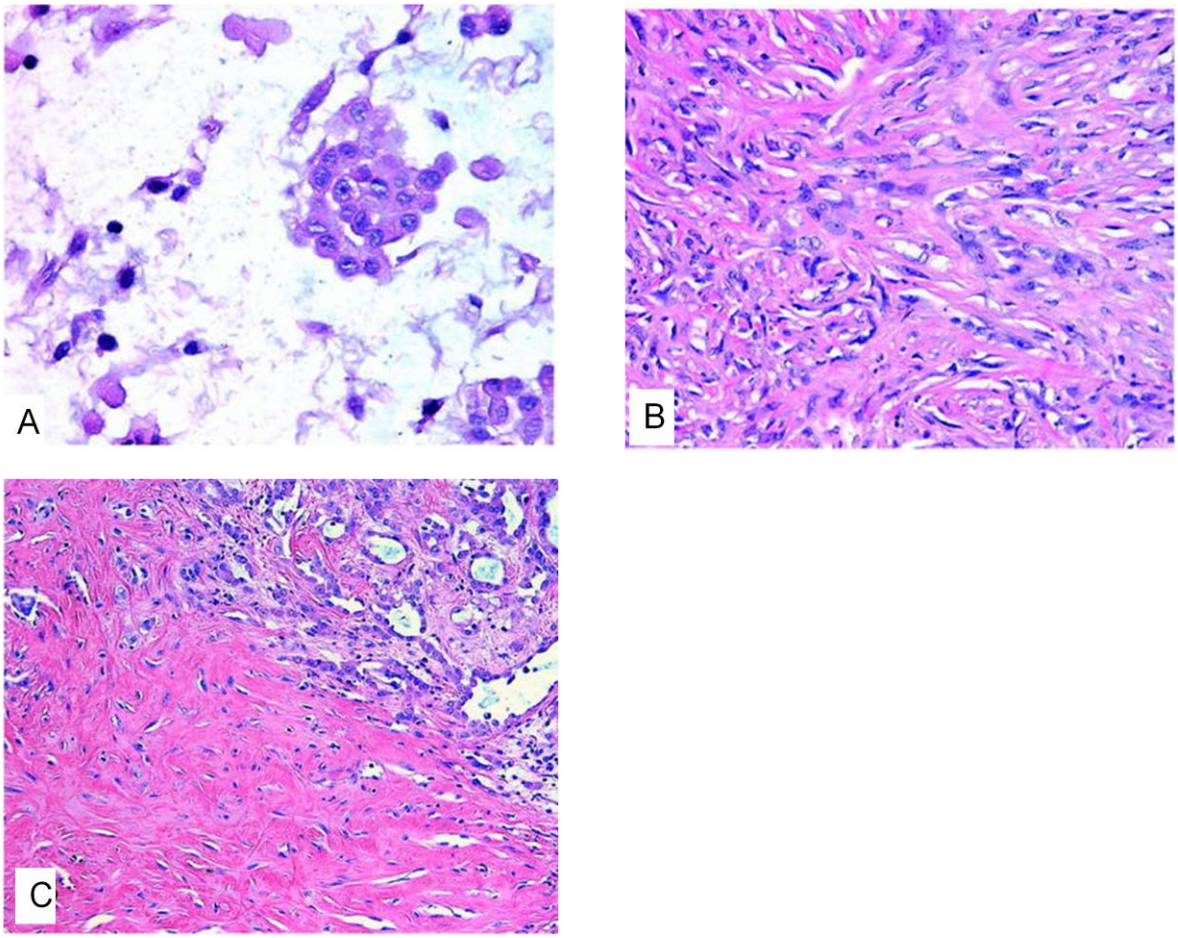

Figure 1.3 Histological microphotograph (original magnification X 40) of epithelioid, sarcomatoid and biphasic mesothelioma. **Figure** (A) demonstrated hematoxylin-eosin staining of epithelioid mesothelioma with clusters of cubiodal shaped epithelial mesothelioma cells. **Figure** (B) demonstrated hematoxylin-eosin staining of sarcomatoid mesothelioma with spindle shaped cells lying in a disorganised way**. Figure** (C) demonstrated hematoxylin-eosin staining of biphasic mesothelioma with epithelioid pattern in top right and sarcomatoid pattern in the bottom left. Reprinted from Allen TC. Recognition of histopathologic patterns of diffuse malignant mesothelioma in differential

diagnosis of pleural biopsies. Arch Pathol Lab Med 2005;129:1415-20 with permission from Archives of Pathology & Laboratory Medicine. Copyright 2005. College of American Pathologists (Allen 2005).

1.4 **Staging**

An ideal staging system of MPM is difficult to define as MPM has an unusual growth pattern. It tends to grow along the parietal and visceral pleura invading the surrounding structures making it difficult to access the volume and spread of the tumour. In view of this many staging systems by various authors such as Butchart, Mattson, Sugarbaker, Chahinian have been suggested, but the most common staging system used is based on the TNM staging suggested by International Mesothelioma Interest Group (IMIG) (Rusch 1995). These are outlined in **Table 1.1**.

Table 1.1 IMIG TNM staging for malignant pleural mesothelioma.

Primary Tumour (T)	
T1	
T1a	Tumour limited to the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura No involvement of the visceral pleura
T1b	Tumour involving the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura Scattered foci of tumour also 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 . Confluent visceral pleural tumour (including the fissures) or extension of tumour from visceral pleura 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, and visceral) 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 one or more 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
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
Metastasis (M)	
MX	Presence of distant metastases cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present
Stage	
Stage 1a Stage 1b	T1aN0M0 T1bN0M0
Stage II	T2N0M0
Stage III	Any T3N0 Any N1M0 Any N2M0
Stage IV	Any T4 Any N3 Any M1

1.5 **Prognostic biomarkers**

Identification of prognostic biomarkers may assist clinicians in appropriate prognostic evaluation and treatment planning of patients with MPM. One of the earliest studies to evaluate the prognostic factors in MPM was the Surveillence, Epidemiology and End Results Program review, which evaluated 1,475 patients with MPM and demonstrated that age, sex, stage, treatment and geographical area predicted patient survival (Spirtas,

Connelly et al. 1988). Various prognostic markers, both clinical and biomarkers have been identified in many studies (Ambrogi, Mineo et al. 2012). The CALGB and the EORTC groups developed scoring systems to discriminate between good and poor prognostic groups in patients receiving systemic treatments (Curran, Sahmoud et al. 1998; Herndon, Green et al. 1998). The CALGB group defined six patient groups based on the performance status, age, weight loss, chest pain, haemoglobin and WBC levels (Herndon, Green et al. 1998). The median survival of the six groups (poor to good prognosis) ranged from 1.4 (95% CI 0.5 – 3.6) months to 13.9 (95% CI 11.1 – 31.4) months and 1 year survival ranged from 0 to 63% (95% CI 46% – 77%). The EORTC group divided patients into two prognostic groups depending upon the sex, performance status, histology (probability of diagnosis and histological subtype) and WBC count. The median survival of the poor and good prognosis groups was 5.5 months and 10.8 months respectively, and 1 year survival was 12% (95% CI 4-20) and 40% (95% CI 30-50) respectively (Curran, Sahmoud et al. 1998). A recent multivariate analysis of an international database of 3101 patients from 15 centers, developed by the International Association for the Study of Lung Cancer Staging Committee showed that age (<50 versus >65 years; HR 1.31, p=0.0006), sex (HR 1.28, p=0.0002), TNM staging (stage IV versus I HR 1.86, p= <0.0001), histology (epithelial versus others; HR 1.7, p= <0.001) and the intent of surgery (palliative versus curative; HR 1.71, p= <0.0001) significantly influenced survival (Rusch, Giroux et al. 2012). Various biomarkers have also been analysed as a prognostic biomarkers, but none have been successfully translated into clinical practice (**Table 1.2**) (Ambrogi, Mineo et al. 2012).

Table 1.2 Biomarkers associated with poor prognosis in MPM. Adapted and modified from (Ambrogi, Mineo et al. 2012)

Functions	Poor prognostic Biomarkers	
	Direct relationship	Inverse relationships
Growth Factors	COX-2	
	MM2 & MM9	
	EGF	
	VEGF	
	PLGF	
	FGF	
	PDGFR	
	HGF	
Apoptosis	Bcl-xL	TRAIL
	PI3K	BAX
	Glucose transporter 1	PTEN loss
Cell cycle	p53	p27
	MIB-1/Ki67	p27 kip 1
Others	ERK	Calretinin
	NFkβ	D2-40
	ERCC1	
	Aquaporin 1	
Serum Biomarkers	MPF	Glycoprotein 90K
	Osteopontin	

1.6 **Treatment options**

1.6.1 **Surgery**

The surgical options available to treat MPM are very limited due increased incidence of regional and distant recurrence. Due to the nature of its growth and spread pattern, obtaining a completely clear (R0) resection margin is extremely difficult. Palliative pleurectomy/ decortication (P/D) and extrapulmonary pneumonectomy (EPP) are the commonest procedures used for surgically removing the tumour. Video-assisted thoracoscopy is also used for diagnostic and palliative purposes. P/D involves removal of parietal and visceral pleura, pericardium and diaphragm. EPP involves en block removal of parietal and visceral pleura, lung, mediastinal lymph nodes, pericardium, and diaphragm. The choice of surgical modality is controversial due to lack of trial data and is dependent of the fitness level of patient, co morbidities, extent of disease and availability of multimodality treatments such as chemotherapy and radiotherapy (Tsao, Wistuba et al. 2009). The MARS trial in UK randomised patients to receiving either trimodality treatment with induction chemotherapy followed by EPP and radiation therapy *versus* no EPP (Treasure, Lang-Lazdunski et al. 2011). A total of 112 patients were registered and 50 were randomised to EPP (n=24) or no EPP (n=26). The hazard ratio for overall survival was 2.75 (p=0.016) in favour of no EPP group. The median survival in EPP group was 14.4 months and in the no EPP group was 19.5 months. The quality of life seemed to be lower in EP group (n=12) compared to EPP group (n=19), but this was not statistically significant.

1.6.2 **Radiotherapy**

Radiotherapy can be used in MPM in adjuvant setting post EPP or prophylactically at the surgical manipulation site to prevent local recurrence. A Phase II trial has shown that adjuvant radiotherapy (54 Gy) added to EPP results in reduced local recurrence rates and improved survival in stage I and II disease (Rusch, Rosenzweig et al. 2001). The potential benefit of prophylactic radiotherapy to the procedural sites of surgical pleural manipulation is debateable. Currently the Phase III Trial of Prophylactic Irradiation of Tracts in Patients with Malignant Pleural Mesothelioma Following Invasive Chest Wall Intervention trial (PIT) and the Surgical and large bore pleural procedures in malignant pleural mesothelioma and radiotherapy trial (SMART) are ongoing in the UK to ascertain the benefits of prophylactic tract radiotherapy in MPM.

1.6.3 **Chemotherapy**

Most of the chemotherapy based studies in MPM conducted in the 20th century were often, non-randomised Phase II trials with very few patients enrolled into those trials. The response rates of single agent chemotherapy rarely exceeded 20%. A meta-analysis of 83 clinical trials (88 treatment arms) published between 1965 and 2001 was published in 2002 (Berghmans, Paesmans et al. 2002). Of these 83 trials, 80 were single arm Phase II trials and three were randomised Phase II trials. This study grouped the studies into four groups depending upon the treatment arms; Cisplatin based regimes without Doxorubicin (n=20); Doxorubicin based regimes without Cisplatin (n=8); Cisplatin plus Doxorubicin (n=6); other chemotherapy drug regimes (n=54). The most active single agent was Cisplatin and the combination of Cisplatin and Doxorubicin demonstrated the highest response rate (28.5%; p=<0.001). Combination chemotherapy was better than single agent chemotherapy (22.6% *versus* 11.6%; p=<0.001).

The current standard for systemic treatment of advanced MPM is combination chemotherapy with Cisplatin and Pemetrexed or Raltitrexed (Stahel, Weder et al. 2009). A large Phase III trial in 456 patients showed that the combination chemotherapy with Cisplatin and Pemetrexed compared to Cisplatin alone had a better response rate (41.3% *versus* 16.7%; $p < 0.0001$), improved progression free survival (5.7 months *versus* 3.9 months; $P = 0.001$) and median overall survival (12.1 months *versus* 9.3 months; $p = 0.02$) (Vogelzang, Rusthoven et al. 2003). Therefore this regimen is considered as standard first line treatment for advanced MPM in most parts of the world. Administration of Pemetrexed has previously been associated with severe, unpredictable and sometimes life threatening toxicities such as myelosupression and gastrointestinal toxicity, which occurred as a result of Folate deficiency. Pre administration of Folate along with Vitamin B12 during the course of Pemetrexed administration significantly reduced the toxicities (Adjei 2004). Baseline deficiency of Folate and Vitamin B12 results in increased homocysteine levels. Raised pre-treatment homocysteine levels may be a more sensitive measure of functional folate status rather than RBC or serum folate levels and may more accurately predict toxicities from Pemetrexed (Niyikiza, Baker et al. 2002). The combination of Raltitrexed with Cisplatin has also been demonstrated to improve overall survival, when compared with Cisplatin alone (11.4 months *versus* 8.8 months; $p = 0.048$), in a Phase III randomised trial of 250 patients (van Meerbeeck, Gaafar et al. 2005). Raltitrexed and Cisplatin may be more cost effective compared to Pemetrexed and Cisplatin with similar efficacy in the first line treatment of MPM (Woods, Paracha et al. 2012).

Currently there is no widely approved salvage regimen after failure of first line treatment and all patients are encouraged to enter into clinical trials (Ceresoli, Zucali et al. 2010).

1.6.4 **Targeted therapy**

1.6.4.1 **Epidermal Growth Factor Receptor (EGFR) inhibitors**

The role of EGFR and its inhibition in MPM is discussed in Chapter 2.

1.6.4.2 **PTEN/AKT/MTOR inhibitors**

The role of PTEN/AKT/MTOR and its inhibition in MPM is discussed in Chapter 3.

1.6.4.3 **Vascular Endothelial Growth Factor inhibitor**

Vascular Endothelial Growth Factor (VEGF) and Vascular Endothelial Growth Factor receptor (VEGFR) plays an important role in tumour angiogenesis (See appendix B for list of all drugs and targets for the targeted biological agents used). VEGF combines with Type 1(Flt-1) and 2(KDR) VEGFR resulting in activation of various intracellular signalling pathways giving rise to endothelial cell proliferation and new vessel formation. The significant role played by VEGF- VEGFR pathway has made it a major target for the development of anti-cancer drugs. The first VEGF inhibitor antibody that has shown promising results in clinical trials and has been approved by the FDA and was Bevacizumab (Avastin®; Genentech Inc). Bevacizumab is currently used in colorectal cancer, non small cell lung cancer, glioblastoma and renal cell cancer.

VEGF has shown to be expressed in the serum as well as in the tumour samples of MPM patients with level of VEGF increasing in advanced disease (Linder, Linder et al. 1998; Konig, Tolnay et al. 1999; Ohta, Shridhar et al. 1999; Konig, Tolnay et al. 2000; Aoe, Hiraki et al. 2006; Yasumitsu, Tabata et al. 2010). Bevacizumab in combination with Erlotinib (OSI-774, Tarceva®, OSI Pharmaceuticals) did not show any significant responses in 24 MPM patients (Jackman, Kindler et al. 2008). Bevacizumab in combination with chemotherapy such as Cisplatin and Gemcitabine also did not demonstrate any

significant clinical benefit (T. Karrison, H. L. Kindler et al. 2007). However, when Bevacizumab was combined with Cisplatin and Pemetrexed in the U.S. Intergroup Phase II trial in 45 patients the median overall survival seen was 15.3 months (95% CI, 11.3-23.5) which was higher than 12.1 months seen in patients receiving Cisplatin and Pemetrexed in the study by Volgelzang *et al* (2003) (Vogelzang, Rusthoven et al. 2003; S. M. Radaideh 2010). The French MAPS trial was a randomised Phase II/III multicentre trial involving 111 patients comparing the addition of Bevacizumab to Cisplatin and Pemetrexed with Cisplatin and Pemetrexed only. There was an improvement in six month disease control rate in favour of the Bevacizumab arm (73.5% *versus* 43.2%; P=0.01) (G. Zalcman, J. Margery et al. 2010).

VEGFR and multi-targeted tyrosine kinase inhibitors such as Vatalanib (PTK787, Bayer & Novartis), Sorafinib (Nexavar®, Bayer), Semaxanib (SU5416, SUGEN) and Thalidomide have shown limited activity in MPM (Zucali, Ceresoli et al. 2011). A Phase II trial of the oral pan-VEGFR tyrosine kinase inhibitor, Cediranib (AZD2171, Astra Zeneca), after failure of first line platinum based chemotherapy in 54 patients, demonstrated 9% response rate and 34% of patients had stable disease (Garland, Chansky et al. 2011).

1.6.4.4 **Ribonuclease inhibitor**

Ranpirnase (Onconase®, Tamir Biotechnology, NJ) is an enzyme that breaks down RNA and inhibits protein synthesis. A Phase II trial of Ranpirnase in MPM in 105 patients demonstrated partial response in four patients, minimal regression in two patients and 35 patients had stable disease. The 1 year survival rate was 34.3% and 2 year survival rate was 21.6% which were higher than that of the historical control (n=337) of 27% and 12% respectively (Mikulski, Costanzi et al. 2002). In a larger Phase III randomised (unpublished) study Ranpirnase in combination with Doxorubicin was compared to

Doxorubicin alone in 413 patients. There was no difference seen in the overall survival between the two groups (11.1 *versus* 10.7 months; HR 1.02, 95% CI 0.82-1.26). However in 130 patients who were pretreated with one line of therapy there was a modest survival benefit seen (10.5 *versus* 9 months; HR 1.49, 95% CI 1.02-2.17) (M. Reck, M. Krzakowski et al. 2009).

1.6.4.5 **Histone deacetylase inhibitors**

Histones exist in acetylated or deacetylated form catalysed by histone acetyltransferase and histone deacetylase (HDAC). Equilibrium between the two forms of the histone is maintained by these enzymes. When histone proteins in deacetylated form are combined to DNA, the transcription of DNA is significantly inhibited acting as transcription repressors. Histone deacetylase inhibitors (HDACi) induce cell apoptosis by expressing genes associated with apoptosis and cell cycle arrest (Xu, Parmigiani et al. 2007). Vorinostat (Zolinza®, Merck) is an HDACi approved by FDA for use in patients with cutaneous T-cell lymphoma. In the largest ever randomised Phase III VANTAGE 014 trial of Vorinostat in MPM recruiting 661 patients, Vorinostat was compared to placebo in patients pretreated with chemotherapy. This study did not demonstrate any benefit of Vorinostat in MPM with median overall survival being 30.7 week in the Vorinostat arm compared to 27.1 weeks in placebo arm (p =0.858) (Krug, Kindler et al. 2011).

The anti-epileptic drug Valproic acid has shown to be an HDACi (Gottlicher, Minucci et al. 2001). A Phase II trial evaluating the effect of Valproic acid in combination with Doxorubicin in pretreated patients demonstrated a modest benefit. The response rate was 16%, disease control rate was 36% and overall survival was 6.7 months (95% CI 4.9 – 8.5) in the cohort of 45 patients enrolled in the study (Scherpereel, Berghmans et al. 2011).

1.7 **Conclusion**

Over the past decade there has been significant improvement in the treatment of MPM with combination chemotherapy with Cisplatin and Pemetrexed or Raltitrexed being the first line therapy. Despite there being an impressive 40% response rate the survival is still at best just over a year. There are no approved second line chemotherapy regimens. Current clinical trials of targeted treatment options have failed to show any meaningful benefit in MPM with maximum response being less than 20%. In particular EGFR, MTOR and VEGF inhibitors, which have shown significant activity in other solid tumour types and are approved for use by FDA, have failed to impress and change practise in MPM. The largest ever MPM clinical trial of 661 patients evaluating the HDACi Vorinostat, did not demonstrate any benefit. In view of the poor survival benefit from first-line chemotherapy, and the lack of subsequent effective treatment options, there is a need for the development of more effective treatment modalities for patients with MPM.

Chapter 2. Targeted Epidermal Growth Factor Receptor Therapy in Malignant Pleural Mesothelioma – Where do we stand?

Agarwal V, Lind MJ, Cawkwell L. Targeted epidermal growth factor receptor therapy in malignant pleural mesothelioma: Where do we stand? Cancer treatment reviews 2011;37:533-42.

2.1 **Epidermal Growth Factor Receptor**

Novel targeted therapies have been successful in other tumour types. One of the most commonly studied targets in cancer therapeutics is the epidermal growth factor receptor (EGFR). Epidermal growth factor (EGF) was first discovered by Stanley Cohen in 1960 during his study of nerve growth factor in mouse sub-maxillary glands and subsequently, in 1975, he confirmed the presence of plasma membrane receptors in human fibroblasts (Cohen 1960 March; G. Carpenter 1975). EGFR was isolated in 1982 as a 170 kDa transmembrane glycoprotein with an EGF binding site on the extracellular surface (Cohen, Ushiro et al. 1982). The structure of EGFR was found to be the human equivalent of the mammalian v-erb-B oncogene protein from the avian erythroblastosis virus. Unlike the human EGFR, the v-erb-B oncogene protein did not have the extracellular EGF binding domain thereby demonstrating that the intracellular domain may play an important role in tumourigenesis (Downward, Yarden et al. 1984). EGFR belongs to the human epidermal growth factor receptor (HER) family, which has four structurally related receptor tyrosine kinases ErbB1 (EGFR), ErbB2 (HER2), ErbB3 and ErbB4 (Salomon, Brandt et al. 1995). The EGFR protein consists of 1186 amino acids. The receptor structure consists of extracellular, transmembrane and intracellular domains (**Figure 2.1**). The extracellular domain consists of cysteine-rich clusters, which form the ligand-binding domain. Upon binding with ligands such as EGF or transforming growth factor alpha (TGF-α), the EGFR monomers form homodimers by dimerisation with another EGFR or heterodimers with another receptor of the HER family. The intracellular domain has tyrosine kinase activity. Dimerisation of the EGFR results in structural rearrangement of the intracellular tyrosine kinase domain. Adenosine triphosphate (ATP) is then recruited into the catalytic domain, resulting in its auto-phosphorylation. This leads to the activation of a cascade of

intracellular signal transduction pathways resulting in cell proliferation, anti-apoptosis, invasion and metastasis (Bogdan and Klambt 2001; Hynes and Lane 2005; Citri and Yarden 2006). Among a host of various intracellular signalling pathways stimulated by EGFR, the major pathways activated are the RAS/RAF/MAPK pathway resulting in cell proliferation, metastasis and invasion, and the PI3K/AKT/MTOR pathway resulting in inhibition of apoptosis (Ciardiello and Tortora 2008) (**Figure 2.2**).

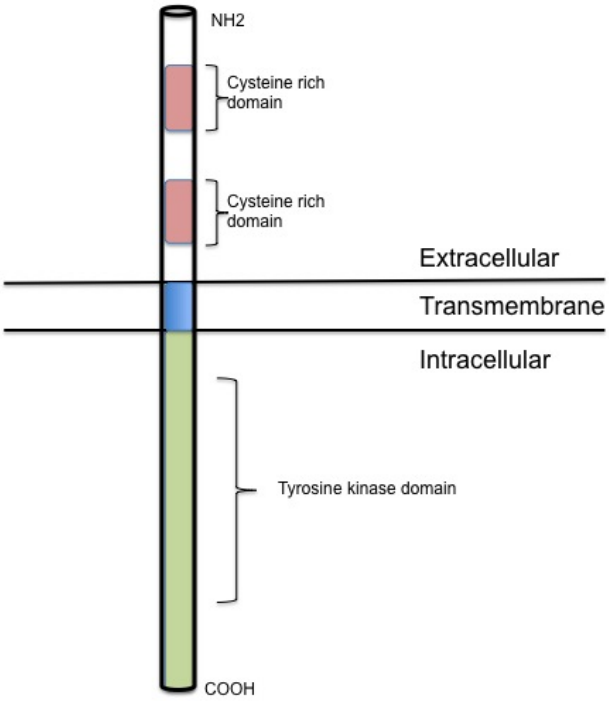

Figure 2.1 Structure of EGFR. This figure shows the structure of EGFR with the extracellular component demonstrating the ligand binding cysteine rich domain, the transmembrane domain and the intracellular tyrosine kinase domain.

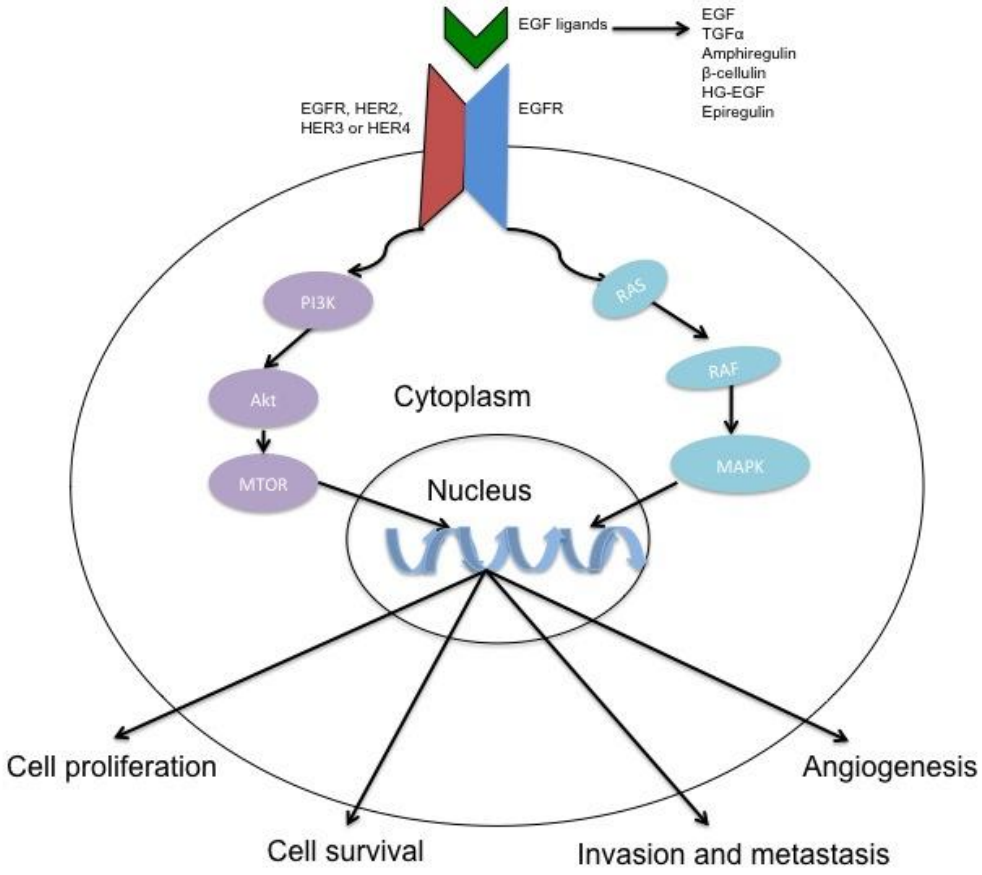

Figure 2.2 EGFR signal transduction pathways. Upon EGFR stimulation by EGF ligands, the RAS/RAF/MAPK pathway and the PI3K/AKT/MTOR pathway is activated resulting in the transcription of various genes leading to cell proliferation, cell survival, invasion, metastasis and angiogenesis. (Adapted from Ciardiello F and Tortora G. N Engl J Med 2008; 358:1160-1174).

EGFR is physiologically expressed in various epithelial cells such as skin, hair follicles, mammary glands, liver and the gastrointestinal tract (Herbst and Shin 2002). It plays an important role is normal cellular functions such as proliferation, migration, differentiation and cell survival and is essential for embryonic development and tissue homeostasis in adults (Wieduwilt and Moasser 2008; Reiss and Saftig 2009). Dysregulation of EGFR may be associated with an oncogenic phenotype. Cancer cells often secrete EGFR ligands

initiating an autocrine loop resulting in activation of EGFR and oncogenesis (de Larco and Todaro 1978; Yarden and Sliwkowski 2001). Over-expression of EGFR has been seen in a number of tumour types including breast, ovarian, glioblastoma, bladder, pancreas, prostrate, renal, lung, colon and head and neck (Gullick 1991; Yarden and Sliwkowski 2001; Herbst and Shin 2002; Holbro and Hynes 2004). EGFR expression in normal cells range from 40,000 to 100,000 receptors per cell (Herbst and Shin 2002). In malignant cells the number of EGFRs per cell increases significantly; for example in breast cancers up to 2 million EGFRs per cell have been reported. Various studies have analysed the prognostic relevance of EGFR expression with conflicting results. It is currently unclear if expression of EGFR necessarily equates to activation of EGFR and neoplastic progression. Expression of EGFR may or may not be associated with EGFR gene amplification (Bredel, Pollack et al. 1999; Bhargava, Gerald et al. 2005). The EGFR gene is amplified in oligodendrogliomas (Fallon et al, 2004), glioblastomas (Marquez et al, 2004), lung carcinomas (Baselga and Arteaga, 2005; Giaccone, 2005), gastric carcinomas (Takehana et al, 2003), and breast carcinomas (Takehana, Kunitomo et al. 2003; Al-Kuraya, Schraml et al. 2004; Fallon, Palmer et al. 2004; Marquez, Wu et al. 2004; Bhargava, Gerald et al. 2005). Amplification of the EGFR gene may be associated with truncation mutations of the EGFR extracellular domain, as seen in gliomas, which result in constitutive activation of EGFR and oncogenesis (Humphrey, Wong et al. 1990; Ekstrand, James et al. 1991; Nishikawa, Ji et al. 1994; Pedersen, Meltorn et al. 2001). Mutations in the EGFR TK domain may result in constitutive activation of EGFR and induce carcinogenesis (discussed in 2.2.1.1).

2.2 **Targeted EGFR therapy**

Two classes of EGFR inhibitors have been developed: anti-EGFR monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) (See appendix B for list of all drugs and targets for the targeted biological agents used). The anti-EGFR mAbs, including Cetuximab (IMC-C225, Erbitux®, ImClone Systems Incorporated) and Panitumumab (ABX-EGF, Vectibix®, Amgen), target the extracellular domain of EGFR and compete with the ligand for binding, thus reducing tyrosine kinase phosphorylation. The EGFR TKIs, including Gefitinib (ZD1839, Iressa®, AstraZeneca) and Erlotinib, compete with ATP and inhibit EGFR auto-phosphorylation (**Figure 2.3**). EGFR inhibitors currently in clinical practice are Erlotinib, Gefitinib, Lapatinib GW572016, Tyverb®, GlaxoSmithKline) Cetuximab and Panitumumab (**Table 2.1**).

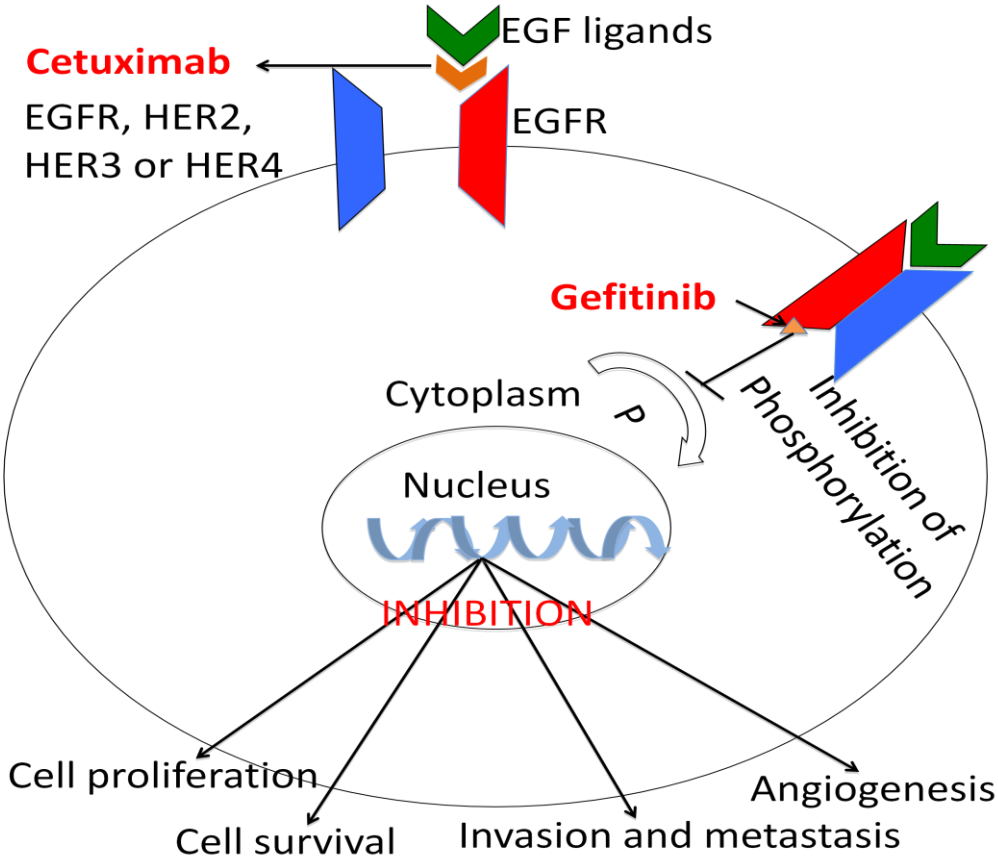

Figure 2.3 Mechanism of action of EGFR inhibitors. This figure shows the action of monoclonal antibodies such as Cetuximab at the extracellular domain of EGFR and that of tyrosine kinase inhibitor such as Gefitinib at the intracellular domain (Ciardiello and Tortora 2008).

Table 2.1 EGFR inhibitors in clinical practice.

Drugs	Target	Dose	Indication
Erlotinib	EGFR tyrosine kinase inhibitor	150 mg daily orally	NSCLC refractory to platinum based chemotherapy First line treatment in advanced pancreatic cancer in combination with Gemcitabine
Gefitinib	EGFR tyrosine kinase inhibitor	250 mg daily orally	Licensed as first line therapy for advanced stage NSCLC with positive EGFR mutation
Lapatinib	Dual EGFR and HER2 tyrosine kinase inhibitor	1250 mg daily every 3 of 4 weeks orally	With Capecitabine in HER2 Positive advanced or metastatic breast cancer refractory to Herceptin
Cetuximab	Anti-EGFR monoclonal antibody	400 mg/m2 loading dose followed by 250 mg/m2 weekly, intravenously	In combination with radiation for local or locally advanced HNSCC or platinum refractory metastatic HNSCC In metastatic colorectal cancer after failure of Oxaliplatin and Irinotecan based regimens.
Panitumumab	Anti EGFR Monoclonal Antibody	6mg/kg IV every 2 weeks	In metastatic colorectal cancer after failure of Oxaliplatin and Irinotecan based regimens.

2.2.1 **EGFR in non small cell lung cancer**

Non small cell lung cancer (NSCLC) demonstrates a high frequency of EGFR over-expression when compared to normal pleura (Rusch, Baselga et al. 1993; Franklin, Veve et al. 2002; Hirsch, Varella-Garcia et al. 2003). Nakamura *et al* (2006) performed a meta-analysis of the survival impact of EGFR over-expression in NSCLC which included 2972 patients from 18 studies (Nakamura, Kawasaki et al. 2006). EGFR over-expression was seen in 58% squamous carcinomas, 39% adenocarcinomas, 38% large cell carcinomas, and 32% of a miscellaneous category but EGFR over-expression did not show any significant impact on survival (HR 1.14; 95% CI, 0.97 – 1.34; p = 0.103) (Nakamura, Kawasaki et al. 2006).

2.2.1.1 **Role of TKIs and EGFR gene mutations in NSCLC**

Both Gefitinib and Erlotinib have shown a modest response in the order of 8-19% in NSCLC (Fukuoka, Yano et al. 2003; Kris, Natale et al. 2003; Perez-Soler, Chachoua et al. 2004; Shepherd, Rodrigues Pereira et al. 2005; Thatcher, Chang et al. 2005). However it was noted that sub-groups of patients (those who were of Asian origin, female or non-smokers and those with tumours of the adenocarcinoma histological type) had a higher likelihood of response to EGFR TKI therapy (Thatcher, Chang et al. 2005). This led to the investigation of potential molecular biomarkers of response to EGFR TKI therapy. Lynch *et al* (2004) hypothesised that patients who had a good response to Gefitinib had somatic mutations in the EGFR gene and hence sequenced the entire coding region (Lynch, Bell et al. 2004). They identified somatic mutations in 8/9 tumours from patients with Gefitinib-responsive lung cancer but no mutations were identified in any of 7 tumours from patients who did not have any response to Gefitinib ($p < 0.001$) (Lynch, Bell et al. 2004). These activating mutations in the tyrosine kinase domain were associated with increased kinase

activity and conferred susceptibility to TKIs. Paez *et al* (2004) conducted a genome-wide screen of receptor tyrosine kinases and found mutations in the EGFR gene in 15/58 NSCLC samples from Gefitinib-naïve patients in Japan and in 1/61 samples from Gefitinib-naïve patients in the United States of America (Paez, Janne et al. 2004). They also evaluated 9 patients who had been treated with Gefitinib and reported that 5/5 tumour samples which showed a response to Gefitinib therapy carried a mutation in the EGFR. No mutations were found in any of 4 tumours which had not responded to Gefitinib therapy ($p = 0.0027$) (Paez, Janne et al. 2004). It is now known that EGFR gene is located in chromosome region 7p12 and mutations in NSCLC cluster in exons 18-21, which are within the tyrosine kinase domain (**Figure 2.4**) (Sharma, Bell et al. 2007). Approximately 45% of mutations are in-frame deletions involving amino acids L746 to A750 in exon 19 and a further 45% of mutations create the substitution L858R within exon 21 (Sharma, Bell et al. 2007). The remaining mutations mainly occur in exons 18 and 20. These mutations are not found in normal lung tissues (Shigematsu, Lin et al. 2005; Chan, Gullick et al. 2006).

The presence of these activating mutations in the tyrosine kinase domain increases the kinase activity and locks the EGFR in a constitutively active state with tumour growth dependent on EGFR signalling (Gazdar, Shigematsu et al. 2004; Yun, Boggon et al. 2007). TKIs bind more avidly to mutant EGFR compared to wild type due to marked rotational change in the tyrosine kinase domain of the mutant receptor, thereby making it more responsive to their inhibitory effects (Tracy, Mukohara et al. 2004; Yun, Boggon et al. 2007).

Activating mutations in the tyrosine kinase domain (exons 18-21) were shown to be present in approximately 10% (25/262) of NSCLC samples in the United States and 30% (122/419) in the East Asian population (Pao and Miller 2005). They were found to be more frequent

in adenocarcinoma and in tumours from patients who were non-smokers, female, or of Asian ethnic origin (Paez, Janne et al. 2004; Pao and Miller 2005; Shigematsu, Lin et al. 2005). These characteristics were similar to those patients who demonstrated a good clinical response to Gefitinib or Erlotinib therapy in Phase III trials (Shepherd, Rodrigues Pereira et al. 2005; Thatcher, Chang et al. 2005). A recent meta-analysis of somatic EGFR gene mutation as a predictive biomarker for response to single agent TKI therapy in NSCLC evaluated data from 59 clinical trials of Erlotinib or Gefitinib therapy. Among the 3101 patients, 1020 (32.9%) had tumours with an EGFR gene mutation and the analysis demonstrated that mutant EGFR was predictive of response to single agent TKI therapy (sensitivity 0.78; 95% CI, 0.74 - 0.82; specificity 0.86; 95% CI, 0.82 - 0.89) (Dahabreh, Linardou et al.).

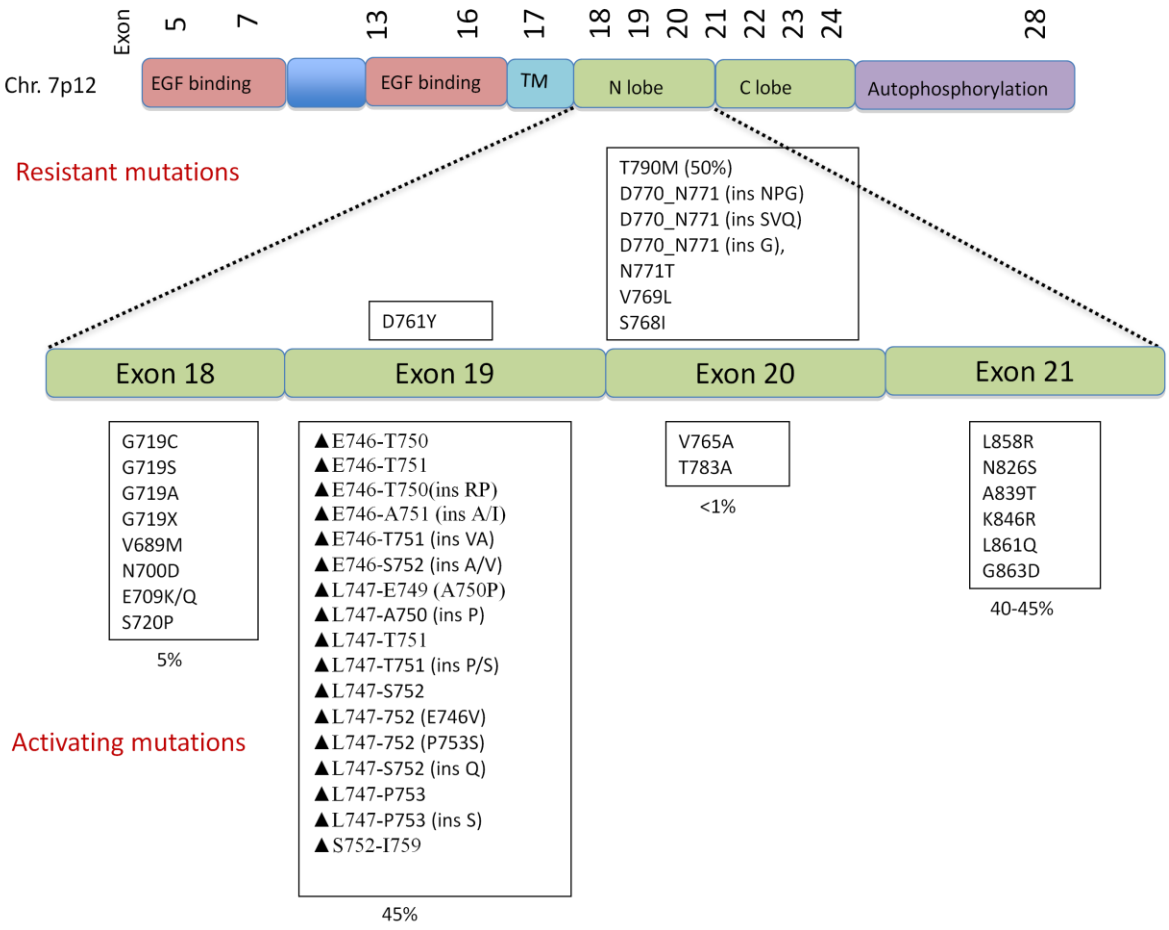

Figure 2.4 EGFR mutations in NSCLC. This figure demonstrates the structure of EGFR gene. The commonest mutations were seen with exons 19 and 21 resulting in activating mutations of the TKI domain. (Adapted and modified from (Sharma, Bell et al. 2007). TM= transmembrane

2.2.1.2 **KRAS gene mutations**

KRAS is a GTPase, which lies downstream of the EGFR and activates the RAS/RAF/MAPK and PI3K/AKT/MTOR pathways. Mutations in the KRAS gene may result in constitutive downstream activation of the EGFR signalling pathways resulting in lack of sensitivity to both TKIs and monoclonal antibodies targeting EGFR, both of which act upstream of KRAS. KRAS gene is located on chromosome 12p12 and consists of 6 exons (www.atlasgeneticsoncology.org). KRAS mutations are commonly seen in codon 12 (GGT) and codon 13 (GGC) of exon 2 and codon 61 (CAA) of exon 3 (van Krieken, Jung et al. 2008) (**Figure 2.5**). Codon 12 and 13 code for glycine and codon 61 codes for Glutamine in wild type KRAS. Single point mutations of the bases lead to amino acid exchange in the KRAS protein resulting in reduction of Ras GAP GTPase activity. This locks the KRAS protein in the active Ras-GTP conformation resulting in KRAS being in a constitutively active state (Ellis and Clark 2000). Mutated KRAS occurs in approximately 20% of NSCLC samples and a meta-analysis of 17 studies (1008 NSCLC patients) where Erlotinib or Gefitinib therapy had been used, revealed that response to EGFR TKI treatment was highly unlikely if the tumour carried a KRAS mutation (Linardou, Dahabreh et al. 2008). A recent extended meta-analysis of 22 studies (1470 patients with NSCLC) again reported that mutated KRAS was associated with a poor response to EGFR TKI therapy (3% response *versus* 26% response for wild type KRAS) (Qiu, Mao et al. 2010).

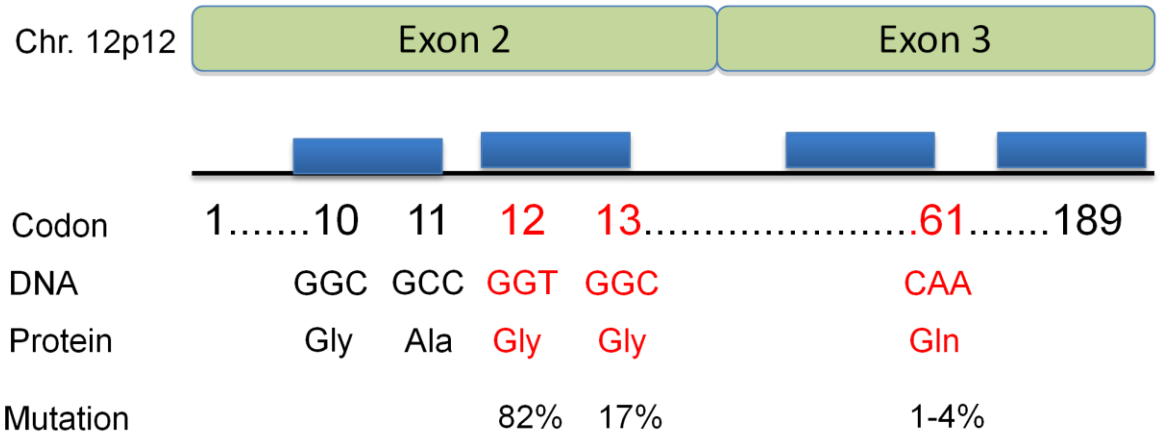

Figure 2.5 KRAS gene. Numbers in red are codons that may result in missense mutations resulting in alteration of the KRAS protein thereby inhibiting the Ras GAP GTPase activity. Adapted and modified from (van Krieken, Jung et al. 2008).

2.2.2 **Anti-EGFR monoclonal antibody therapy in NSCLC**

Cetuximab has been shown to give a modest response in NSCLC when combined with chemotherapy, resulting in small but statistically significant benefit (Robert, Blumenschein et al. 2005; Butts, Bodkin et al. 2007; Rosell, Robinet et al. 2008; Ettinger 2010). The large FLEX Phase III trial in advanced NSCLC randomised 1125 patients with EGFR-positive tumours to either chemotherapy alone (Cisplatin and Vinorelbine) or chemotherapy with Cetuximab. The combination arm showed a minimal overall survival benefit (median 11.3 months *versus* 10·1 months; p=0.044) (Pirker, Pereira et al. 2009).

2.2.3 **Anti-EGFR monoclonal antibody therapy in colorectal cancer**

In colorectal cancer the response rates with single agent anti-EGFR mAb therapy were a modest 9 to 12% however, when combined with chemotherapy, this increased to approximately 20% (Cunningham, Humblet et al. 2004; Saltz, Meropol et al. 2004; Folprecht, Lutz et al. 2006; Lenz, Van Cutsem et al. 2006; Tabernero, Van Cutsem et al.

2007; Van Cutsem, Peeters et al. 2007). Several clinical trials and studies have demonstrated that colorectal cancers which carry a mutation in the KRAS oncogene, are significantly associated with resistance to anti-EGFR mAb therapy (Cunningham, Humblet et al. 2004; Saltz, Meropol et al. 2004; Lenz, Van Cutsem et al. 2006; Van Cutsem, Peeters et al. 2007; Karapetis, Khambata-Ford et al. 2008; De Roock, Claes et al. 2010). Mutated KRAS is present in approximately 40% of colorectal cancers, is associated with poor prognosis and induces resistance to anti-EGFR mAb treatment (either alone or in combination with chemotherapy) (Andreyev, Norman et al. 2001; De Roock, Piessevaux et al. 2008; Linardou, Dahabreh et al. 2008). Poor response to anti-EGFR mAb therapy is also related to BRAF mutations (Linardou, Dahabreh et al. 2008; Laurent-Puig, Cayre et al. 2009; Hawkes and Cunningham 2010).

2.2.4 **Predictive biomarkers for targeted EGFR therapy**

The association of EGFR and KRAS gene mutations with response to targeted EGFR therapy has been discussed above. Additional gene mutations in the EGFR downstream signalling pathways may also lead to resistance to EGFR therapy (**Figure 2.6**).

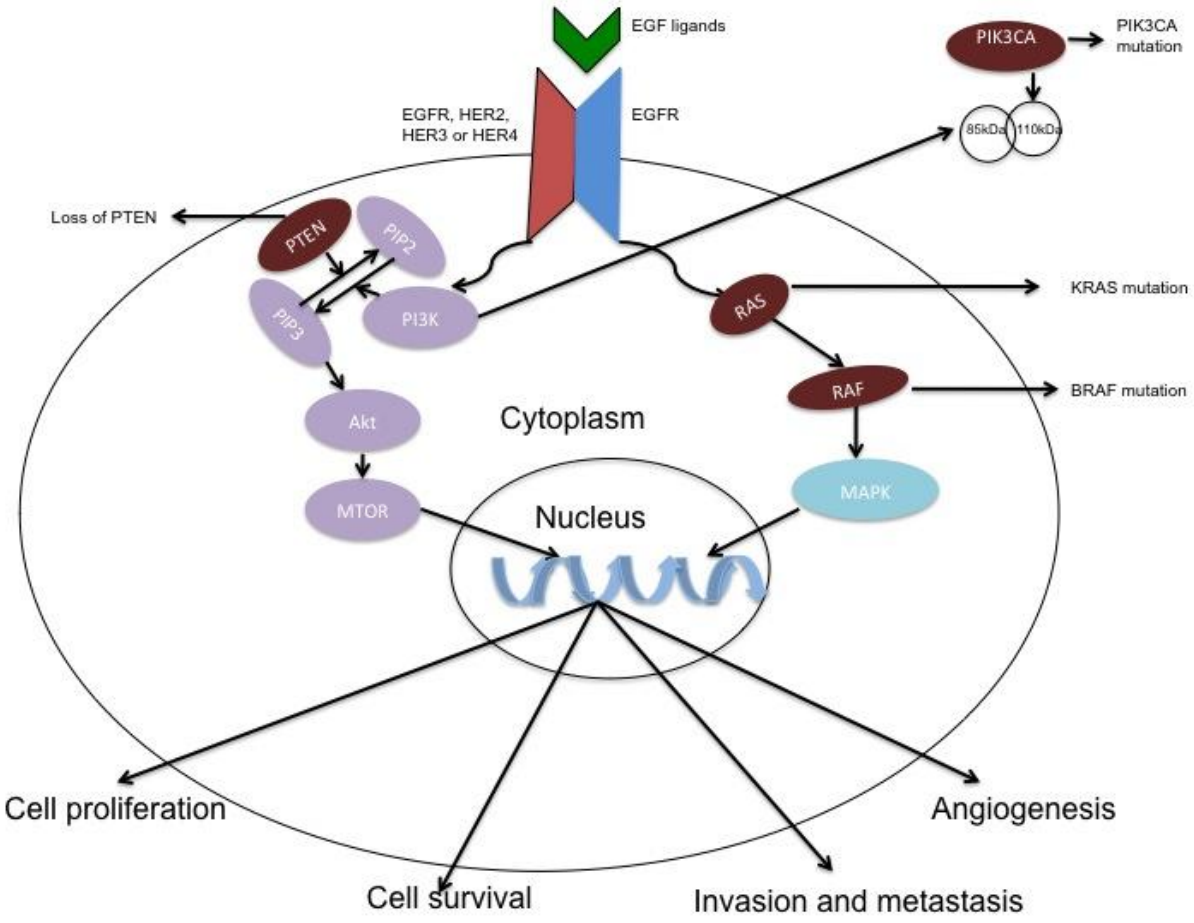

Figure 2.6 Biomarkers predictive of EGFR resistance. This figure demonstrates the biomarkers that lie downstream of EGFR, the mutation of which can result in inducing resistance to EGFR inhibitors. PIK3CA encodes for the 110 subunit of PI3K and its mutation results in persistent activation of PI3K. PTEN protein enables dephosphorylation of PIP3 to PIP2 and the loss of PTEN results in PIP3 remaining in a phosphorylated form resulting in persistent activation of the AKT. Mutation of RAS and RAF result in persistent activation of the RAS/RAF/MAPK pathway.

The BRAF gene is a member of the RAF family of serine/threonine kinases and lies downstream of RAS in the RAS/RAF/MAPK pathway. BRAF gene is located on chromosome 7q34 and is composed of 18 exons (www.atlasgeneticsoncology.org). All activating mutations are seen within the kinase domain and in 90% of the cases thymine is substituted with adenine at nucleotide 1799 in exon 15. This leads to valine being substituted for by glutamate at codon 600 (V600E) (Davies, Bignell et al. 2002; Yuen, Davies et al. 2002; Tan, Liu et al. 2008). The presence of BRAF mutations is associated with poor progression-free and overall survival in colorectal cancer and has been associated with resistance to anti-EGFR mAb therapy (Di Nicolantonio, Martini et al. 2008; Loupakis, Ruzzo et al. 2009; Tol, Nagtegaal et al. 2009; De Roock, Claes et al. 2010).

Activation of the PI3K protein by phosphorylation leads to downstream activation of AKT in the PI3K/AKT/MTOR pathway (Markman, Atzori et al. 2010). The p110α catalytic subunit of PI3K is encoded by the PIK3CA gene. Colorectal carcinomas with mutations in PIK3CA have been reported to show increased resistance to anti-EGFR mAb therapy (Perrone, Lampis et al. 2009; Sartore-Bianchi, Martini et al. 2009; De Roock, Claes et al. 2010).

The PTEN tumour suppressor protein antagonises the activity of PI3K in the PI3K/AKT/MTOR pathway and inactivation of PTEN may lead to constitutive activation of PIK (Zhang and Yu 2010). In colorectal cancers the loss of the PTEN protein is associated with increased resistance to EGFR mAbs (Frattini, Saletti et al. 2007; Perrone, Lampis et al. 2009; Sartore-Bianchi, Martini et al. 2009).

Wild type KRAS status is known to be a good predictive biomarker for response to EGFR mAbs in metastatic colorectal cancer. However not all wild type tumours respond and the additional analysis of mutations in the BRAF, NRAS and PIK3CA genes in tumours

demonstrating wild type KRAS was found to improve the prediction of response to Cetuximab (De Roock, Claes et al. 2010). It has been suggested that colorectal tumours which are "quadruple negative" for mutations in the KRAS, BRAF, PIK3CA and PTEN genes would benefit most from EGFR mAb therapy (Bardelli and Siena 2010).

2.2.5 **EGFR over expression in MPM**

EGFR is over-expressed in 44 to 97% of MPM samples as demonstrated by various immunohistochemical studies (**Table 2.2**). Immunohistochemistry is a subjective technique and can lack reproducibility due to the use of different antibodies, scoring systems and methodologies. Some research groups reported that there was no relationship between EGFR over-expression and outcome in patients with MPM (Destro, Ceresoli et al. 2006; Okuda, Sasaki et al. 2008; Gaafar, Bahnassy et al. 2010) whilst others have reported that EGFR over-expression is associated with improved outcome (Dazzi, Hasleton et al. 1990; O'Byrne, Edwards et al. 2004; Edwards, Swinson et al. 2006). However EGFR over-expression in MPM is seen more commonly in the epithelial histological subtype, which is associated with improved patient survival, and EGFR over-expression is not an independent prognostic marker (Dazzi, Hasleton et al. 1990; Edwards, Swinson et al. 2006).

Table 2.2 EGFR over expression in MPM by immunohistochemistry

Study	Total samples	Histological subtype	Antibody used	Scoring	Positive for EGFR expression	Prognostic marker
Dazzi *et al*(Dazzi, Hasleton et al. 1990)	34	Epithelial – 16; Biphasic – 9; Sarcomatoid - 9	F4 monoclonal antibody	Positive - greater than or equal to 5%	Total- 23/34 (68%) Epithelial -14/16 (87.5%); Biphasic – 2/9 (22%); Sarcomatoid - 7/9 (78%)	No
Destro *et al*(Destro, Ceresoli et al. 2006)	61	Epithelial – 50; Biphasic – 9; Sarcomatoid - 2	EGFrAb-10 (clone 111.6) (Neomarkers, Union City, CA)	Negative (0-10%); Low expression (11-50%); High expression (>50%)	Total (Low + high expression) – 34/61 (56%)	No
(CALGB 30101)(Govindan, Kratzke et al. 2005)	28	Not reported	EGFr antibody clone 31G7 (*Zymed* Laboratories, Inc)	All samples were scored as 0+, 1+, 2+, and 3+	Total (2+ and 3+) - 27/28 (97%)	No
Edwards *et al*(Edwards, Swinson et al. 2006)	168	Epithelial – 98; Biphasic – 37; Sarcomatoid - 33	EGFR.113 (Novocastra Laboratories Ltd., Newcastle, UK)	Positive - greater than or equal to 5%	Total – 74/168 (44%) Epithelial -58/98 (59%); Biphasic – 13/37 (35%); Sarcomatoid – 3/33 (9%)	No
Garland *et al*(Garland, Rankin et al. 2007)	57	Not reported	EGFr antibody clone 31G7 (*Zymed* Laboratories, Inc)	All samples were scored as 0+, 1+, 2+, and 3+	Total (2+ and 3+) - 43/57 (75%)	No
Rena *et al* (Rena, Boldorini et al. 2011)	83	Epithelial – 57; Biphasic – 20; Sarcomatoid - 6	EGFR antibody clone H11 (Dako, Denmark)	All samples were scored as 0+, 1+, 2+, and 3+	Total – 70/83 (84%) Epithelial -30/57 (52%); Biphasic – 8/20 (40%); Sarcomatoid – 0/6	No

2.2.6 **EGFR TKI therapy in MPM**

Gefitinib inhibits EGF-induced phosphorylation of downstream signal transduction pathways and the growth of MPM cells *in vitro* (Janne, Taffaro et al. 2002). Another EGFR TKI, PD153035, was shown to reduce motility and invasion in MPM cell lines (Cole, Alleva et al. 2005). Based on the *in vitro* studies showing activity of EGFR TKIs in MPM cells, two Phase II trials were conducted (**Table 2.3**). The CALGB 30101 Phase II trial enrolled 43 chemotherapy-naïve patients with pleural (n = 42) or peritoneal (n = 1) mesothelioma and a performance status of 0 - 1. All patients received 500mg Gefitinib once a day until disease progression or unacceptable toxicity was observed. The primary end point of the study was the percentage of patients who remained alive and progression-free three months after the start of Gefitinib treatment. The 3-month progression-free survival was 40% (95% CI, 25 - 56%) (Govindan, Kratzke et al. 2005). This was compared with a similar historic control group of 337 chemotherapy-naïve patients with malignant mesothelioma from the Cancer and Leukemia Group B database, which incorporated 7 Phase II trials (Herndon, Green et al. 1998). The 3 month failure-free survival rate among patients with good performance status (275/337 patients) was approximately 60%. This was higher than that seen in the Gefitinib trial (40% progression-free survival at 3 months) and the authors therefore concluded that single-agent Gefitinib was not active in malignant mesothelioma (Govindan, Kratzke et al. 2005). In the second Phase II trial, 63 chemotherapy-naïve patients with advanced or recurrent MPM and a performance status of 0 - 1 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 responses seen, although 14/33 (42%) had stable disease

(Garland, Rankin et al. 2007). The median progression-free survival of 2 months in this study was significantly lower than that associated with standard first-line Pemetrexed/Cisplatin chemotherapy (5.7 months; 11 (Vogelzang, Rusthoven et al. 2003). The authors therefore concluded that single-agent Erlotinib was not effective in MPM.

In both the studies evaluating Gefitinib and Erlotinib in MPM, the EGFR protein was shown to be expressed by immunohistochemistry in the majority of the tissue samples, but its expression did not predict response to TKI. EGFR gene amplification by and mutation of the EGFR TKI domain, which have shown to predict response to TKIs, were not assessed (Lynch, Bell et al. 2004; Paez, Janne et al. 2004; Hirsch and Witta 2005). Furthermore, in both studies the radiological response was assessed using the Response Evaluation Criteria In Solid Tumours (RECIST) criteria. This may have had a significant impact on the analysis of the response rates in both studies. Response assessment of tumour therapy in MPM is difficult due to the growth pattern of MPM. MPM grows as a 'rind'around the pleural surface and hence uni- or bi-dimensional tumor measurement may not be suitable for adequate tumor response assessment. The RECIST criteria specify a single dimensional measurement of the tumor's longest diameter and sets thresholds of tumour response evaluation (complete response is disappearance of all tumour; partial response is reduction by >30% of sum of the longest diameter of all lesions; stable disease is reduction <30% and progression <20%; progressive disease as progression by >20% of sum of the longest diameter of all lesions) (Therasse, Arbuck et al. 2000). Due to the nature of the growth of MPM, the RECIST criteria should be modified from the longest diameter to tumour thickness perpendicular to chest wall or mediastinum when evaluating tumour response in MPM (Byrne and Nowak 2004). Symptomatic benefit and quality of life data was not assessed in either of the trials as it may have contributed to our assessment of

clinical benefit to the patients. Both studies were non randomised, Phase II trials, with small patient numbers. The primary end point in the study by Govindan et al was the percentage of patients who remained progression free after 3 months and in the study by Garland et al was one year survival. These end points were compared with historical controls which may not accurately predict the outcome in the same patient population. A randomised study comparing the TKI with either best supportive care and assessing survival data such as overall survival would have given a better understanding of the efficacy of the drug. However, a randomised trial with extensive follow up would have had significant cost and resource implications. In both studies, the TKIs were used as first line agents in chemo naive patients. The efficacy of TKIs in combination with chemotherapy or as second line treatment has not been assessed.

Table 2.3 Phase II studies showing the response rates and survival analysis of EGFR TKIs in malignant mesothelioma.

	Response rate				Median overall survival (Months), 95% CI	Median progression free survival (Months), 95% CI	1 year survival (%), 95% CI
	CR	PR	SD	PD			
Gefitinib(Govindan, Kratzke et al. 2005)	1/43 (2%)	1/43 (2%)	21/43 (49%)	15/43 (35%)	6.8, (3.5 - 10.3)	2.6, (1.5 - 4.2)	32, (21 - 50)
Erlotinib (Garland, Rankin et al. 2007)	0/33	0/33	14/33 (42%)	15/33 (45%)	10, (5 - 13)	2, (2 - 4)	43, (31-55)

CR – complete response; PR – partial response; SD – stable disease; PD – progressive disease; CI – confidence interval

2.2.7 **EGFR gene mutations in MPM**

Cortese *et al* (2006) identified mutations in the tyrosine kinase domain of the EGFR gene in 19/99 lung adenocarcinoma samples but in none of 66 MPM samples (Cortese, Gowda et al. 2006). Similarly, Destro *et al* (2006) failed to identify exon 18-21 mutations in 16 MPM samples (Destro, Ceresoli et al. 2006) and Okuda *et al* (2008) failed to identify exon 18-21 mutations in 25 MPM samples from a Japanese population (Okuda, Sasaki et al. 2008). Therefore it would be reasonable to extrapolate that the absence of EGFR activating mutations in MPM may be one of the causes of resistance to EGFR TKI therapy in MPM.

2.2.8 **KRAS gene mutations in MPM**

A number of studies have explored the presence of KRAS gene mutations in MPM. Metcalf *et al* (1992) screened 20 mesothelioma cell lines for KRAS gene mutations at codons 12, 13 and 61 and found no mutations (Metcalf, Welsh et al. 1992). Kitamura *et al* (1998; 2002) did not identify KRAS mutations in MPM samples from 7 Japanese and 12 American patients (Kitamura, Araki et al. 1998; Kitamura, Araki et al. 2002). Ni *et al* (2000) also examined 17 mesothelioma samples for KRAS mutations and did not identify any mutations (Ni, Liu et al. 2000).

2.2.9 **BRAF gene mutations in MPM**

In MPM, BRAF gene mutations were not present in 53 tumours and 6 cell lines studied (Dote, Tsukuda et al. 2004).

2.2.10 **PIK3CA gene mutations in MPM**

Suzuki *et al* (2009) studied 21 mesothelioma cell lines and did not find any PIK3CA gene mutations (Yutaro Suzuki, Hideki Murakami et al. 2009).

2.2.11 **Loss of PTEN in MPM**

Discussed in detail in section 3.1.1.

2.3 **Cross-talk between EGFR and other pathways**

There is known to be significant cross-talk between the EGFR pathway and other receptor signalling pathways. Resistance to EGFR inhibition therapy may be due to the over-expression of an alternative receptor which compensates for the loss of EGFR function. Such "by-pass" mechanisms may therefore account for resistance to EGFR inhibition therapy and highlight the possible requirement for multi-receptor blockade therapy. Downstream proteins within the EGFR signalling pathway, such as PI3K and AKT (Chapter 3) are utilised by other tyrosine kinase receptor growth factor pathways, including the c-MET receptor and IGF-1R pathways (Engelman and Janne 2008; Eyzaguirre, Buck et al. 2008; Kono, Marshall et al. 2009). In MPM, Jagadeeswaran *et al* (2006) reported over-expression of the c-MET protein in 82% of 66 MPM tissue samples and in 0/21 samples of normal pleura using immunohistochemistry (Jagadeeswaran, Ma et al. 2006). In mesothelioma cell lines, treatment with the small molecule c-MET inhibitor SU11274 lead to dose-dependent growth inhibition (Jagadeeswaran, Ma et al. 2006). IGF-1R has been shown to be important in the malignant phenotype of MPM (Hoang, Zhang et al. 2004) and treatment of mesothelioma cell lines with the IGF-1R inhibitors NVP-AEW541(Novartis) and AG1024 resulted in dose-dependent growth inhibition (Whitson, Jacobson et al. 2006; Kai, D'Costa et al. 2009). AG1024 was also shown to enhance the cytotoxic effect of Cisplatin in mesothelioma cells (Kai, D'Costa et al. 2009).

Significant cross talk exists between the EGFR and COX-2 pathways (Dannenberg, Lippman et al. 2005). COX-2 is an inducible enzyme, which converts Arachidonic acid to prostaglandins (PG) and its role in MPM is discussed in detail in Section 4.1. Increased

EGFR stimulation and subsequent activation of intracellular MAPK protein promotes transcription of COX-2 which then produces PG including PGE2. PGE2 can trans-activate the EGFR, promoting cell proliferation and migration, and also increases the expression of amphiregulin which is a ligand of the EGFR (**Figure 2.7**). The activation of EGFR via the COX-2 pathway can form a positive feedback loop resulting in increased production of COX-2/PGE2, which in turn trans-activates the EGFR pathway. In MPM, both EGFR and COX-2 are frequently over-expressed and, since significant cross-talk exists between the two pathways, targeting both of these pathways may form a rationale for treatment. In mesothelioma cell lines, treatment with EGFR inhibitors or COX-2 inhibitors leads to cytotoxicity (Janne, Taffaro et al. 2002; O'Kane, Eagle et al. 2010) but the *in vitro* effects of combined inhibition, targeting both the EGFR and COX-2 pathways, are as yet unknown. Although no clinical studies in MPM have investigated a combination therapy with EGFR TKI inhibitors and COX-2 inhibitors, a Phase I study of the COX-2 inhibitor Celecoxib (Celebrex®, Pfizer) combined with Gefitinib in recurrent or metastatic squamous cell carcinoma of the head and neck was well tolerated and had an encouraging response rate of 22% (Wirth, Haddad et al. 2005). In NSCLC the combination of Celecoxib or COX-2 inhibitor Rofecoxib (Vioxx®, Merck) with Gefitinib showed good tolerability but response rates were comparable to single agent Gefitinib (Gadgeel, Ruckdeschel et al. 2007; O'Byrne, Danson et al. 2007) Another Phase I study combined Celecoxib with Erlotinib in 22 patients with advanced NSCLC and reported a partial response rate of 33% (Reckamp, Krysan et al. 2006).

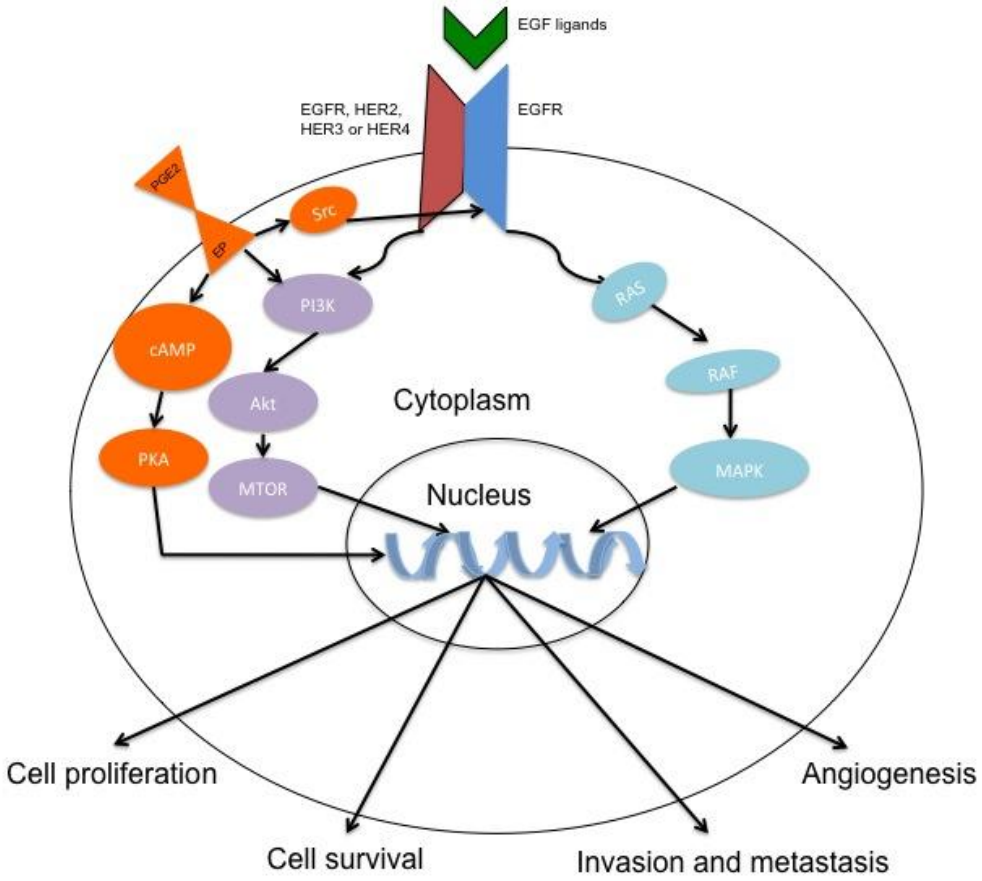

Figure 2.7 Activation of EGFR pathway by PGE2. Activation of the EP receptor by prostaglandin E2 (PGE2) results in activation of the EGFR via its action on Src. It also signals directly through the PI3K/AKT/MTOR network, which is downstream of the EGFR, and through cAMP and PKA resulting in a malignant phenotype (Dannenberg, Lippman et al. 2005).

2.4 **Conclusions**

EGFR is over-expressed in MPM. *In vitro* studies have demonstrated EGFR TKI therapy (Gefitinib) can induce cytotoxic effects in MPM cell lines. However, two small Phase II trials have shown that EGFR TKIs (Gefitinib and Erlotinib) may not be clinically effective in MPM (Govindan, Kratzke et al. 2005; Garland, Rankin et al. 2007). However, both trials were Phase II, non-randomised trials with small patient numbers (n=43; n=63) and hence definite conclusions cannot be drawn from these trials. The mechanisms of resistance to EGFR TKI therapy in MPM could be varied, and may be associated with the absence of activating mutations in the TK domain of EGFR. Other resistance mechanisms may include loss of the PTEN protein, resulting in persistent activation of PI3K/AKT/MTOR pathway, or transactivation of EGFR by COX-2/PGE2. Currently, there are no *in vitro* or *in vivo* published studies assessing the effect of anti-EGFR mAbs in MPM. The anti-EGFR mAbs are not dependent on the presence of activating mutations at the tyrosine kinase domain of EGFR and it is yet unknown whether mAbs will have a cytotoxic effect in MPM. However, loss of PTEN protein and transactivation of the EGFR by COX-2/PGE2 may induce resistance to these mAbs. *In vitro* studies to investigate the effects of anti-EGFR mAbs in MPM are required to assess their cytotoxic effects in MPM. Combination therapy using an anti-EGFR mAb with a COX-2/PGE2 inhibitor or an AKT/MTOR inhibitor may show enhanced therapeutic potential when compared to single agent mAb therapy.

Chapter 3. PTEN/AKT/MTOR pathway in Mesothelioma – Does it have a role to play?

Publication

Agarwal V, Lind M J, Cawkwell L. PTEN/AKT/MTOR pathway in Mesothelioma – Does it have a role to play. Awaiting Submission

3.1 **PTEN/PI3K/AKT/MTOR pathway**

The PI3K/AKT/MTOR pathway plays an important physiological role in transmitting proliferative signals from cell surface growth receptors such as EGFR, Platelet derived growth factor receptor (PDGFR), Insulin growth factor receptor (IGFR), Fibroblast growth factor receptor (FGFR) and Hepatocyte growth factor (HGF) to the nucleus via their interaction with various other proteins. This signalling results in the control of gene expression and protein synthesis thereby controlling growth, proliferation and metabolism of the cell. (Wang and Proud 2006; Zhou, Luo et al. 2010). The dysregulation of PI3K/AKT/MTOR pathway has shown to play an important role in many tumour types (Chiang and Abraham 2007; Guertin and Sabatini 2007). This pathway can be activated by stimulation of PI3K by receptor tyrosine kinases in response to growth factor stimuli or by loss of tumour suppressor gene PTEN (phosphatase and tensin homolog) resulting in hyperphosphorylation and constitutive activation of AKT (**Figure 3.1**). (Manning and Cantley 2007). Phosphorylation of AKT (pAKT) results in activation or inhibition of a host of downstream targets resulting in cell survival, cell proliferation and protein synthesis. AKT blocks apoptotic proteins such as the BIM, BAX, BAD, FOXO family of transcription factors resulting in pro-apoptosis and cell survival. Phosphorylation and retention of p27 in the cytoplasm by pAKT causes cell cycle progression (Fujita, Sato et al. 2002; Shin, Yakes et al. 2002). The NFkβ pathway is also activated by pAKT resulting in cell survival (Nidai Ozes, Mayo et al. 1999; Madrid, Wang et al. 2000). Phosphorylation of the tumour suppressor TSC2 by pAKT disrupts the critical balance between TSC2 and TSC1 leading to activation of MTOR.

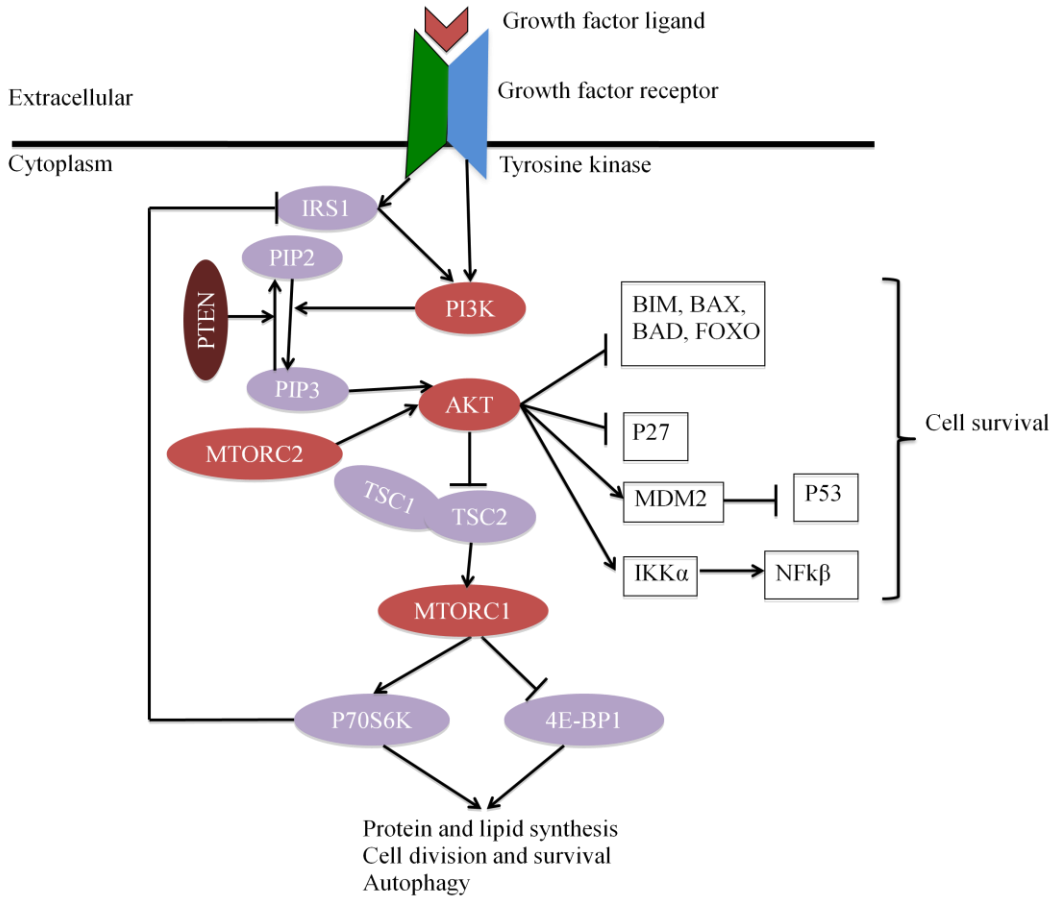

Figure 3.1 PI3K/AKT/MTOR signal transduction pathway, feedback inhibition and their downstream targets. Growth factors when stimulated by growth factor ligands result in enhanced tyrosine kinase activity. Receptor tyrosine kinases activate PI3K by direct binding or by phosphorylation of IRS1 which binds to PI3K. PI3K converts PIP2 to PIP3 resulting in phosphorylation of AKT. PTEN converts PIP3 back into PIP2 thereby negatively regulating the phosphorylation of AKT. Phosphorylation of AKT results in inhibition of downstream targets such as BIM, BAX, BAD, FOXO, p27 and TSC2 and activation of MDM2 (inhibiting p53), IKKα and NFkβ. Disruption of the critical balance between TSC2 and TSC1 leads to activation of MTORC1 which further phosphorylates p70S6K and inhibits 3E-BP1. IRS1 is inhibited by p70S6K thereby negatively regulating the activation of PI3K/AKT/MTOR pathway. MTORC2 can also phosphorylate AKT resulting in activation or inhibition of downstream targets.

MTOR can be assembled into two different complexes MTORC1 and MTORC2 (**Figure 3.2**) (Guertin and Sabatini 2007). Both the MTOR complexes share the MTOR and mLST8/GβL proteins. In addition MTORC1 contains the regulatory associated protein of MTOR (Raptor). MTORC2, in addition to MTOR and mLST8/GβL, is constituted of Rapamycin insensitive companion of MTOR (Rictor), S1N1 and PROTOR/PRR5 (Vilar, Perez-Garcia et al. 2011). MTOR protein is made up of up to 20 tandem HEAT repeats at the amino acid terminal and mediates protein-protein interactions (Yang and Guan 2007). This is followed by a FAT domain, the FRB domain, kinase domain and the FATC domain located at the C-terminus of the protein. The FRB domain provides the docking site for Rapamycin- FKBP12 complex and the FAT and FATC modulate the kinase activity. Phosphorylation of the kinase domain results in the activation of the downstream effectors of MTOR. Rapamycin binds to FKBP12 protein and this Rapamycin- FKBP12 complex binds to the FRB domain of MTOR in MTORC1 thereby inhibiting its downstream signalling. The exact mechanism of how Rapamycin- FKBP12 acts is currently not known, but may be due to inhibition of MTOR autophosphorylation, inhibition of intrinsic kinase activity or preventing MTOR protein from interacting with its substrates (Yang and Guan 2007). However, Rapamycin- FKBP12 complex does not bind to the FRB domain of MTOR in MTORC2 thereby making MTORC2 insensitive to the effects of Rapamycin (Jacinto, Loewith et al. 2004; Sarbassov, Ali et al. 2004).

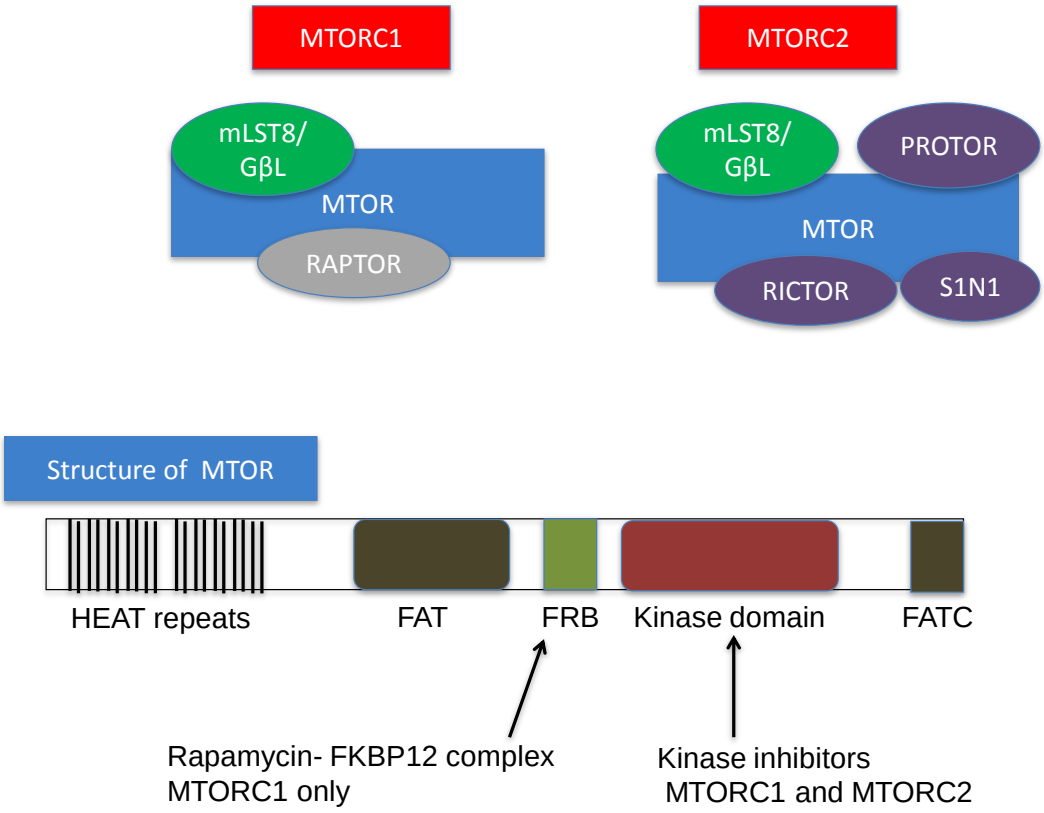

Figure 3.2 Structure of MTORC1 and MTORC2 complexes and the structure of MTOR protein. Adapted and modified from (Yang and Guan 2007). MTORC1 is made up of MTOR, RAPTOR and mLST8/GβL proteins. MTORC2 is made up of MTOR, mLST8/GβL, RICTOR and S1N1 and PROTOR proteins. MTOR protein is made up of upto 20 tandem HEAT repeats that mediate protein-protein interactions, the FAT domain, the FRB domain, the kinase domain and the FATC domain. The FRB domain provides the docking site for Rapamycin- FKBP12 complex in MTORC1.

Alteration of the critical balance between TSC2 and TSC1 by pAKT results in phosphorylation of MTORC1. Activation of MTORC1 phosphorylates downstream targets such as 4E-BP1and p70S6K resulting in protein and lipid synthesis and cell survival. Both MTORC1 and p70S6K can phosphorylate and degrade IRS1 thereby acting as a negative feedback loop to control PI3K/AKT/MTOR pathway activation (Shi, Yan et al. 2005). MTORC2 has recently shown to have a critical role to play in the development of prostate cancer cell lines that are devoid of PTEN (Guertin, Stevens et al. 2009). MTORC2 phosphorylates and activates AKT resulting in activation of its downstream targets (Sarbassov, Guertin et al. 2005). The presence of PTEN would counteract the stimulatory effect of MTORC2 on AKT.

3.1.1 **Over expression of AKT/MTOR and loss of PTEN proteins in MPM**

Immunohistochemical analysis of expression or loss of biomarkers within the PI3K/AKT/MTOR pathway has been studied in MPM (**Table 3.1**). The first study to look at loss of PTEN protein and expression of pAKT protein and pMTOR protein was by Altomare *et al* (2005), who demonstrated that PTEN protein was lost in 2/26 tumour samples and pAKT protein and pMTOR protein was expressed in 17/26 and 24/26 tumour samples respectively when analysed by immunohistochemistry. The PTEN exons 2-8 in M43 cells (MPM cell line) was also analysed using single-strand conformation polymorphism assay and PTEN gene was found to be absent. Western blot confirmed the loss of PTEN protein in the M43 cells (Altomare, You et al. 2005). In another study by Garland *et al* (2007), immunohistochemical analysis showed that PTEN protein was lost in 3/19 tumour samples and pAKT protein and pMTOR protein was expressed in 16/19 and 12/19 tumour samples respectively (Garland, Rankin et al. 2007). A large study of 341 tumour samples by Opitz *et al* (2008) demonstrated that PTEN protein was lost in 211/341

tumour samples. The loss of PTEN protein strongly correlated with poor median overall survival in 129 patients evaluated (PTEN absent 9.7 months *versus* PTEN present 15.5 months; log rank test p=0.0001) (Opitz, Soltermann et al. 2008) This association of loss of PTEN protein with poor survival was independent of histology (epithelioid vs non epithelioid; p=0.003), age (<62 years vs >62 years; p=0.3) and surgery (yes vs no; p=0.09) in multivariate cox regression analysis. Another recent study by Watzka *et al* (2010) showed that pAKT protein was expressed in 65/74 tumour samples when analysed by immunohistochemistry (Watzka, Setinek et al. 2010). A tissue microarray of 37 MPM samples showed that pAKT protein was expressed in all of them (Wilson, Barbone et al. 2008). In another study by Suzuki *et al* (2009) in MPM cell lines, loss of PTEN protein was seen in 2/21 cell lines (ACC-MESO-1 and Y-MESO-25) and AKT protein activation was seen in 13/21 cell lines (Yutaro Suzuki, Hideki Murakami et al. 2009).

Despite there being significant variations in the reported frequency of loss of PTEN protein in MPM ranging from 8% to 62%, expression of pAKT protein and pMTOR protein has been seen in a majority of all tumour samples in the studies conducted. Loss of PTEN was shown to be a significant indicator of poor prognosis in the study by Opitz *et al* (2008) (Opitz, Soltermann et al. 2008). Expression of pAKT was not found to be of any prognostic significance (Watzka, Setinek et al. 2010). Expression of MTORC2 and the prognostic value of MTOR (MTORC1 and MTORC2) in MPM has to be best of our knowledge not yet been reported in the literature.

Table 3.1 Expression of PTEN/pAKT/pMTOR assessed by Immunohistochemistry.

Study	**Protein**	**No of tissue samples**	**Expression**	**Prognostic value**
Altomare *et al* (Altomare, You et al. 2005)	pAKT pMTOR PTEN	26	17 (65%) 24 (92%) Lost in 2 (8%)	N/A
Garland *et al*(Garland, Rankin et al. 2007)	PTEN pAKT pMTOR	19	Lost in 3 (16%) 16 (84%) 12 (74%)	N/A
Opitz *et al*(Opitz, Soltermann et al. 2008)	PTEN	341	Lost in 211 (62%)	Poor prognostic (PTEN- 9.7 months *versus* PTEN+ 15.5 months; p=0.003; n=126)
Wilson *et al* (Wilson, Barbone et al. 2008)	pAKT	37	37 (100%)	N/A
Watzka *et al* (Watzka, Setinek et al. 2010)	PAKT	74	65 (88%)	Not prognostic

3.1.2 **Inhibition of the PTEN/PI3K/AKT/MTOR pathway in MPM**

Various *in vitro* studies in MPM cell lines have been done looking at the inhibition of the PTEN/PI3K/AKT/MTOR pathway. The PTEN/PI3K/AKT/MTOR pathway can be manipulated by either counteracting the loss of PTEN or inhibiting over expressed proteins such as PI3K, AKT or MTOR. Multi targeted therapies can also be used to target multiple proteins within the pathway to enhance the therapeutic effect.

3.1.2.1 **PTEN over expression**

Since loss of PTEN protein results in phosphorylation and activation of AKT, the up regulation of PTEN protein may decrease phosphorylation of AKT. To assess the effect of over expression of PTEN protein in MPM, a study transfected two MPM cell lines (REN and I-45) with adenovirus type 5 vectors AdPTEN and studied their effects. PTEN gene mutation status of both cell lines was unknown and a basal level of PTEN protein was present, which on transfection with AdPTEN was significantly increased. Forced over expression of PTEN protein resulted in decreased phosphorylation of AKT and increased apoptotic death of the cell (Mohiuddin, Cao et al. 2002). In another study, wild type as well as mutant PTEN was introduced in PTEN deficient MPM cell line (M43 cell line) using adenovirus (Altomare, You et al. 2005). Cells with wild type PTEN and not with mutant PTEN disrupted cell cycle progression and reduced AKT phosphorylation.

3.1.2.2 **PI3K / AKT / MTOR inhibition**

In vitro studies looking at the inhibition of the PI3K / AKT /MTOR pathway in MPM cell lines and MPM tumour fragment spheroids have been evaluated by various authors (See appendix B for list of all drugs and targets for the targeted biological agents used). Tumour fragment spheroids are original tumour fragments which are grown in a three dimensional

structure thereby maintaining the original characteristics of the tumour. The PI3K inhibitor LY294002 inhibits the ATP binding of the catalytic subunit of PI3K and has been used to inhibit the PI3K / AKT pathway in MPM (**Figure 3.3**). LY294002 has been shown to reduce phosphorylation of pAKT in MPM cell lines (M43 cell lines) as well as in tumour fragment spheroids (Altomare, You et al. 2005; Wilson, Barbone et al. 2008). Rapamycin inhibits MTORC1 by binding to FK-binding protein 12 (FKBP12) and has shown to reduce phosphorylation of p70S6K, downstream of MTORC1, in M43 cell lines as well as in tumour fragment spheroids (Altomare, You et al. 2005; Wilson, Barbone et al. 2008). Inhibition of MTORC1 by Rapamycin has been shown to reduce the negative feedback loop exerted via the IRS1/PI3K pathway and thereby increasing the phosphorylation of AKT in mesothelioma tumour fragment spheroids (Barbone, Yang et al. 2008).

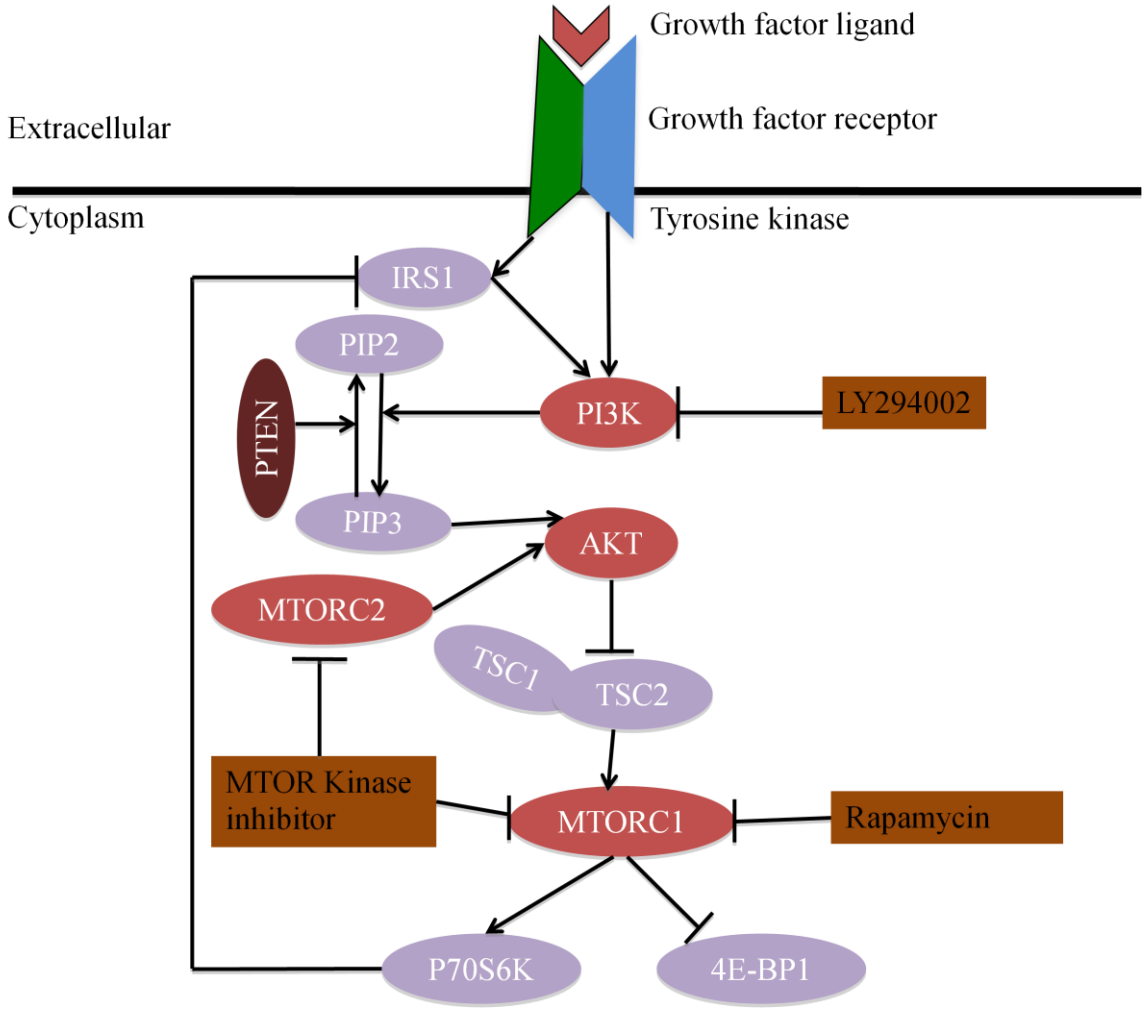

Figure 3.3 Inhibitors of PI3K/AKT/MTOR signal transduction pathway. LY294002 inhibits PI3K, Rapamycin inhibits MTORC1 and MTOR kinase inhibitors inhibit MTORC1 and MTORC2.

Altomare *et al* (2005) studied the effect of LY294002, and Rapamycin in the M43 cell line (Altomare, You et al. 2005). Treatment of M43 cells with LY294002 resulted in 3.3 fold rise in sub-G1 cell population and with Rapamycin resulted in increased G1 cell population by 30% demonstrating apoptosis and cell cycle arrest at G1 Phase respectively.

In the study by Kim *et al* (2005), the effect of potent apoptotic inducer TNF related apoptosis-inducing ligand (TRAIL) plus protein synthesis inhibitor Cycloheximide was

analysed in MPM monolayer cell lines (M28, REN, MS-1) and MPM tumour fragment spheroids along with the additive effect of LY294002 and Rapamycin (Rapamune®, Wyeth pharmaceuticals) (Kim, Wilson et al. 2005). All tumour spheroids were stained for cytokeratin and cleaved caspase 3 after treatment. Cell apoptosis was quantified in the tumour spheroids as the percentage of all cytokeratin-positive cells with staining of cleaved caspase 3. Cell apoptosis in treated mononuclear cell lines were measured by characteristic nuclear morphology compared to the untreated sample. TRAIL plus Cycloheximide induced complete apoptosis (treated mean apoptosis score 94%; SEM 6% *versus* untreated mean apoptosis score 6%; SEM 4%) in monolayer MPM cell lines, but MPM tumour fragment spheroids demonstrated significant apoptotic resistance (treated mean apoptosis score 32%; SEM 4% *versus* untreated mean apoptosis score 7%; SEM 1%). The addition of LY294002 and Rapamycin to TRAIL plus Cycloheximide overcame the resistance to apoptosis in tumour fragment spheroids. LY294002 induced apoptosis in tumour fragment spheroids on its own and also enhanced the apoptotic effect of TRAIL plus Cycloheximide (treated mean apoptosis score 47; SEM 6%). Rapamycin did not have any effect on the tumour fragment spheroids on its own but enhanced the apoptotic effect of TRAIL plus Cycloheximide (treated mean apoptosis score 50; SEM 17%). In another study by the same group, Rapamycin, but not LY294002 (alone and in combination with Rapamycin), enhanced the apoptotic effect of TRAIL plus Cycloheximide in tumour fragment spheroids. Similar results were seen when Cycloheximide was replaced with Gemcitabine to mimic a more clinically relevant setting (Wilson, Barbone et al. 2008). The effect of Rapamycin overcoming resistance to apoptosis in tumour fragment spheroids may suggest that the resistance may be mediated through the MTOR protein.

Rapamycin has also been shown to affect the interaction of MPM cells with the extracellular matrix, thereby effecting cell invasiveness and metastasis. Ranzato *et al* (2009) studied the effect of Rapamycin in cell motility of MPM cells (MM98, REN, BR95, HMC-htert) on fibronectin and concluded that Rapamycin decreased cell spreading, motility and invasion by inhibiting the MTORC1 (Ranzato, Grosso et al. 2009).

3.1.2.3 **Inhibition of multiple targets**

The PI3K inhibitor LY294002 when combined with Cisplatin resulted in enhanced inhibition of cell growth in MPM cell lines (M43 and M17) and induction of apoptosis (M43 cell line) compared to either agent alone (Altomare, You et al. 2005). *In vitro* studies with ribonuclease enzyme Ranpirnase (Onconase®, Tamir Biotechnology, NJ) (Section 0)) in MPM cell lines have shown that Ranpirnase had significant cytotoxic effect on SV40 negative MPM cell lines by induction of apoptosis (MB cells and MMO cells) but not in SV40 positive cell lines (MPP and MCAT cell lines) (Ramos-Nino, Vianale et al. 2005). SV40 positive cell lines were found to have increased pAKT compared to SV40 negative MPM cell lines. The MB cell line (SV40 negative) and MPP cell line (SV40 positive) were treated with LY294002 alone, Ranpirnase alone and in combination. MB cells showed a higher response rate to Ranpirnase and a lower response rate to LY294002. However, MPP cells showed a higher response rate to LY294002 and a lower response rate to Ranpirnase. Survival of both the cell lines was reduced when Ranpirnase and LY294002 were used in combination.

In another *in vitro* study, the combination of Rapamycin with Cisplatin in MPM cell lines (MS257, MS924, MS248, MS589, H2052 and JMN1B) resulted in inhibition of cell proliferation and enhanced apoptosis in MS924, MS248, MS589 and JMN1B cells compared to either agent alone, but not in MS589 and H2052 cell when analysed using cell

proliferation MTS assays (Section 5.2.2.3) (Hartman, Esposito et al. 2010). MS257, MS589, H2052 and JMN1B were more sensitive to single agent Cisplatin compared to MS924 and MS248 cells. MS924, MS248 and JMN1B demonstrated loss of cell viability when treated with single agent Rapamycin, but not MS257, MS589 and H2052 cells. MS589 and H2052 cells which were resistant to the effect of single agent Rapamycin were also resistant to the combined effect of Rapamycin and Cisplatin.

In MPM tumour fragment spheroids targeting multiple targets in the PI3K/AKT/MTOR signal transduction pathway by combining LY294002 and Rapamycin did not yield any additional benefit compared to Rapamycin alone (Wilson, Barbone et al. 2008).

3.1.3 **Clinical trials with MTOR inhibitors in MPM**

A Phase I study of the Rapamycin rapalog Temsirolimus® (CCI-779, Wyeth pharmaceuticals) did not show any response in two patients with MPM (Raymond, Alexandre et al. 2004). Currently there are two Phase II trials ongoing which are looking at the effect of another Rapamycin rapalog Everolimus® (RAD001, Novartis, Basel) in MPM. The first trial is being conducted by the Southwest Oncology Group (Clinical Trials Identifier: NCT00770120), which is currently recruiting patients who have progressed on first line platinum based chemotherapy and is assessing the effect of Everolimus in patients with unresectable MPM. The primary outcome is to measure four month progression free survival and the secondary outcomes are to measure response rate, overall survival and toxicity. The second Phase II trial is being conducted by Memorial Sloan-Kettering Cancer Centre to assess the effect of Everolimus in patients who have progressed on standard chemotherapy in MPM (Clinical Trials Identifier: NCT01024946) . The primary outcome is to determine the rate of clinical benefit at 16 weeks. The secondary outcomes are response rate and toxicity.

3.1.4 **Discussion**

The pAKT and pMTOR proteins are frequently up regulated in MPM (65% to 100%) and may induce resistance to apoptosis resulting and cell survival. Targeting the PI3K/AKT/MTOR pathway may have a therapeutic effect in MPM, which is otherwise a very poor prognostic disease with a fatal outcome.

In vitro studies looking at inhibiting PI3K as well as MTORC1 in mesothelioma cell lines as well as tumour fragment spheroids have at best shown to have a modest response. The effect of LY294002 in tumour fragment spheroids is debatable with one study by Kim *et al* (2005) demonstrating some effect but not when repeated by Wilson *et al* (2008) (Kim, Wilson et al. 2005; Wilson, Barbone et al. 2008). This lack of benefit by PI3K inhibitor in MPM could be because of AKT activation by other means such as MTORC2.

The modest response to inhibition of MTORC1 by Rapamycin in MPM, as well as other cancers, may be as a result of inhibition of the negative feedback loop of the MTOR pathway (MTOR/p70S6K/IRS1/PI3K) resulting in increased activation of AKT which stimulates cell survival and proliferation (Chiang and Abraham 2007). One of the potential ways to overcome feedback activation of AKT by MTORC1 inhibition would be to block MTORC1 as well as PI3K/AKT. However, the combination of PI3K inhibitor LY294002 and MTORC1 inhibitor Rapamycin did not have any enhanced therapeutic effect when tested in mesothelioma tumour fragment spheroids (Wilson, Barbone et al. 2008). One of the potential reasons for this may be direct activation of AKT by loss of PTEN and presence of MTORC2. Combined inhibition of MTORC1 and MTORC2 may be an improved therapeutic strategy. Inhibition of MTORC2 may further limit the feedback activation of AKT by MTORC1 inhibition (Gupta, Ansell et al. 2009). Second generation MTOR kinase inhibitors have been developed which compete with ATP at the catalytic site

of MTOR protein and inhibit both MTORC1 and MTORC2 (**Figure 3.2**) (Yang and Guan 2007; Liu, Thoreen et al. 2009). The catalytic domains of MTOR protein and p110α subunits of PI3K are structurally related. Therefore inhibition of MTOR kinase may also inhibit PI3K thereby further reducing the AKT activation produced by MTORC1 inhibition. Various MTOR kinase inhibitors such as OSI-027 (Osi Pharmaceuticals) and AZD-8055 (Astra Zeneca) and dual PI3K / MTOR kinase inhibitors such as NVP-BEZ235 (Novartis) and XL-765 (Exelisis) are currently in early Phase trials (Vilar, Perez-Garcia et al. 2011).

In vitro studies with PI3K/MTORC1 inhibitors, when combined with chemotherapy, have shown to have an enhanced therapeutic potential. PI3K/MTOR inhibitors may overcome resistance to apoptosis mediated through the PI3K/AKT/MTOR signal transduction pathway thereby sensitising the cells to the cytotoxic effects of chemotherapy resulting in an enhanced therapeutic outcome.

So far there have been no clinical studies reported looking at the therapeutic effect of PI3K/MTOR inhibitors as single agents, multi targeted therapies or in combination with chemotherapy in MPM. The two clinical trials with Everolimus discussed above are still recruiting patients and the outcomes are awaited. Further studies need to be done to better understand the role of PI3K/MTOR inhibitors in the clinical setting in MPM. The role of MTORC2 in the absence of PTEN in MPM needs to be further delineated and the effect of combined inhibition of MTORC1 and MTORC2 needs to be studied.

Chapter 4. Role of Arachidonic acid metabolites in MPM

Arachidonic acid is a polyunsaturated fatty acid present in the phospholipid of cell membrane. It is metabolised by the Cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes resulting in the production of prostanoids, Leukotrienes, hydroxyeicosatetraenoic acids (HETE) and hydroperoxyeicosatetraenoic acids, which have been implicated in inflammation and carcinogenesis.

4.1 **Cyclooxygenases**

Cyclooxygenases, also known as Prostaglandin-endoperoxidase synthases, are a family of myeloperoxidases which catalyse the rate limiting step of Prostaglandin biosynthesis from Arachidonic acid (Chandrasekharan and Simmons 2004; Sobolewski, Cerella et al. 2010). COX's are present as homodimers. Each monomer is made up of three different structural domains. The amino terminal of the protein contains a single EGF binding domain, which functions as a dimerisation domain, followed by four amphipathic helices that anchors the protein to the membrane (Chandrasekharan and Simmons 2004). The third domain is the catalytic domain involved in the metabolism of Arachidonic acid. COX's are normally located at the luminal aspect of the endoplasmic reticulum and nuclear membrane. There are 3 isoforms of COX's. COX-1 is a glycoprotein encoded by a gene on chromosome 9. It is ubiquitously and constitutively expressed and plays an important role in tissue homeostasis, cytoprotection and cell signalling (Smith 1989). COX-2 is also a glycoprotein with its primary structure similar to that of COX-1 and is encoded by a gene on chromosome 1. It is inducible and is regulated by cytokines and growth factors in inflammation and oncogenesis. COX-2 is up regulated in various cancers such as colon, lung, breast, stomach, bladder and mesothelioma (Marrogi, Pass et al. 2000; O'Kane, Cawkwell et al. 2005). COX-3 is a splice variant of COX-1, present mainly in brain and

spinal cord and its function is currently not clear (Sobolewski, Cerella et al. 2010). COX's are bifunctional enzymes with cyclooxygenase (bis-dioxygenase) and peroxidase activity. Arachidonic acid is converted to PGG2 by cyclooxygenase activity of COX's and the peroxidase activity reduces PGG2 to PGH2. Both the reactions are coupled and functionally interconnected.

4.1.1 **Prostanoid biosynthesis**

Arachidonic acid is released from the membrane phospholipids by Phospholipase A2. Once released Arachidonic acid is oxygenated to PGG2 and reduced to PGH2 by COX's. PGH2 is then further converted to PGE2, PGF2, PGD2, PGI2 and Thromboxane A2 (TXA2) (**Figure 4.1**). These PG's then exert their effect by binding to their cell surface receptors in an autocrine or paracrine manner. Among these PG's PGE2 is implicated in carcinogenesis (Menter, Schilsky et al. 2010).

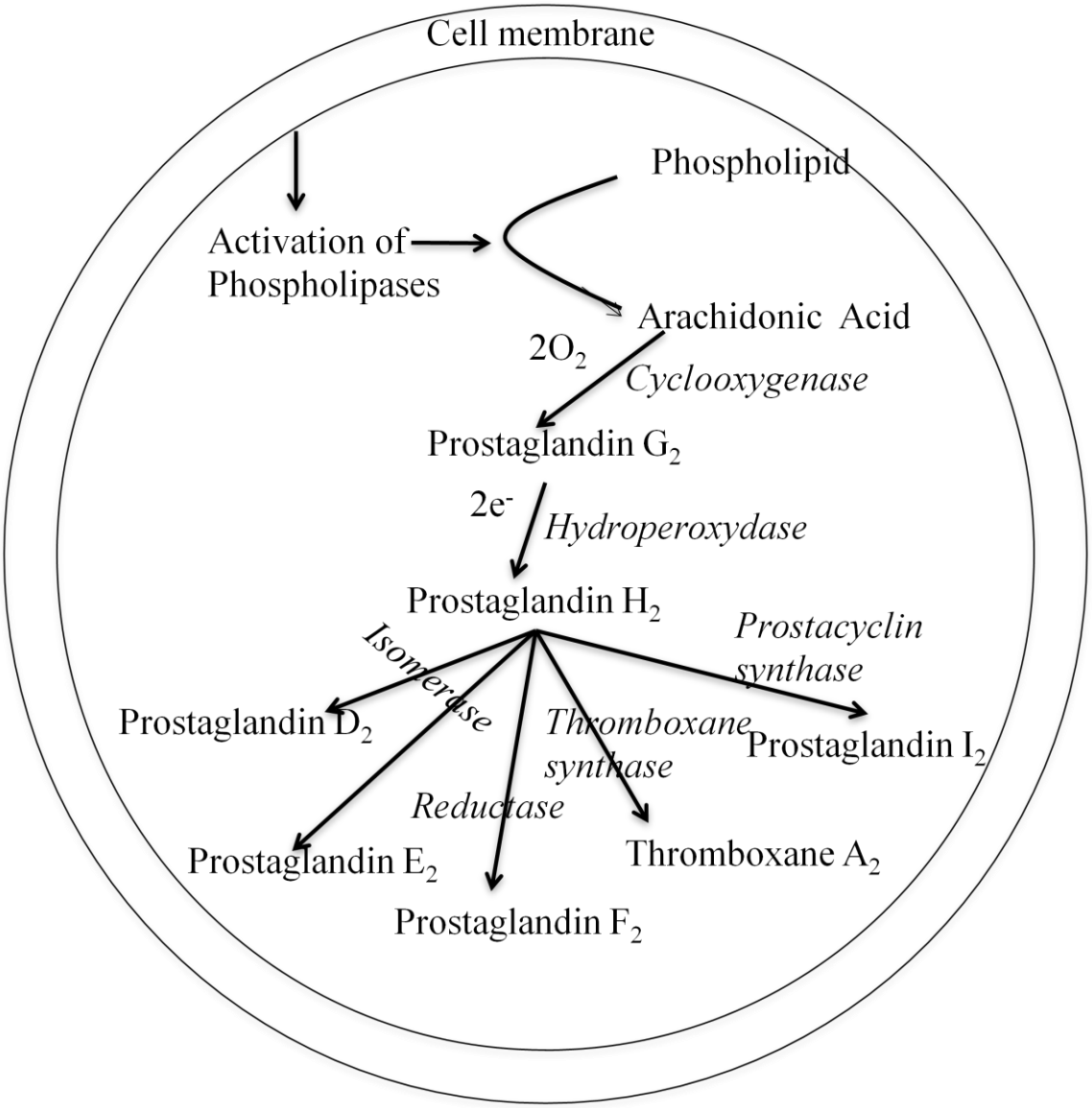

Figure 4.1 Synthesis of Prostanoids from Arachidonic acid. Adapted from (Smith 1989).

4.1.2 **COX-2 / PGE2 and cancer**

PGE2 plays an important role in carcinogenesis and like COX-2, is up regulated in many cancers including colon, lung, breast, head and neck cancers and is associated with poor prognosis (Wang and Dubois 2010). PGE2 and thus COX-2 can induce tumourogenesis by enhancing cell proliferation, survival and metastasis via a variety of cell signalling

pathways (Sobolewski, Cerella et al. 2010). Significant cross talk exists between the COX-2/PGE2 and EGFR pathway resulting in transactivation of EGFR and activation of its downstream signalling pathways (Section 2.3 and **Figure 2.7**) (Dannenberg, Lippman et al. 2005). Increased EGFR stimulation and subsequent activation of the intracellular MAPK result in increased transcription of COX-2, which then produces PG's including PGE2. PGE2 can transactivate EGFR, promoting cell proliferation and migration, and also increases expression of amphiregulin, which is a ligand of EGFR. The activation of EGFR by COX-2/PGE2 can form a positive feedback loop resulting in increased production of COX-2/PGE2, which in turn transactivates the EGFR pathway.

4.1.3 **Role of COX-2 inhibition in MPM**

In MPM, COX-2 protein has shown by immunohistochemistry to be over expressed in 59% to 100% of tumour samples (**Table 4.1**) (Marrogi, Pass et al. 2000; Edwards, Faux et al. 2002; Baldi, Santini et al. 2004; O'Kane, Cawkwell et al. 2005). The COX-2 inhibitor NS398 has been shown to have dose and time dependent antiproliferative activity in the mesothelioma cell line (VAMT-1) when compared to a normal mesothelial isolate (CHTN 18833) (Marrogi, Pass et al. 2000). COX-2 inhibitor Celecoxib has shown to inhibit MPM cell growth (MPP89, H-Meso and Ist-Mes 1) (Catalano, Graciotti et al. 2004). The COX-2 inhibitor DuP-697 has demonstrated significant cytotoxicity in mesothelioma cell lines (MSTO-211H and NCI-H2052) and enhanced the cytotoxic effect of Pemetrexed from 4 to 26 fold in the cell lines (O'Kane, Eagle et al. 2010). In mice oral administration of Rofecoxib significantly reduced the growth of mesothelioma (**Table 4.2**) (DeLong, Tanaka et al. 2003) (See appendix B for list of all drugs and targets for the targeted biological agents used).

Table 4.1 COX-2 over expression in MPM assessed by immunohistochemistry.

Study	n =	Histological subtype	Antibody used	COX-2 Positive	Prognostic marker
Marrogi *et al* (Marrogi, Pass et al. 2000)	30	Epithelial – 23; Biphasic – 4; Sarcomatoid - 3	C22420; (Transduction Laboratories, Lexington, KY)	30/30 (100%)	Not reported
Edwards *et al* (Edwards, Faux et al. 2002)	18	Not reported	SC-1745 (Santa Cruz)	18/18 (100%)	Yes – poor survival (p=0.0005)
Baldi *et al* (Baldi, Santini et al. 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* (O'Kane, Cawkwell 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)

Table 4.2 Cytotoxic effects of COX-2 inhibitors in MPM.

Study	Drugs	Samples	Methodology	Comments
Marrogi *et al* (Marrogi, Pass et al. 2000)	NS398	MPM cell line (VAMT-1)	Cell count and FACS	
DeLong *et al* (DeLong, Tanaka et al. 2003)	Rofecoxib	Mouse model	Tumour size in mouse	Only in small tumours
Catalano *et al* (Catalano, Graciotti et al. 2004)	Celecoxib; NS398; Indometacin	MPM cell lines (MPP89,H-Meso,1st-Mes1); Mouse model	Cell Proliferation Assay (MTS)	Celecoxib > NS398 > Indometacin
O'Kane et al (O'Kane, Eagle et al. 2010)	flurbiprofen; DuP-697;	MPM cell lines (MSTO-211H, NCI-H2052, NCI-H2452)	Cell Proliferation Assay (MTT)	Potentiates the cytotoxic effects of Pemetrexed

4.1.4 **Role of combined COX-2 and EGFR inhibition in MPM**

In MPM, both EGFR and COX-2 are over expressed and may have a significant role to play in survival and metastasis of this disease. Since significant cross talk exists between the two, targeting both the pathways by combining their respective inhibitors may form a rationale for the treatment. In MPM cell lines, both EGFR inhibitors Gefitinib (Janne, Taffaro et al. 2002; Catalano, Graciotti et al. 2004) and COX-2 inhibitors Celecoxib, NS398 and DuP-697 (O'Kane, Eagle et al. 2010) have shown to be cytotoxic when used individually. Combined inhibition of five MPM cell lines (NCI-2452, MPP89, Ist-Mes-1, Ist-Mes-2 and MSTO-211) with Gefitinib and Rofecoxib showed synergistic effect in only the Ist-Mes-2 cell line (Stoppoloni, Canino et al. 2010). It is yet unknown whether the combination of EGFR inhibitors (Monoclonal antibodies and TKIs) and COX-2 inhibitors would enhance the cytotoxic effects of either agent when used alone.

4.2 **Lipoxygenases**

Three isoenzymes exist for Lipoxygenase, 5LOX, 12LOX and 15LOX. 5LOX and 12LOX have been implicated in carcinogenesis.

4.2.1 **Leukotriene biosynthesis**

Arachidonic acid is converted into Leukotriene (LT) A4 by its interaction with 5LOX and 5LOX activating protein (FLAP). LTA4 can be converted into 5-HETE, hydrolysed into LTB4 or LTC4. LTC4 is then converted to LTD4, which is then converted into LTE4 (**Figure 4.2**). Arachidonic acid can also be converted into 12-HETE by its interaction with 12LOX (Wang and Dubois 2010). The LT's are produced by leukocytes, epithelial cells and endothelial cells and play an important role in mounting an inflammatory response.

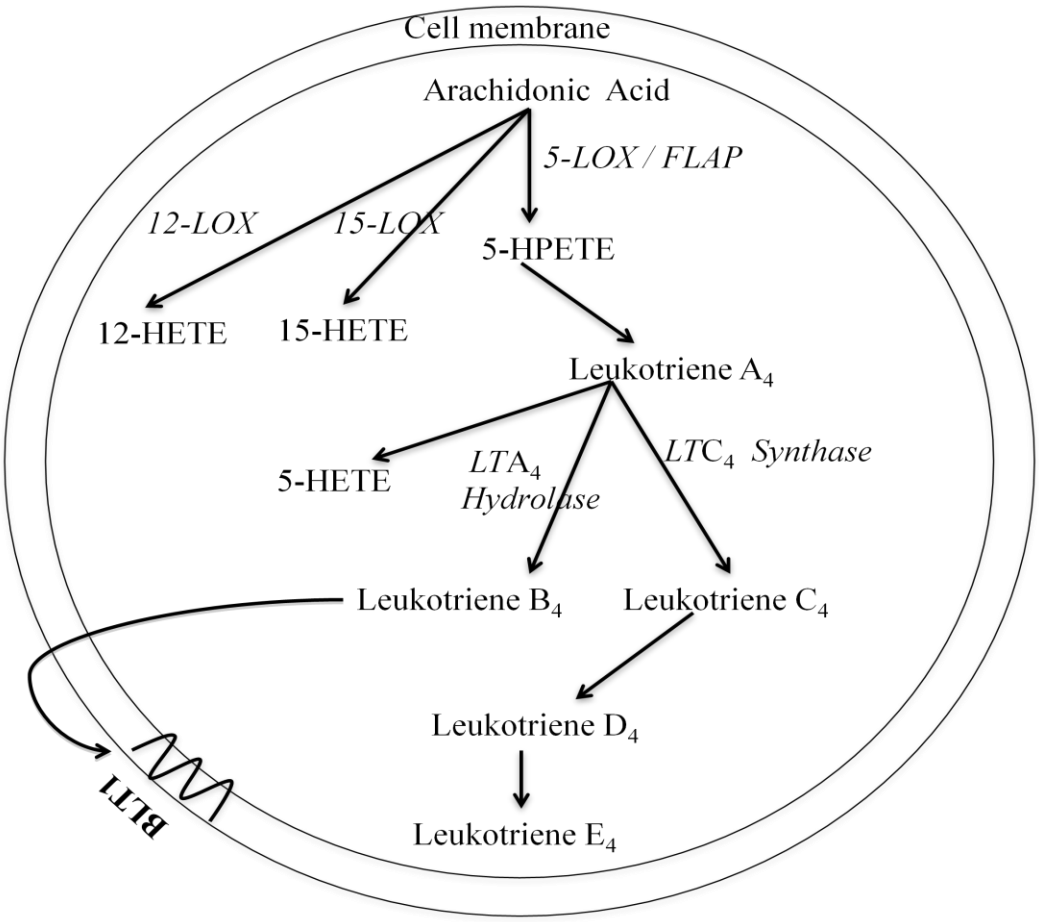

Figure 4.2 Synthesis of Leukotriene from Arachidonic acid.

4.2.2 **LOX and Cancer**

Expression of 5LOX and 12LOX has recently been associated with carcinogenesis of various solid tumours. 5LOX and 12LOX are generally absent in normal epithelia, but are expressed in various epithelial cancers such as colon, oesophageal, lung, prostate, bladder, oral, melanoma, renal and breast (Natarajan, Esworthy et al. 1997; Gupta, Srivastava et al. 2001; Hennig, Ding et al. 2002; Winer, Normolle et al. 2002; Jiang, Douglas-Jones et al. 2003; Ohd, Nielsen et al. 2003; Yoshimura, Matsuyama et al. 2003; Chen, Wang et al. 2004; Matsuyama, Yoshimura et al. 2004; Yoshimura, Inoue et al. 2004; Hoque, Lippman

et al. 2005; Li, Sood et al. 2005; Barresi, Grosso et al. 2007).

LTB4 has been shown to cause cell proliferation and survival in colon cancers. LTB4 mediated its action via the cell surface receptor BLT1, which activates MAPK pathway. In pancreatic cancers LTB4 / BLT1 activates the PI3K-AKT and the MAPK pathway resulting in cell survival (**Figure 4.3**) (Wang and Dubois 2010). 12HETE interacts with the NFkB pathway resulting in antiapoptosis and cell survival (Kandouz, Nie et al. 2003). 12HETE activates protein kinase C and interact with various growth factors resulting in angiogenesis, invasion and metastasis (Honn, Tang et al. 1994).

4.2.3 **Role of LOX inhibition in MPM**

So far there has been only one study published evaluating the role of LOX in MPM cell lines. The three mesothelioma cell lines (created by infecting normal mesothelial cells with SV40) expressed both 5LOX and 12LOX when assessed by RT-PCR as opposed to normal mesothelial cells which expressed 12LOX only. The 5LOX inhibitor AA-861, the generalised LOX inhibitor NDGA and LOX antisense oligonucleotides, significantly inhibited mesothelioma cell proliferation in these cell lines (Romano , Catalano et al. 2001).

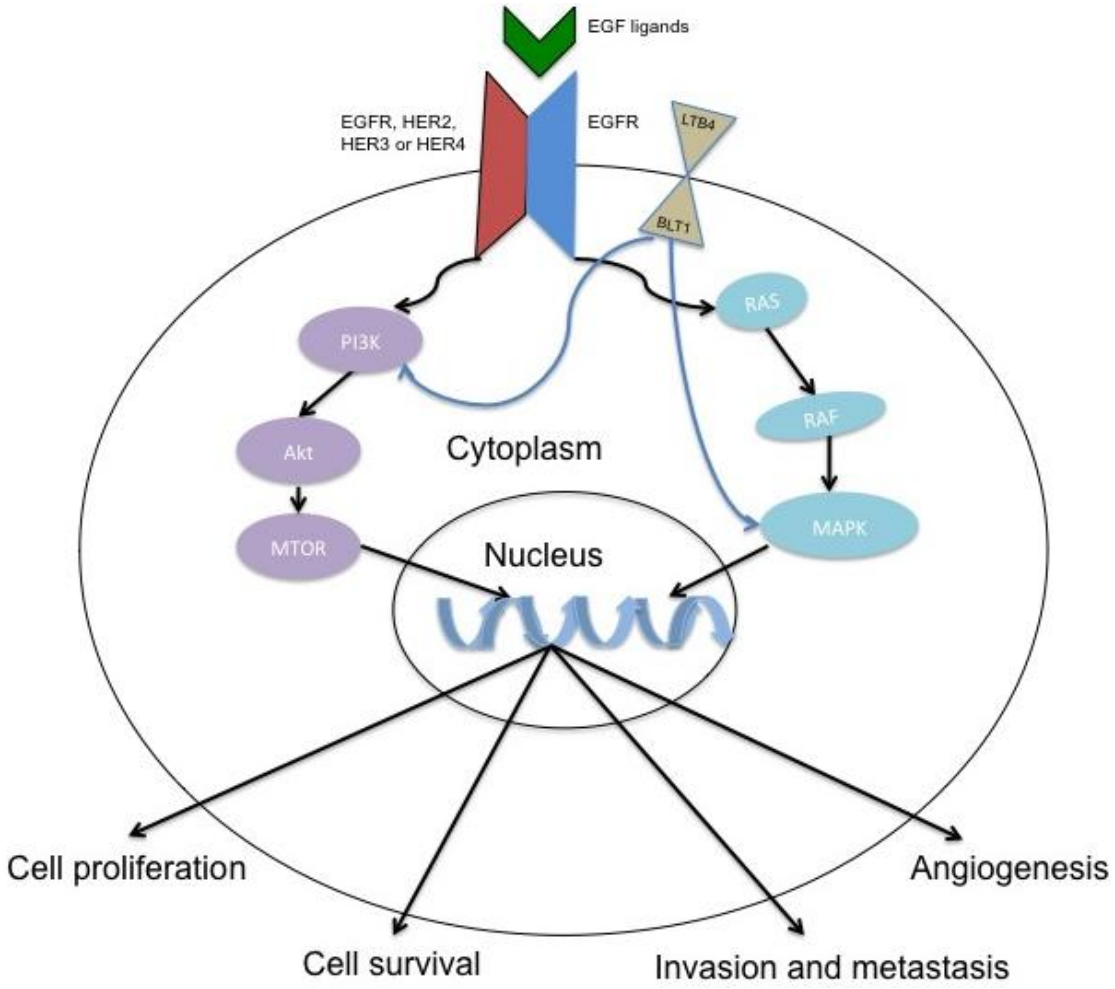

Figure 4.3 Activation of EGFR signalling pathway by LTB4. LTB4 activates BLT1 receptor and their signal transduction pathway leads to activation of EGFR downstream pathway. BLT1 can be activated by its ligand LTB4 resulting in the activation of PI3K and MAPK. (Adapted from (Wang and Dubois 2010)).

4.3 **Dual COX-2 and 5LOX inhibitors**

Both COX-2 and 5LOX have shown be up regulated in a variety of solid tumours such as colon, prostrate, pancreas, oral and oesophageal cancers and combined inhibition of both COX-2 and 5LOX have shown to have an enhanced therapeutic effect in *in vitro* studies

(Tucker, Dannenberg et al. 1999; Chen, Wang et al. 2004; Hoque, Lippman et al. 2005; Li, Sood et al. 2005; Ye, Wu et al. 2005; Cianchi, Cortesini et al. 2006; Zhi, Wang et al. 2006). Currently there are no published studies demonstrating the anti tumour effect of dual COX-2 and 5LOX inhibitors in MPM.

4.4 **Discussion**

COX-2 plays an important role in carcinogenesis and found to be expressed in many solid tumours including MPM. COX-2 inhibitors such as Celecoxib, NS398 and DuP-697 have been shown to inhibit proliferation of MPM in cell lines and mouse models. Studies done in our laboratory have previously demonstrated that COX-2 inhibitors not only inhibit MPM cell lines, but also potentiate the cytotoxic effect of Pemetrexed chemotherapy. Significant cross talk exists between the COX-2 and EGFR pathway therefore targeting both the pathways simultaneously may form a rationale for the treatment. Combined inhibition of COX-2 (Rofecoxib) and EGFR (Gefitinib) has shown synergistic effect in one MPM cell line (Ist-Mes-2) (Stoppoloni, Canino et al. 2010). Currently there are no published reports evaluating the combination of anti-EGFR monoclonal antibodies and COX-2 inhibitors. Further studies are needed to evaluate the anti-tumour effect of EGFR and COX-2 inhibition.

The LOX enzymes (5LOX and 12LOX) are expressed in a variety of solid tumours. It is currently not known whether the LOX enzyme is expressed in MPM and this needs further evaluation.

Chapter 5. Principles of Laboratory techniques

5.1 **Translational research**

Translational research in medicine involves conducting basic research in the laboratory (bench) and translating significant findings into clinical practice (bedside). This bench to bedside approach is the hallmark of translational research and needs close collaboration between the researchers and clinicians to obtain quick and meaningful results. With recent advances in molecular technologies, we are now able to study the pathophysiology of cancer cells and the effect of drugs on the cells in a much greater detail. There are various assays available which can analyse the effect of drugs on cells and the use of methods such as proteomics can further enhance our understanding of the mechanism of action of these drugs.

5.2 **Cellular assays**

There are various methods by which the effect of drugs in cells can be studied *in vitro*. They can be broadly classified into cytotoxicity assays and cell proliferation assays. When assessing the amount of cell lysis or death due to a drug, cytotoxicity assays are preferred and when assessing the amount of cell proliferation or reduction in cell proliferation, cell proliferation assays are used.

5.2.1 **Cytotoxicity assays**

Cytotoxicity assays are performed to assess the cell killing property of a compound or drug. Cell death usually results in alteration of cell membrane permeability resulting in release of intracellular substances such as lactate dehydrogenase (LHD) or uptake of extracellular substances such as Trypan blue, which would otherwise not take place in viable cells with intact cell membrane (**Figure 5.1**). Trypan blue can be taken up by the damaged cell membrane. Individual cells can then be counted using a haemocytometer or flow cytometry

to assess the cytotoxic effect of a drug, as the cells stained with trypan blue would be the dead cells. As this involves analyzing single cells, it is laborious, time consuming and not suitable for high volume assays. LDH is a stable cytoplasmic enzyme that is present in most of the cells and is released in the medium when the cell membrane is damaged. This LDH can then be quantified using commercially available kits.

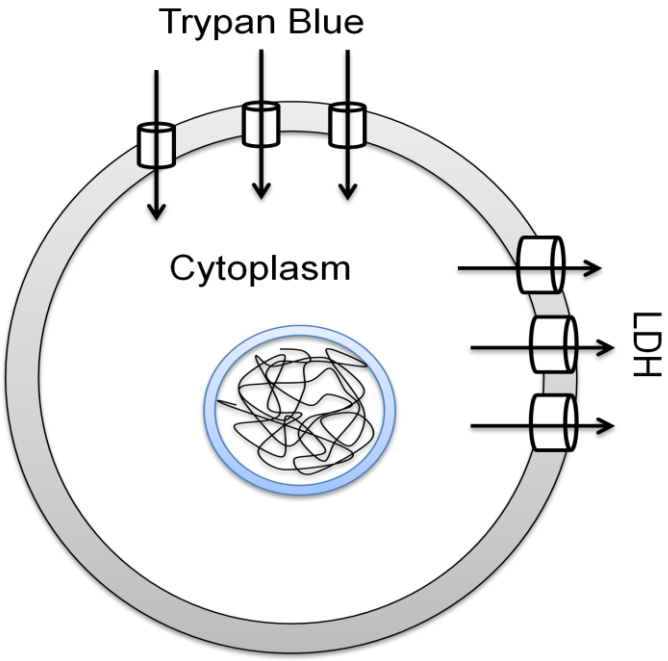

Figure 5.1 Cytotoxicity assays. Alterations in plasma membrane permeability in dead cells result in the release of LDH or uptake of Trypan blue which can be measured.

5.2.2 **Cell proliferation assays**

Cell proliferation assays are based on measuring the number of viable or healthy cells proliferating in a given medium. There are various ways in which cell proliferation can be

measured such as clonogenic assays, DNA synthesis assays and mitochondrial activity assays.

5.2.2.1 **Clonogenic assays**

Clonogenic assays are cell survival assays which measure the ability of a single cell to form a colony which is defined as more than 50 cells. It measures the ability of every cell to multiply indefinitely and form colonies. Cells are seeded in appropriate media before and after treatment and the colonies counted after 1-3 weeks. This assay is the method of choice for analysing dose survival curves for radiation induced cell damage and certain cytotoxic drugs (Franken, Rodermond et al. 2006). As this assay relies on counting individual colonies, it is time consuming and laborious and it is not practical for high output assays.

5.2.2.2 **DNA Synthesis Assays**

These assays measure the synthesis of DNA as a surrogate marker for cell proliferation as DNA replication is a prerequisite for cell multiplication. Prelabelled DNA precursors can be added onto the cells, which are taken up by the dividing cells and incorporated into their DNA. These precursors can then be quantified and the amount of precursors incorporated into the DNA will be directly proportional to the multiplying cells. Thymidine analogue 5-bromo-2'-deoxy-uridine (BrdU) can in incorporated into the DNA of replicating cells instead of Thymidine. The cultured proliferating cells are incubated with BrdU for optimum duration during which BrdU is incorporated into the DNA. The incorporated BrdU can then be detected using commercially available immunodetection kits, which has enzyme, conjugated anti BrdU antibody and can be detected by a substrate reaction and quantified by chemiluminescent or colorimetric assays.

5.2.2.3 **Mitochondrial activity assays**

Viable cells are metabolically active as energy is required for cellular growth and functions. Metabolically active cells when incubated with tetrazolium salts are reduced to colored formazan and can be quantified using Enzyme linked immunosorbent assay (ELISA) plate reader. Since most of the cellular metabolism occurs in the mitochondria, this is a measure of mitochondrial activity in viable cells. Dead cells are not metabolically active and do not have any effect on the tetrazolium salts. The intensity of the colour change due to the production of formazan salts is directly proportional to the number of viable cells in the medium. As a result, mitochondrial activity assays can also be used to assess the cytotoxic capability of a drug, as cell death would reduce the intensity of the colour change. There are various tetrazolium salts commercially available, but the most widely used salts are the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and MTS salts (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Mosmann 1983; Cory, Owen et al. 1991). Certain anti-neoplastic drugs such as Epirubicin, Paclitaxel, Doxetaxel, and Cisplatin can directly interact with MTT salts and reduce it to Formazan, thereby overestimating the amount of cell proliferation (Rao and Knaus 2008).

MTT is a water-soluble tetrazole, which is yellow in colour. MTT is reduced by mitochondrial reductase enzymes present in metabolically active (viable) cells into formazan salt, which is purple in colour and is not soluble in water, hence forming crystals (**Figure 5.2**). These crystals can be solubalised using lysis buffer (40%SDS, 40%DMF, pH 6.7) and quantified by reading the absorbance spectrophotometrically at wavelength 570 nm using an ELISA plate reader.

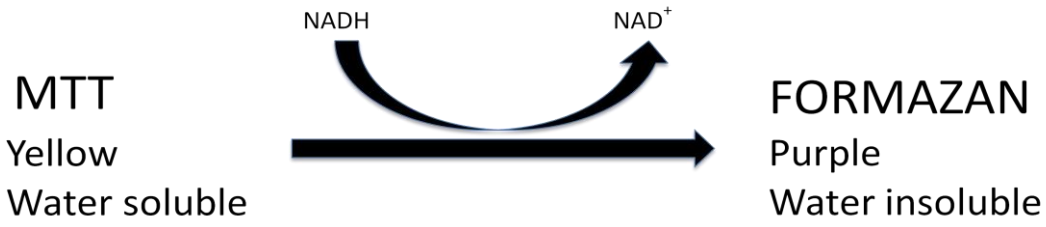

Figure 5.2 Chemical reaction demonstrating conversion of MTT to Formazan.

MTS is another alternative colorimetric assay, which uses a different reagent. MTS produces formazan in the presence of an electron coupling reagent phenazine methosulfate. This formazan is a water-soluble product and can be quantified by reading its absorbance spectrophotometrically at a wavelength of 492 nm.

Use of MTS reagent has various advantages over the use of MTT reagent. Formazan products produced as a result of reduction of the MTT reagent are not water-soluble and form crystals, which require a lysis buffer to solubilise resulting in cell death. Due to cell death only a single reading at a time point can be taken. However, the MTS reagent in the presence of phenazine methosulfate produces water-soluble formazan products. It requires fewer steps than the MTT experiment and reduces variability. Also since the use of MTS reagent is not cytotoxic, multiple readings at various time intervals can be taken.

5.3 **Discussion**

To analyse the effect of a drug in cell lines both cytotoxic as well as proliferative assays can be used. However, for assessing the effect of drugs which act on specific targets, cell proliferation assays are better than cytotoxicity assays as these drugs do not necessarily induce cell death, but may be cytostatic. Since the cytotoxicity assays rely on alteration in cell membrane permeability, it underestimates the cytotoxic effects of some drugs that may cause cell death due to its intracellular effect. In our study we would be assessing the

cytotoxic effects of drugs that may cause death by inhibiting intracellular signalling pathways and may not induce alteration in cell permeability. In view of this, using cytotoxicity assays based on alternation is cell permeability was not used in our study.

For the use of high throughput assays when analyzing the effect of multiple drugs in multiple cell lines clonogenic assays are impractical, as they require counting of individual colonies making them laborious and time consuming. The use of micro plates containing 96 wells in each plate is the most widely use format when multiple assays are required. These plates have 12 wells placed horizontally in 8 rows. They lend themselves to rapid analysis of high sample count and simultaneous assessment of various drugs, multiple dilutions of a drug or multiple cell lines. As the use of 96 well plates require a typical reaction volume of 100 µl to 200 µl per well, it uses significantly less media and drugs making them cost effective. The absorbance of the colour change in the media of the plates can be measured easily using an ELISA plate reader.

DNA synthesis assays and the mitochondrial activity assays can be performed using the micro plate format for rapid and effective analysis. For the purpose of our study, the use of mitochondrial activity assays is preferred as it can also measure the antiproliferative and the cytotoxic capabilities of anti-neoplastic drugs simultaneously as opposed to DNA synthesis assays, which are antiproliferative. We preferred MTS assays due to its ease of use and less variability in the experiments compared to the MTT assays.

5.4 **Proteomics**

Once the anti-tumour effect of a certain drugs is identified, it is important to explore the mechanism of action of the drug. Novel proteomic platforms can be used to identify the molecular mechanism of action of drugs. There are various global analysis methods by which a cancer cell can be studied such as genomics, transcriptomics and proteomics (**Figure 5.3**).

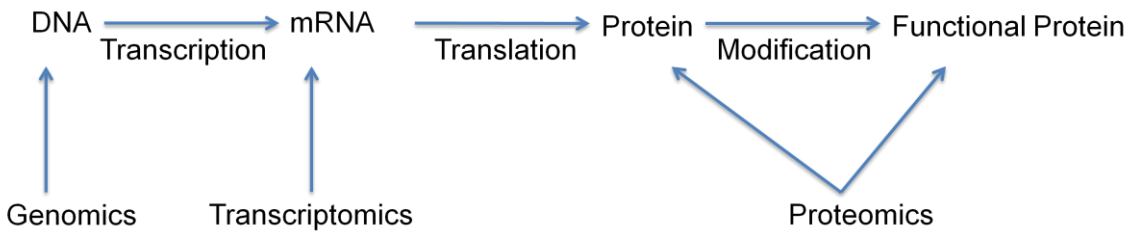

Figure 5.3 Global analysis methods. Global methods by which a cancer cell can be studied at various time intervals depending upon its progression from DNA to functional proteins are Genomics, which is based on the study of DNA; Transcriptomics, which is based on the study of RNA expression; and Proteomics, which is based on the study of proteins expression.

A genome is the entire DNA that is present in an organism and contains all the genes. These genes code for all proteins that are required by the organism. The Human Genome Project has now identified all the genes present in human DNA. The DNA is transcribed into mRNA (messenger ribonucleic acid) that is then translated into proteins, which after posttranslational modifications form the functional end result. The genome is relatively static and mutations in the genes may not have any functional relevance. Genes can also code for more than one protein. The levels of mRNA and proteins are dynamic. The global

study of mRNA expression, called transcriptomics, is usually microarray based and used for gene expression profiling. This explores the relative levels of mRNA expression from genes that are known to be associated with a disease. However, this does not take into consideration any posttranslational modification of the proteins translated or protein degradation. The levels of mRNA and protein expression may not correlate at any one time point as the mRNA may not be translated into protein or the peak in protein expression may lag behind the peak in mRNA production.

In view of the above drawbacks, the global study of the proteome, called proteomics, may provide a more realistic picture of the functional aberrations within a cancer cell. Proteomics is a large-scale, global, high throughput, technique for the study of the proteome. The study of proteins and its interactions within a cancer cell using proteomic techniques is called oncoproteomics. Oncoproteomics can be used in understanding complex molecular changes that occur in a cancer cell and help us in identifying mechanisms of drug actions, drug resistance and toxicities. It can also be used to identify potential new targets for diagnosis and treatment. They may also be useful to identify biomarkers to individualise treatment strategies for patients.

5.4.1 **Antibody Microarray**

Antibody microarray is a technique used in oncoproteomics that can compare protein expression profiles in two different samples such as a sample treated with a drug compared to its control. An antibody microarray is a collection of antibodies spotted in pairs onto a nitrocellulose coated glass microscope slide. It is not however, a global technique as it is reliant on the number of antibodies that can be spotted on the slide, but this can be many hundreds. The two different protein samples are labelled with different coloured dyes, mixed together in equal quantities and incubated with the slide. Depending upon the

amount of protein present in each labelled sample, protein expression is detected upon competitive binding of the protein to its corresponding antibody on the slide. The amount of each dye present on individual antibody is directly proportional to the amount of protein present in each sample. The signal intensity can be measured using a fluorescent scanner and fold change of the differential expression calculated depending upon the signal intensity. A fold change of more than 1.8 is considered significant (Hodgkinson, Elfadl et al. 2011). The antibodies spotted onto the slide can relate to a specific biological function such as apoptosis or a cell signalling pathway such as EGFR pathway and hence can be targeted to analyse a specific outcome. There are various outcomes that can be measured such as defining signal transduction pathways, molecular classification of tumours, drug sensitivity and resistance, molecular mechanism of action of drugs, etc.

5.4.2 **Data mining**

Depending upon the number of antibodies spotted in antibody microarray experiments, the results of the differentially expressed proteins can run into hundreds, which needs to be analysed in a logical and scientifically meaningful manner depending upon their biological functions, interaction with each other and with other proteins within a specific signalling pathway. There are various commercially available software that can enable us to analyse the results, such as, Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com/), STRING (http://string-db.org/), GeneGo MetaCore (http://www.genego.com/metacore.php), Ariadne Pathway Studio (http://www.ariadnegenomics.com/products/pathway-studio/), etc. Among these, IPA is the most widely used software (Muller, Schrotter et al. 2011).

Ingenuity Pathway Analysis is web-based database launched in 2003. It maintains a database of genes and proteins along with their biological and chemical relationships,

interactions, pathways, drug targets and biomarkers. This database is called Ingenuity pathways knowledge base (IPKB) and is manually curated and regularly updated based on published data in scientific literature. The genes of the differentially expressed proteins identified by antibody microarray analysis can be analysed depending upon its interaction and signalling pathways. This allows for better understanding of the biological functions, protein interaction and cell signalling pathways of the differentially expressed proteins identified in high throughput proteomic experiments.

5.5 **Identification of individual proteins**

Various antibody based techniques can be used to identify specific proteins of interest in cells as well as tissues. Identification of individual proteins can help validate proteins found using high throughput proteomic techniques as well as ascertain the expression or absence of certain proteins in cell lines or tissue samples. Some of the antibody based techniques that are widely used are Western blot, ELISA, Flow cytometry and Immunohistochemistry.

5.5.1 **Western blot**

Western blot can be used to identify proteins which are separated based on their molecular weight. The proteins within a sample are separated by electrophoresis based on their molecular weight and then transferred onto a nitrocellulose membrane (**Figure 5.4**). The nitrocellulose membrane has a high affinity for protein binding and hence the free protein binding sites need to be blocked using non-fat milk or Bovine serum albumin. An antibody specific to the protein in question can now be added which would bind to the protein if present. This antibody can be tagged with a label or a secondary antibody can be added which can be detected using chemiluminescence or fluorescence techniques. To ensure equal loading of different proteins in the same membrane, the membrane can be probed

using housekeeping proteins such as α tubulin or GAPDH which should demonstrate constant level of expression and can act as loading controls. Western blotting is generally used for qualitative purposes, but the expressed proteins can then be quantified using a densitometer. This will enable identification of proteins that are differentially expressed in different samples such as drug treated *versus* control. Quantification of proteins using a densitometer may not be accurate due to various pitfalls in the procedure and at best can be considered as a semi-quantitative (Gassmann, Grenacher et al. 2009). Digitalisation of the western blot film can alter the signal intensity depending upon the quality and illumination of the scanner. Other steps such as colour of the film, background staining and overlapping bands can all influence the accuracy of the protein quantification. The accuracy of protein quantification can be improved by using appropriate external and internal controls and with the creation of standard curves, which can be used to calibrate protein expression levels.

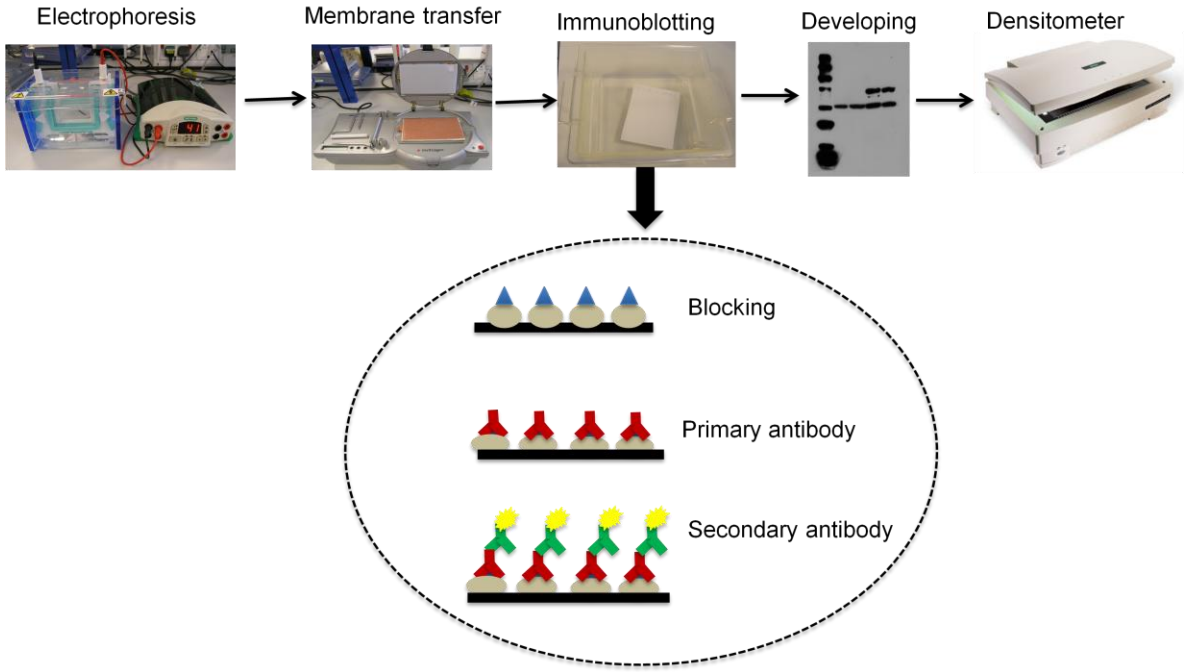

Figure 5.4 Schematic diagram of a western blot procedure. Proteins lysate are loaded onto a gel and separated by electrophoresis. The proteins are transferred from the gel to a nitrocellulose membrane. The membrane is blocked by using non-fat milk and then incubated with primary and then subsequently secondary antibody which is tagged and can be detected by chemiluminescence technique. The film can then be scanned and analysed using a densitometer.

5.5.2 **Enzyme Linked Immunosorbent Assay (ELISA)**

In ELISA the specific antibody is immobilised onto a microtitre plate. The protein lysate is added onto the plate and the specific protein of interest if present binds to the antibody. A secondary antibody coupled with an enzyme can then be added to bind with the fixed antibody. A substrate specific to the enzyme is added which then changes colour and can be read using a plate reader thereby enabling the identification and quantification of the

protein of interest. The intensity of the colour is directly proportional to the amount of protein present.

5.5.3 **Flow cytometry**

Flow cytometry measures the optical characteristics of a cell as well as the fluorescent characteristics of the fluorescent dyes (fluorochrome) attached to the cell. Fluorochromes (eg: R-phycoerythrin) are dyes that can be tagged onto a specific antibody against a protein of interest. Flow cytometers are made up of a fluidics chamber, light source and the optical and fluorescence detection mirrors (**Figure 5.5**). The cell population once injected into the flow cytometer is arranged into a single file inside a sheath due to the linear flow of fluid surrounding the sheath. This enables a single cell to pass through an interrogation point where the light source (laser) then intersects the cell. The cells then scatter the light depending upon their physical properties such as the size and the granular content. The optical mirrors are placed at various positions to absorb the light scatter. One of the mirrors is placed in the line of the light source which measures the forward scatter (FSC) based on the size of the cell and one perpendicular to the light source which measures the side scatter (SSC) based on the granularity of the cell. The fluorescent mirrors detect the fluorescence of the fluorochrome attached to the cell.

If the presence of a particular protein on the cell surface or inside a cell needs to be identified, then an antibody specific to the protein of interest, conjugated with a fluorochrome, is incubated with the cells forming an antibody-protein complex. When this cell is passed through the interrogation point the fluorochrome will be excited upon contact with the light source of a particular wavelength due to the light being absorbed by the fluorochrome. Some of the energy of the excited fluorochrome is released as heat due to the internal conformational change of the fluorochrome. When the fluorochrome returns to its

normal resting stage the rest of the energy is released as fluorescence. The wavelength of the fluorescence released is longer compared to the wavelength of the lights used to excite the fluorochrome due to the loss of some of the energy as heat. The wavelength of the fluorescence of the dye emitted can be measured using a variety of fluorescent mirrors and filters which allow light of particular wavelengths to pass through. When light comes in contact with the fluorescent detectors a small voltage is generated which can be log amplified and displayed on a histogram. The data can be quantified depending upon the number of cells that are positive for the protein of interest.

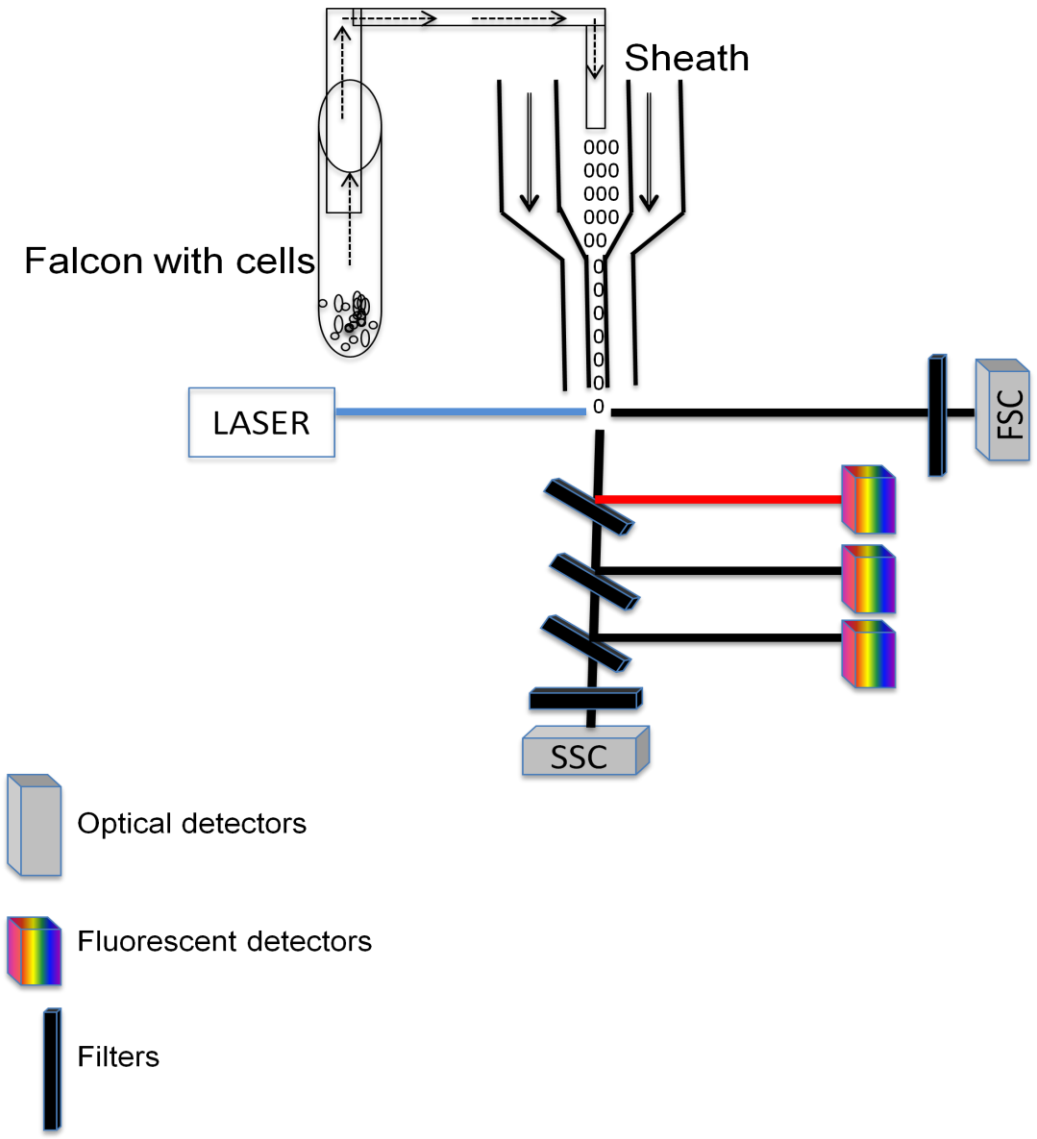

Figure 5.5 Components of a flow cytometer. The cell suspension is injected into the fluidics system containing the sheath with a laminar flow which arranges the cells in a single file. The cell is intersected by a light source from a laser. The light source is scattered by the cell, passed through the filters and then detected by optical and fluorescent detectors. One of the mirrors is placed in the line of the light source which measures the forward scatter (FSC) and one perpendicular to the light source which measures the side scatter (SSC).

5.5.4 **Immunohistochemistry**

Immunohistochemistry can be used to identify the presence or absence of proteins in a formalin fixed paraffin embedded clinical tissue samples using antibodies against the protein of interest (**Figure 5.6**). The tissue samples are mounted onto a glass slide. Formalin cross reacts with proteins present in the tissues and alters the chemical characteristics of the proteins. Heating the slides at high temperature (microwave or boiling) can hydrolyse the cross link bonds formed between formalin and the proteins. This process is call antigen retrieval. The non specific binding sites on the proteins are blocked using casein. The slide is then incubated with the primary antibody specific to the protein of interest. The antibody (if optimised) would bind to the protein if present. The slide is then incubated with a biotin conjugated secondary antibody against the primary antibody. The secondary antibody can be detected using enzyme streptavidin conjugate. Horseradish peroxidase (HRP) is a common enzyme used with streptavidin. HRP in the presence of Hydrogen peroxide oxidizes water soluable chromogens such as DAB (3,30-diaminobenzidine tetrahydrochloride) into coloured insoluble precipitates which can be seen visually under a light microscope. Significant inter observer variability exists while interpreting the slides. This may be as a result of different training and experience between the observers, evaluating different regions of a slide, presence of tumour heterogeneity, different scoring methods, use of different microscopes, etc. This user variability can be minimised by standardizing the scoring methodology, appropriate training of the observers and using multiple observers to analyse the slide. Computerised scanning and assessment of the slide may be a more objective and reproducible technique of minimising inter-observer variability.

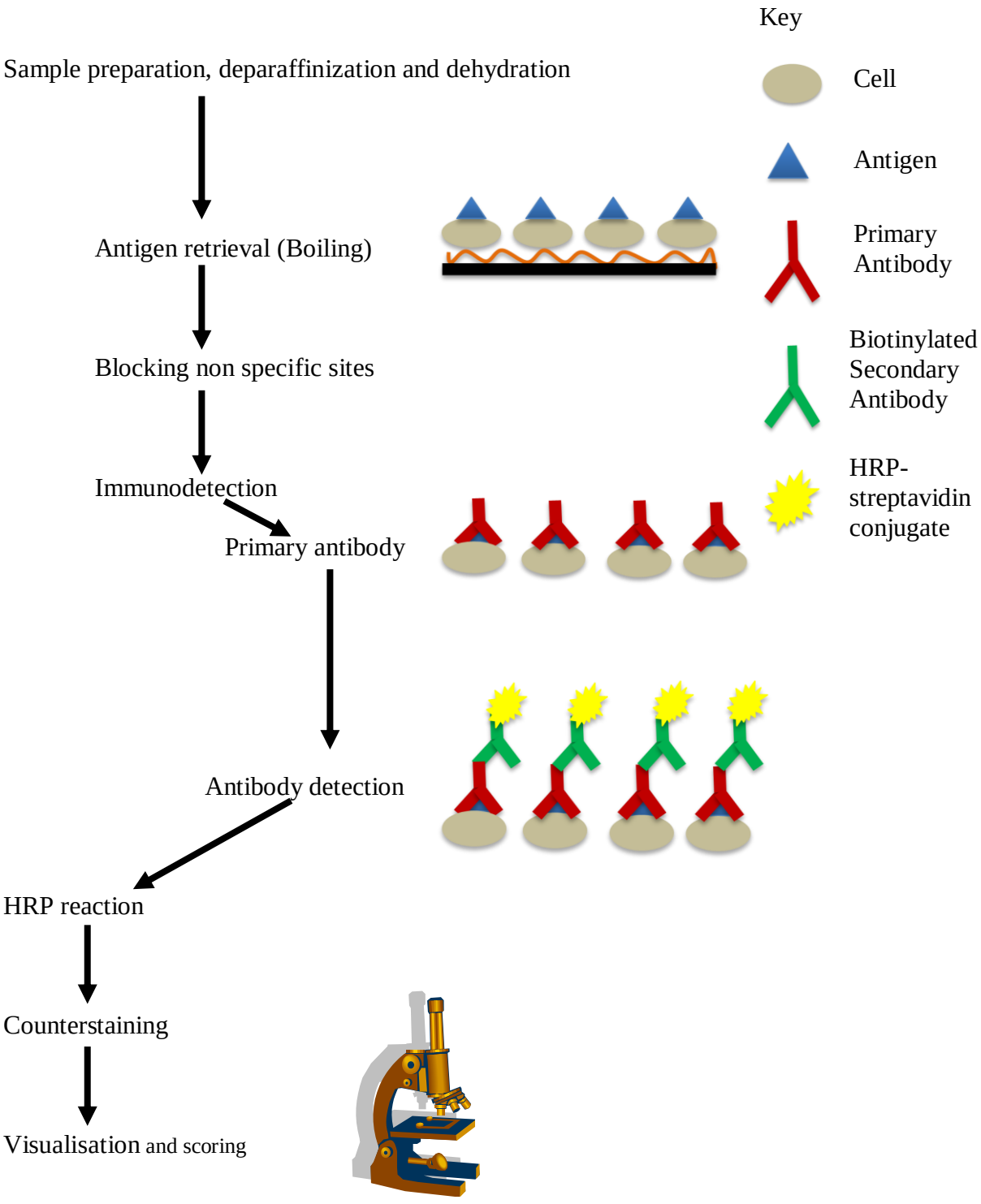

Figure 5.6 Schematic diagram of Immunohistochemistry protocol.

5.6 **Aims**

Despite recent advances in chemotherapy, advanced malignant pleural mesothelioma (MPM) is associated with poor prognosis. With the administration of Cisplatin/Pemetrexed, the median survival of MPM is still only 12 months and currently there is no widely approved salvage regimen after failure of first line treatment. There is a need for development of newer targeted treatment options in MPM. Epidermal Growth Factor Receptor (EGFR), Mammalian target of Rapamycin (MTOR) and Cyclooxygenase-2 (COX-2) are frequently up regulated in MPM and the potential role of Lipoxygenase (LOX) is unclear. The molecular interaction of EGFR, MTOR, COX-2 and LOX are detailed in **Figure 5.7**.

Currently there are no published studies assessing the effect of anti-EGFR monoclonal antibodies such as Cetuximab in MPM. EGFR TKIs such as Gefitinib have previously been shown to be cytotoxic to EGF stimulated MPM cell lines, but early Phase clinical trials using Gefitinib and Erlotinib have not shown any benefit for patients (Janne, Taffaro et al. 2002; Govindan, Kratzke et al. 2005; Garland, Rankin et al. 2007). The presence of activating EGFR mutations has a significant positive impact on the response of NSCLC to EGFR TKIs. However, the presence of these mutations has not been demonstrated in MPM. Similarly the presence of KRAS and BRAF mutations, which induce resistance to EGFR monoclonal antibody therapy have so far not been seen in MPM and needs further evaluation. There is a modest anti-neoplastic effect seen with MTORC1 inhibition using Rapamycin in MPM cell lines, but there are no published studies evaluating the effect of combined MTORC1 and MTORC2 inhibition in MPM (Altomare, You et al. 2005). Also the effect of combining MTOR inhibition with EGFR inhibition in MPM is currently not known. The COX-2 inhibitor DuP-697 has demonstrated cytotoxic effect in COX-2

expressing MPM cell lines and also potentiate the inhibitory effect of Pemetrexed in these cell lines (O'Kane, Eagle et al. 2010). However, the mechanism of action of DuP-697 is currently not well defined. The mechanism of action of DuP-697 needs to be evaluated to further enhance our understanding of how COX-2 inhibitors work in MPM and also identify new targets which can be therapeutically manipulated. COX-2 inhibitors such as Celecoxib and Rofecoxib have been shown to have some anti-tumour effect in MPM and Rofecoxib has also shown to have some synergy with Gefitinib in MPM (Catalano, Graciotti et al. 2004; Stoppoloni, Canino et al. 2010). The effect of COX-2 inhibition when combined with anti-EGFR monoclonal antibodies such as Cetuximab in MPM is currently not known. In MPM, various small studies have shown that PTEN protein is rarely absent, but a large immunohistochemical study of 341 MPM samples demonstrated that PTEN protein was lost in 62% and its loss was associated with poor survival (Opitz, Soltermann et al. 2008). This needs further independent validation as it may have significant impact in our understanding and choice of targeted treatment option in MPM.

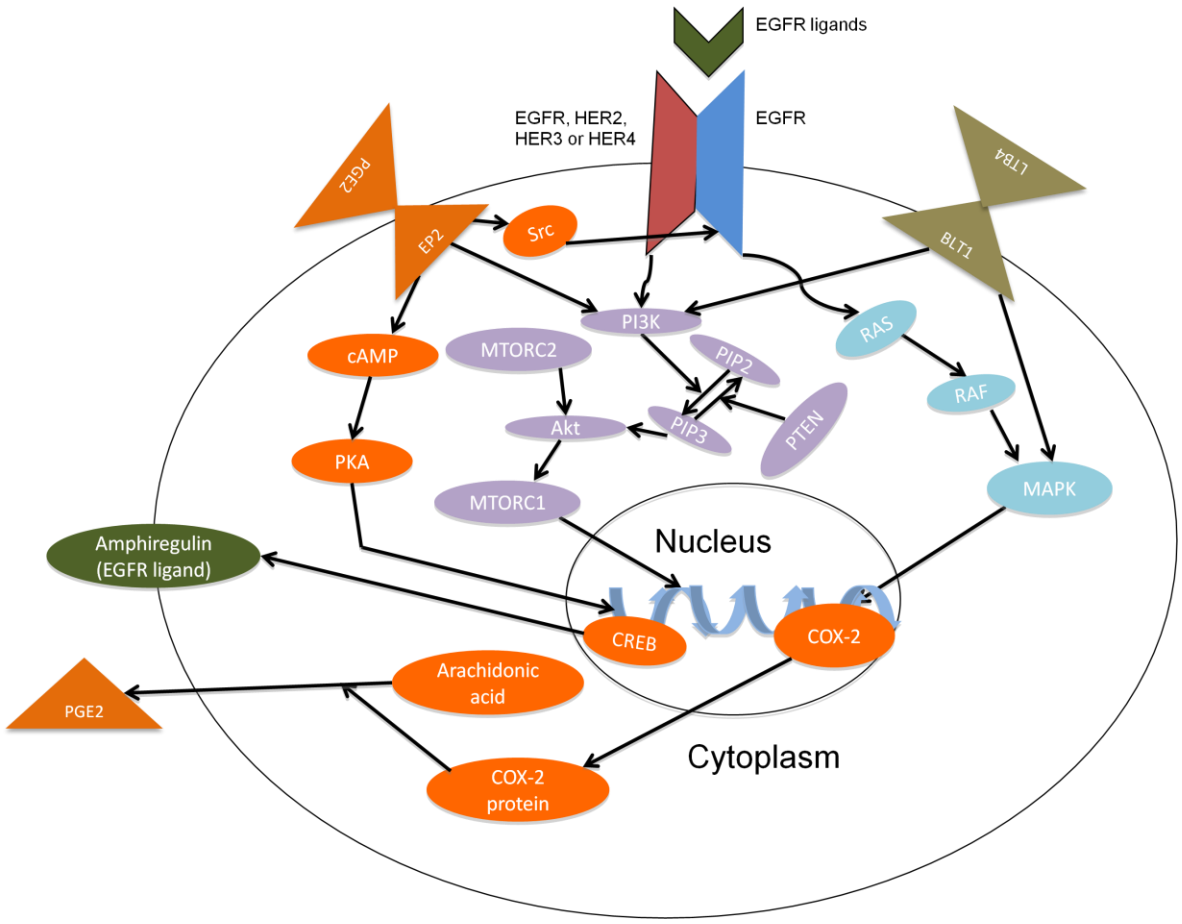

Figure 5.7 The EGFR, MTOR, COX-2 and LOX interactions. This figure demonstrates the interactions between EGFR, MTOR, COX-2/PGE2 and LOX/LTB4 pathways. Activation of EGFR by EGF ligands, including Amphiregulin, results in the activation of PI3K/AKT/MTOR pathway and the RAS/RAF/MAPK pathway. MAPK results in increased transcription of COX-2 gene thereby increasing the production of COX-2 protein which acts on Arachidonic acid to form PGE2. PGE2 activated the EGFR pathway by increasing the production of Amphiregulin via its action on cAMP and PKA, by directly activating PI3K and by activating the EGFR TK domain via Src. This results in a positive feedback loop between COX-2 and EGFR signalling pathways. 5LOX results in the increased production of LTB4 which acts on BLT receptors and activates PI3K and MTOR resulting in activation of PI3K/AKT/MTOR pathway and MAPK pathway.

The specific aims for this project are

- To identify the absence of PTEN protein expression and its prognostic relevance in MPM using immunohistochemistry.
- To demonstrate the expression and prognostic relevance of 5LOXand 12LOX in MPM using immunohistochemistry.
- To evaluate the expression of EGFR and PTEN proteins and the presence of EGFR, KRAS and BRAF mutation in MPM cell lines (NCI-H2052, NCI-H2452, MSTO-211H) and non small cell lung cancer cell lines (A549). A549 cell line was used as control as it has been extensively evaluated in the literature.
- To evaluate the effect of EGFR inhibition using Cetuximab and Gefitinib in MPM cell lines using the MTS assay. The effect of Cetuximab in MPM has not been previously evaluated.
- To evaluate the effect of MTORC1 and MTORC2 inhibition using Rapamycin (MTORC1 inhibitor) and Ku0063794 (MTORC1 and MTORC2 inhibitor) in MPM cell lines using the MTS assay.
- To evaluate the effect of COX-2 inhibition using Celecoxib in MPM cell lines using the MTS assay.
- To identify if the therapeutic effect of anti EGFR inhibition can be enhanced by combining Cetuximab and Gefitinib individually with Rapamycin, Ku0063794 and Celecoxib in MPM cell lines using the MTS assay.
- To study the mechanism of action of COX-2 inhibitor DuP-697 using antibody microarray, IPA, and western blot. This will also be a pilot study to see if antibody microarray can be used to identify mechanism of action of drugs.

Chapter 6. Materials and Methods

6.1 **Materials**

6.1.1 **Cell lines**

The mesothelioma cell lines MSTO-211H (Biphasic, ATCC Number CRL-2081), NCI-H2052 (epithelial, ATCC Number CRL-5915) and NCI-H2452 (epithelial, ATCC Number CRL-5946) were obtained from the American Type Culture Collection (ATCC). The lung cancer cell line A549 (catalogue number 86012804) was used as a control was obtained from the European Collection of Cell Cultures (ECACC). All cell lines were maintained in RPMI 1640 medium (#31870, Invitrogen). RPMI 1640 medium (500 mls) was supplemented with 50 mls of heat inactivated fetal bovine serum (#10106, Invitrogen), 5 mls of 200 mM L-glutamine (#25303, Invitrogen), 5 mls of 100 U/ml penicillin (#15140, Invitrogen) and 5 mls fungizone (#15290, Invitrogen).

6.1.2 **MTS Reagents**

MTS reagent (The CellTiter 96® AQ_{ueous} One Solution Reagent) was obtained from Promega (G3582). The reagent contains a 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 (Section 5.2.2.3) .

6.1.3 **Drugs**

Dimethyl Sulfoxide (DMSO; #D2650, Sigma Aldrich) was used as a drug carrier.

6.1.3.1 **Cetuximab**

Cetuximab was obtained as a Material transfer arrangement from Merck KGaA, Germany (see appendix D).

6.1.3.2 **Mechanism of action**

Cetuximab is a recombinant, chimeric monoclonal antibody directed against the EGFR. It is manufactured by ImClone and Bristol-Myers Squibb under the brand name Erbitux®. Cetuximab binds to the extracellular domain of the EGFR with an affinity approximately 5-10 times that of endogenous ligands, thereby preventing activation, dimerisation and internalization of the receptor. This may result in inhibition of the EGFR signalling and subsequently reduce the EGFR dependent growth of the tumour cells (**Figure 2.3**) Cetuximab also mediates an antibody dependent cell-mediated cytotoxicity towards EGFR-expressing tumour cells (Kurai, Chikumi et al. 2007).

Cetuximab has a molecular mass of 145781.6 g/mol (**Figure 6.1**).

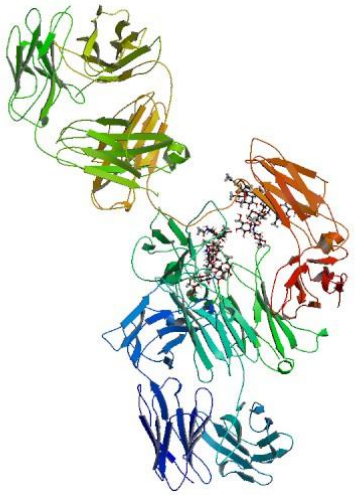

Figure 6.1 3-Dimentional structure of Cetuximab (Image from www.drugbank.ca)

6.1.3.3 **Pharmacokinetics**

Cetuximab when administered intravenously at an initial dose of 400 mg/m2 body surface area had a mean volume of distribution of 2.9 l/m^2 (range; 1.5 to 6.2 l/m2). Cetuximab has a long elimination half life of 70 to 100 hours. The mean plasma Cmax was 243 µg /ml

(Tan, Moore et al. 2006). The mean steady state peak concentration at FDA approved dosing ranges from 168 to 235 μg/mL (Mukohara, Engelman et al. 2005).

6.1.3.4 **Therapeutic indications**

Cetuximab currently has FDA and EMEA approval for the following indications:

- Patients with EGFR expressing, KRAS wild type metastatic colorectal cancer.
- Patients with squamous cell cancer of head and neck.

6.1.3.5 **Gefitinib**

Gefitinib was purchased from Tocris Bioscience (catalogue number 3000).

6.1.3.6 **Mechanism of action**

Gefitinib is a small molecule tyrosine kinase inhibitor directed against the tyrosine kinase domain of the EGFR (**Figure 2.3**). It is manufactured by AstraZeneca under the trade name Iressa®. Gefitinib inhibits the EGFR tyrosine kinase domain by binding to the ATP binding site and inhibiting EGFR autophosphorylation, thereby subsequently inhibiting the EGFR downstream signalling and cellular growth (Kuramitsu, Miyamoto et al. 2009).

6.1.3.6.1 Molecular structure

Gefitinib has a molecular mass of 446.9 g/mol and its chemical formula is $C_{22}H_{24}ClFN_4O_3$ (**Figure 6.2**).

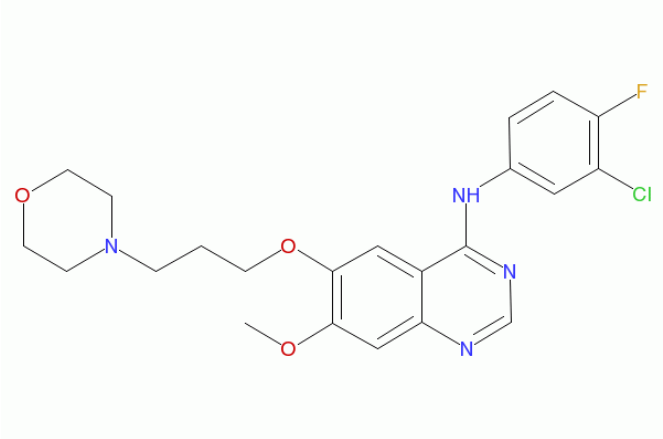

Figure 6.2 Chemical Structure of Gefitinib. (Image from www.tocris.com)

6.1.3.6.2 Pharmacokinetics

Gefitinib is administered orally and peak plasma concentration occurs 3 to 7 hours after administration. It has a mean steady state volume of distribution of 1400 litres and 90% is plasma protein bound. Gefitinib is metabolised extensively in the liver predominantly by cytochrome P450 (CYP) 3A4 and excreted mainly as metabolites via the faeces. Concomitant administration of CYP 3A4 inhibitors such as Itraconazole increased the Gefitinib plasma levels and administration of CYP 3A4 inducers such as Rifampicin reduces the Gefitinib plasma levels (Cohen, Williams et al. 2004). The mean steady state peak concentration at FDA approved dosing was 615 ng/ml (Herbst, Maddox et al. 2002).

6.1.3.6.3 Therapeutic indications

It is approved by FDA and EMEA for use in metastatic Non small cell lung cancer with activating mutations of EGFR-TK domain.

6.1.3.7 **Rapamycin**

Rapamycin was purchased from Tocris Bioscience (catalogue number 1292).

6.1.3.7.1 Mechanism of action

Rapamycin (Wyeth, Madison, NJ), manufactured by Wyeth under the trade name Rapamune®, acts by forming a complex with cytosolic protein FK-binding protein 12 (FKBP12) and binding to MTORC1 thereby resulting in inhibition of its downstream signalling pathway (Huang, Bjornsti et al. 2003). It was initially approved by FDA for use as an immunosuppressant in organ transplant patients for prophylaxis of organ rejection.

6.1.3.7.2 Molecular structure

Rapamycin has a molecular mass of 914.18 g/mol and its chemical formula is $C_{51}H_{79}NO_{13}$.

(**Figure 6.3**).

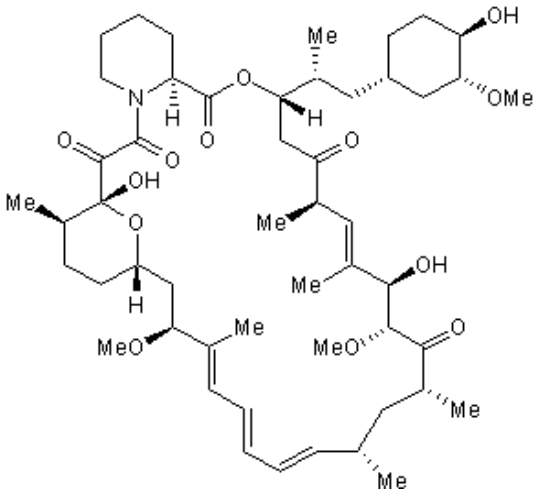

Figure 6.3 Chemical Structure of Rapamycin. (Image from www.tocris.com)

6.1.3.7.3 Pharmacokinetics

Rapamycin is administered orally and metabolised in the liver by CYP 3A4 enzyme. At a maximum tolerated dose of 6mg per day in patients with solid tumours, the plasma Cmax was 27.6 ng/ml and the steady state plasma concentration was 12.7 ng/ml (Jimeno, Rudek et al. 2008).

6.1.3.7.4 Therapeutic Indications

Rapamycin is indicated for the prophylaxis of organ rejection in patients receiving renal transplants. Everolimus (Novartis) is a derivative of Rapamycin, and is approved by FDA and EMEA for use in advanced renal cell cancer and progressive neuroendocrine tumours of the pancreas.

6.1.3.8 **Ku0063794**

Ku0063794 was purchased from Tocris Bioscience (catalogue number 3725).

6.1.3.8.1 Mechanism of action

Ku0063794, synthesised by AstraZeneca, is a MTOR kinase inhibitor and inhibits both MTORC1 and MTORC2. It also suppresses cell growth and induces G1-cell cycle arrest. (Garcia-Martinez, Moran et al. 2009).

6.1.3.8.2 Molecular structure

Ku0063794 has a molecular mass of 465.5 g/mol and its chemical formula is C25H31N5O4 (**Figure 6.4**).

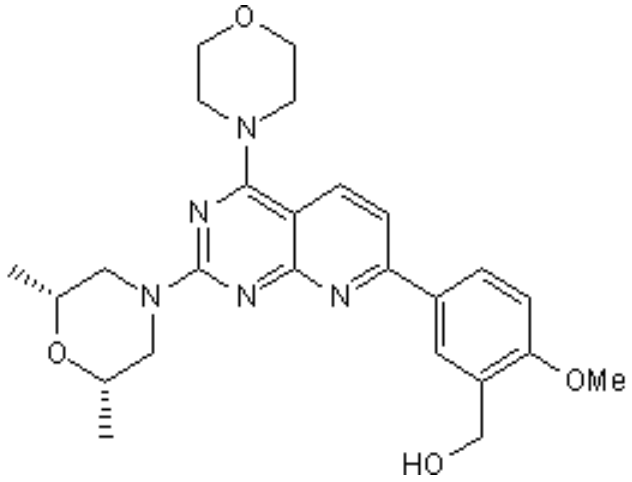

Figure 6.4 Chemical Structure of Ku0063794. (Image from www.tocris.com)

6.1.3.8.3 Pharmacodynamics

Ku0063794 inhibits both MTORC1 and MTORC2 in Human embryonic kidney cells (HEK-293) with an IC50 of approximately 10 nM (Garcia-Martinez, Moran et al. 2009).

6.1.3.8.4 Therapeutic indications

Ku0063794 is a prototype of the MTOR inhibitor AZD8055 currently in Phase I trial in advanced solid tumours (Shimizu, Dobashi et al. 2009).

6.1.3.9 **Celecoxib**

Celecoxib was obtained as a Material transfer arrangement from Pfizer, USA (see appendix D).

6.1.3.9.1 Mechanism of action

Celecoxib, marketed by Pfizer under the trade name Celebrex® and Onsenal®, is a selective COX-2 inhibitor, resulting in inhibition of prostaglandin synthesis. Various studies have also shown that Celecoxib may act as an antineoplastic agent by induction of apoptosis which may be independent of or dependent on COX-2 inhibition (Hsu, Ching et

al. 2000; Han, Leng et al. 2004; Kern, Haugg et al. 2006; Lou, Fatima et al. 2006; Schonthal 2007).

6.1.3.9.2 Molecular structure

Celecoxib has a molecular mass of 381.37 g/mol and its chemical formula is $C_{17}H_{14}F_3N_3O_2S$ (**Figure 6.5**).

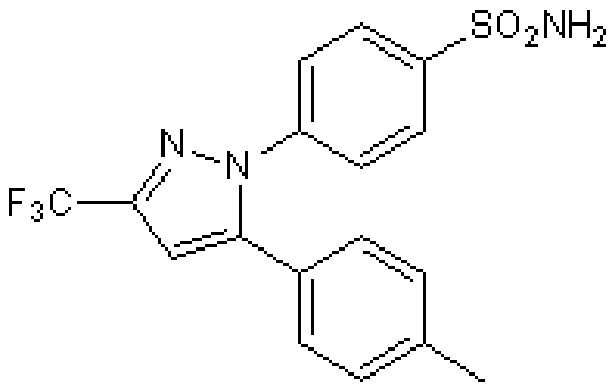

Figure 6.5 Chemical Structure of Celecoxib. (Image from www.tocris.com)

6.1.3.9.3 Pharmacokinetics

Celecoxib is administered orally and has a moderate rate of absorption. It is mainly albumin bound and is eliminated via excretion in urine and faeces. With an oral dose of 400 mg per day, the plasma steady state concentration was found to range from 740 ng/ml to 1000 ng/ml (Grossman, Olson et al. 2008; Sauter, Qin et al. 2008).

6.1.3.9.4 Therapeutic indications

Celecoxib is used as an anti-inflammatory agent. It was initially approved by FDA and EMEA for use in patients with Familial adenomatous polyposis to prevent colon cancers but its approval has been withdrawn due to lack of long term safety and efficacy data.

6.1.3.10 **DuP-697**

DuP-697 was purchased from Tocris Bioscience (catalogue number 1430).

6.1.3.10.1 Mechanism of action

DuP-697 is a selective COX-2 inhibitor (Gierse, Hauser et al. 1995). Studies have also shown that its action as an antiproliferative agent may be independent of COX-2 inhibition and may result in induction of apoptosis via activation of the caspase family (Churchman, Baydoun et al. 2007; Peng, Zhang et al. 2008) .

6.1.3.10.2 Molecular structure

DuP-697 has a molecular mass of 411.3 g/mol and its chemical formula is $C_{17}H_{12}BrFO_2S_2$ (**Figure 6.6**).

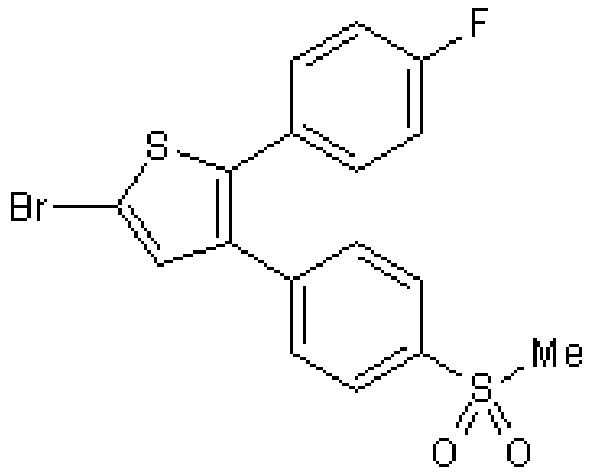

Figure 6.6 Chemical Structure of DuP-697. (Image from www.tocris.com)

6.1.3.10.3 Pharmacodynamics

DuP-697 inhibits COX-2 with an IC50 value of 0.01 µM when compared to COX1 at 0.8µM in cultured Sf21 insect cells (Gierse, Hauser et al. 1995).

6.1.3.10.4 Therapeutic indications

Pre clinical data has shown DuP-697 to have antiproliferative effects in mesothelioma and chronic myeloid leukaemia cells (Peng, Zhang et al. 2008; O'Kane, Eagle et al. 2010).

6.1.4 **Antibodies**

Antibodies for Western blot, Flow cytometry and Immunohistochemistry used are detailed in **Table 6.1**.

Table 6.1 Antibodies for Western blot (WB), Flow cytometry (FC) and Immunohistochemistry (IHC). (RT= Room temperature)

Antibody	Company	Catalogue no	Mol. Wt. (kD)	Host species	Blocking agent	Optimised dilution	Application
Anti rabbit secondary	Santa Cruz	SC2030		goat	milk	1:1000; 1 hour; RT	WB
Anti mouse secondary	Santa Cruz	SC2031		goat	milk	1:1000; 1 hour; RT	WB
Anti alpha tubulin, loading control	Abcam	ab 7291	50	mouse	milk	1:2500; 2 hours; RT	WB
Anti beta actin, loading control	Abcam	ab 8227	40	rabbit	milk	1:2500; 2 hours; RT	WB
GAPDH	Abcam	ab9485	37	rabbit	milk	1:2500; 2 hours; RT	WB
Bid	Abcam	ab32060	22	rabbit	milk	1:300; 16 hours	WB
Bcl xL	Sigma	B9429	27	mouse	milk	1:5000; 2 hours; RT	WB
PTEN	Abcam	ab32199	47	rabbit	milk	1:400; 2 hours; RT	WB
p70S6K	Abcam	ab32359	70	Rabbit	Milk	1:1000; 16 hours; 4^0C	WB

EGFR	Abcam	ab2430	170	Rabbit	Milk	unable to optimise	WB
EGFR	BD Pharminogen	555997		Mouse		Incubate in dark for 30 mins.	FC
PTEN	Abcam	ab32199		rabbit	Normal horse serum	1:50; 2 hours; RT	IHC
5LOX	Abcam	ab39347		rabbit	casein	1:50; 2 hours; RT	IHC
12LOX	Abcam	ab23678		rabbit	casein	1:100; 2 hours; RT	IHC

6.2 **Methods**

6.2.1 **Cell culture**

Cells were cultured in a sterile environment in a class II cell culture hood. All equipment including the cell culture hood, incubator and water bath were cleaned with Virkon and 70% alcohol at regular intervals and the cell culture hood was cleaned prior to every experiment. All other equipment used was sprayed with 70% alcohol prior to placing it into the cell culture hood. Sterile techniques were maintained throughout the procedure to minimise the risk of infection. All cell lines were tested negative for mycoplasma infection.

6.2.1.1 **Thawing of cells**

Cells, which were stored at -80 ^{0}C in DMSO, were placed in a water bath to be thawed quickly. When thawed, the cells were transferred into a screw cap universal tube and 9 mls of media per ml of cells (1:10 dilution) was added very slowly (1-2 mins.) to dilute the DMSO. The cell suspension was then centrifuged (Sigma 2-5 centrifuge) at 400 x *g* over 3 minutes to pellet the cells and remove DMSO, which is toxic to cells. The supernatant was

discarded and the pellet resuspended in fresh prewarmed to 37ºC RPMI media (Section 6.1.1) and incubated into a T25 (25 cm^2) or T75 (75 cm^2) flask depending upon the number of cells. The cells were then placed in a humidified incubator at 37ºC with 5% CO_2.

6.2.1.2 **Culturing of cells**

Cells were cultured and passaged 3 times a week in T25 or T75 flasks. The flasks were first assessed under an inverted microscope for confluency, adherence and infections. In the cell culture hood the old media was removed and 3mls of pre-warmed Trypsin (TrypLE Select; #12563, Invitrogen) was added to dislodge the adherent cells. Cells were kept in the incubator for 3 minutes and tapped gently to dislodge the cells. 7mls of media was added to neutralise the action of Trypsin. The cell suspension was then transferred into a screw cap universal tube and centrifuged at 400 x *g* for 3 minutes. The supernatant was discarded and cell pellet resuspended in media. The cell suspension was divided depending upon confluency and transferred into new flasks.

6.2.1.3 **Freezing cells**

Cells were frozen using freezing media made up of RPMI media containing 10% DMSO. A cell pellet was prepared as per section 6.2.1.2 and 1ml of freezing media added to it slowly. The cell suspension was then transferred to a cryovial and stored at -80ºC.

6.2.2 **Cell counting**

Cell counting was done using a Neubauer haemocytometer (**Figure 6.7**). This is a microscopic slide with a cell counting chamber of 0.1 mm deep cut into it. This chamber has a grid etched into it, which is made up of squares like a graph paper. The main grid is 1mm X 1mm, medium square is 0.2 mm X 0.2 mm (made of 4 X 4 tiny square) and tiny

square is 0.05 mm X 0.05 mm. As the dimensions of the squares are known the volume of the square can be calculated. Each 1 mm X 1 mm square will hold a volume of 10^{-4}ml.

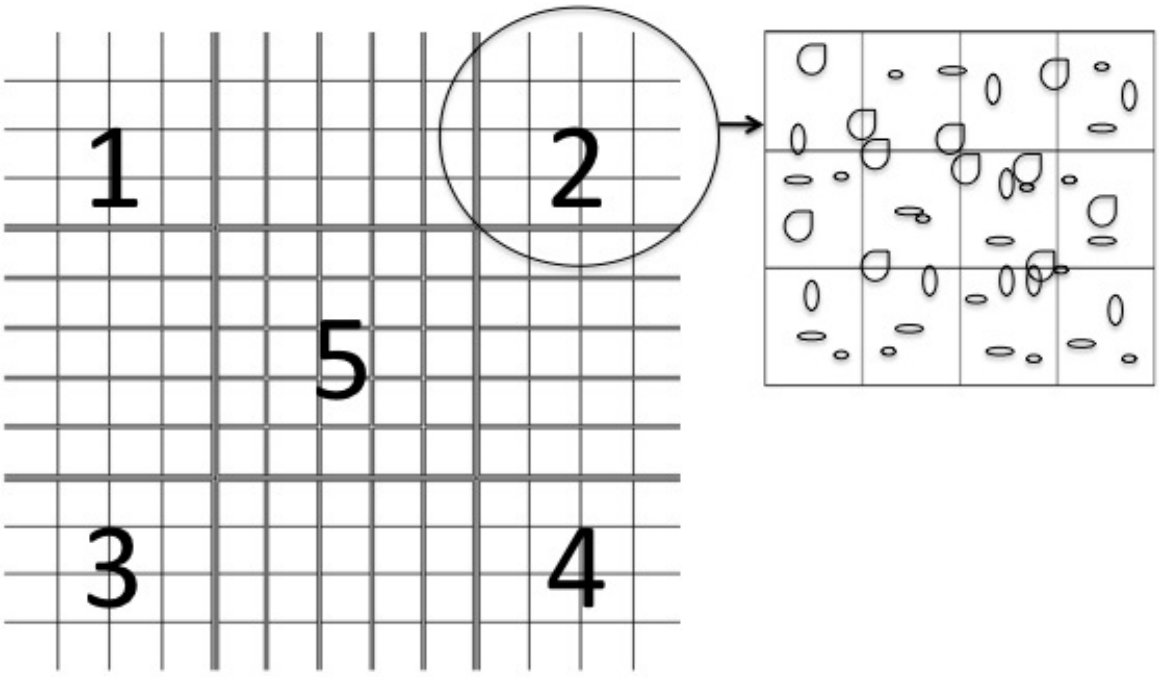

Figure 6.7 Cell counting using Neubauer haemocytometer. Squares 1,2,3,4 and 5 were counted with cell counter and an average of the 5 squares taken. Cells present on the top and left lines of the smaller squares were counted to maintain consistency. Square 2 is highlighted to show cells.

Cells were harvested, pelleted and diluted in 1-2 mls of media. 25 μl of media with suspended cells were taken and added to 25 μl of Trypan blue (0.4% w/v trypan blue in PBS solution) in a 0.5ml eppendorf tube. PBS solution was made up by mixing one tablet of PBS (Gibco™ cat no 18912-014, Invitrogen) in 500 mls of dH_20 and was sterilised by autoclaving at 120 ^{0}C. Adding the cells to trypan blue facilitated visualization and counting of the cells as Trypan blue stains non-viable cells (dead cells) blue. Twenty-five micro litres of the above suspension was taken and pipetted onto the V grove of the slide with cover slip placed on top. The suspension was sucked in by capillary action and filled up the

cell counting chamber. The slide was left for 5 minutes allowing the cells to sediment. Five large squares were counted and an average of the 5 squares taken. Cells overlaying the top and left boarder of the smaller squares were counted and consistency maintained throughout. Each 1mm square should have between 50-150 cells for a more accurate cell count. The average of 5 squares were taken and multiplied by 2 to account for dilution with trypan blue. The value X 10^4 gave the number of cells in 1ml.

Once the number of cell/ml was known, the amount of suspension needed to determine the number of cell required was calculated and made up to 100 μl with RPMI media for each well in a 96 well plate for MTS assay (Section 6.2.4.1). Depending on the number of wells, the amount of cell suspension and media required was calculated first and distributed to the wells accordingly.

6.2.3 **Seeding a 96 well plate with cells**

Seeding cells into a 96 well flat-bottomed plate requires a cell count with a Neubauer haemocytometer. Once the cell count per ml of suspension is known, the amount of suspension to be added into media per well can be calculated.

Example:

Cells counted in chamber 1, 2, 3, 4 and 5 were 61, 82, 46, 54 and 58 cells. The average of these was 60 cells, which was multiplied by 2 to account for dilution with trypan blue. This gave the cell count as 120 X 10^4 cell per ml or 1200 cells /μl. If 10,000 cells per well were required in 6 wells then the amount of cell suspension containing 10,000 cells was calculated to be 8.3 μl (10,000 / 1200 = 8.3μl). If the cells were to be seeded in 6 wells then calculations were done for 8 wells to account for wastage. Hence, for 8 wells 8.3 X 8 = 66.4 μl of cell suspension was required. One hundred micro litres of media was required per well hence for 8 wells we needed 800μl of media. We needed to add 66.4μl of cell

suspension, so the total amount of media required was 800 – 66.4 = 733.6μl. The required amount of media (733.6 μl) was mixed with 66.4μl of cell suspension to give 80,000 cells in 800μl of media. One hundred micro litres of this cell suspension was added to each well to give 10,000 cells / 100μl of media / well.

6.2.4 **Colorimetric Assays**

Colorimetric assays were first described by Mosmann, used in laboratories to measure cell viability and proliferation (Mosmann 1983). MTS assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays (Section 5.2.2.3). MTS reagent, in the presence of an electron coupling reagent phenazine methosulfate, is reduced by mitochondrial reductase enzymes present in metabolically active (viable) cells into formazan products. As only the viable cells can reduce the MTS reagent, cells that are dead will not form formazan products. The formazan products can be quantified by reading the absorbance spectrophotometrically at wavelength 492 nm using a microtitre plate reader.

6.2.4.1 **MTS Assay**

6.2.4.1.1 Protocol

MTS Assay's were performed in a 96 well flat-bottomed plate (**Figure 6.8**).

Day 1: One hundred micro litres of cell suspension (Section 6.2.3) was added in each well and for each drug concentration there were six replicates. One hundred micro litres of media was added in each well along the border of the plate. Distilled H_2O was added to the first well (labelled A1) on the top left hand corner of the plate. Two plates were prepared; Plate A for baseline reading on Day 2 and Plate B for dosing with drugs and reading on Day 5. Both plates were incubated overnight in 37ºC with 5% CO_2 in the incubator.

Day 2-4: MTS reagent (20 µl) (G3582; Promega) was added to the cells seeded on Plate A and incubated for 3 hours in the incubator. The absorbance of Plate A was then read on Day 2 spectrophotometrically at wavelength 492nm using a microtitre plate reader (Labsystems Multiskan MS, Thermo Electron Corporation). The cells seeded in Plate B were dosed with drugs as appropriate on day 2, day 3 and day 4. Media and distilled H_2O was changed in all wells daily. DMSO was used as a drug carrier for Gefitinib, Rapamycin, Ku0063794 and Celecoxib at a final concentration not more than 0.01% diluted in media. Stock solutions for Gefitinib (100mM), Rapamycin (500µM), Ku0063794 (1 mM) and Celecoxib (10mM) were prepared and stored at -20 ºC. All drugs were freshly diluted in media and stored at 4 ºC for not more than 3 days. Cetuximab was provided at a concentration of 2mg/ml, diluted in 10 mM Sodium Phosphate and 145 mM Sodium Chloride (pH 7.2). Cetuximab was stored at 4 ºC and was freshly prepared daily by diluting in media at various concentrations as required.

Day 5: MTS reagent (20 µl) was added into the wells to be read including blanks. The experiment was performed in the dark. The plate was then incubated for 3 hours in 37ºC with 5% CO_2 in the incubator. The plate was then read spectrophotometrically at wavelength 492 nm using a microtitre plate reader and absorbance values obtained.

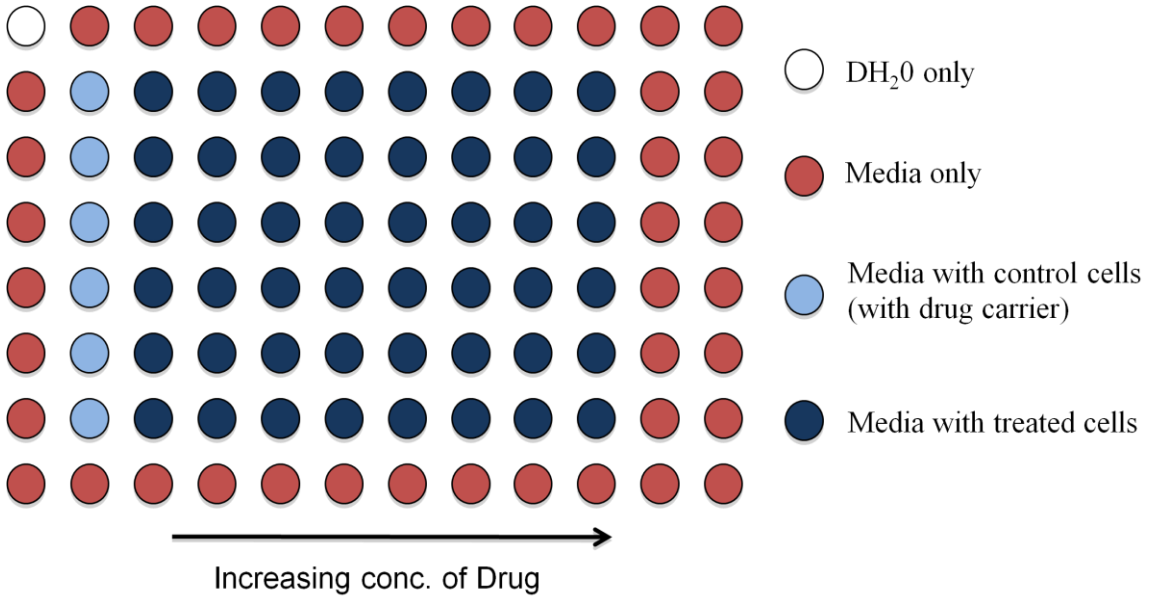

Figure 6.8 MTS assay. This figure demonstrates a 96 well plate showing the layout of media (blank), control and treated cells in six replicates. The drugs were added with increasing concentration from left to right. Media was added along the borders of the flask and distilled water in the top left well.

6.2.4.2 **Calculation of cell proliferation based on MTS reading**

The average values of absorbance from the triplicate wells with cells as well as blanks were calculated. The average of the blanks was subtracted from the average value of wells with cells, which was then taken as the true absorbance reading for those cells.

After determining the absorbance values from base line, for control cells and treated cells, the increase or decrease in the number of cells was established and expressed as a percentage of baseline growth.

a= baseline absorbance

b= control absorbance

c= treated absorbance

Absorbance values were calculated as an average (of 6 replicates) – average of blanks (3 replicates)

Growth of control cells (d) = [(b-a)/a] X 100%

Growth of treated cells (e) = [(c-a)/a] X 100%

Growth of treated cells as a percentage of control cells (f) = [(e-d)/d] X 100 + 100

Using the above formulae the growth of cells treated with various drugs were calculated and expressed as a percentage of baseline or control cells to ascertain the percentage of growth inhibition (see appendix A for an example calculation).

6.2.4.2.1 Statistical analysis

The mean of at least two independent experiments were calculated along with the standard deviation using Microsoft excel 2007. The average and the standard deviation of the percentage of growth inhibition were plotted onto a XY scatter graph. The growth inhibition values were on the Y-axis and the drug concentration on the X-axis. A trend line was added and the concentration at which 50% of the cells were dead was calculated from the equation on the chart.

For the purpose of drug combinations, the half maximal inhibitory concentration of a drug was calculated using GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, California). The drug concentration and the percentage of growth inhibition of all three independent experiments were uploaded onto the software. The drug concentrations were transformed to log scale. The control was set arbitrarily at 2 logs below the lowest drug concentration and reflected 100% normal proliferation. The dose response curve was calculated for each drug in individual cell line and was characterised by a 4 parametric logistic model estimated by a non linear regression analysis. For the purpose of drug

combination studies, the primary drug was used at various dilutions and the secondary drug was added at a fixed concentration (IC50 value) of the drug obtained in the particular cell line.

6.2.5 **Western blot**

6.2.5.1 **Protein extraction**

Proteins were extracted from cell lines using Western blot lysis buffer. Western blot lysis buffer contained:

- 4ml Distilled water (DH_2O)
- 1ml 0.5M TRIS:HCL pH6.8 (Sigma T1503)
- 0.8 ml Glycerol (Sigma G8773) to weigh down the proteins
- 1.6 ml 10% SDS (Sigma L3771) to denature and negatively charge the proteins
- 200 μl 0.05% Bromophenol blue (Sigma B5525) dye to visualise proteins

To one ml of the above solution the following were added:

- 50 μl of 2-Mercaptoethanol (#M-7522, Sigma Aldrich) to denature proteins
- 10 μl Phosphatase Inhibitor Cocktail 1 to maintain phosphorylation of proteins (#P2850, Sigma Aldrich)
- 10 μl Phosphatase Inhibitor Cocktail 2 to maintain phosphorylation of proteins (#P5726, Sigma Aldrich)
- 10 μl Protease Inhibitor to inhibit protein degradation (#80-6501-23, Amersham Biosciences)

Cells were pelleted as per section 6.2.1.2 and washed with cold PBS three times to ensure removal of media. The cells were suspended in 250 μl of the western blot lysis buffer and vortexed for 5 mins. The suspension was then kept overnight (16 hours) in a cold room at 4

^{o}C on an end-over-end rotator. The suspension was then centrifuged at 10,000 xg for 15 minutes at 4 ^{o}C in a microlitres centrifuge. The supernatant was taken and transferred into a pre chilled eppendorf and cell debris discarded. The eppendorf was then stored at -80^{o}C for future use.

6.2.5.2 **Protein quantification**

Proteins were quantified using the RC DC™ (Reducing agent Compatible, Detergent Compatible) protein quantification kit (#500-0119 to -0122, Bio-Rad) which is a colorimetric assay. RC DC™ kit was used due to its compatibility with the western blot lysis buffer. Standard Bovine serum albumin (BSA) protein dilutions were prepared at 0.2, 0.5, 0.75, 1 and 1.5 mg/ml using 2mg/ml BSA stock with dH2O in pre labelled eppendorfs. Protein extracts that needed to be quantified were diluted with dH2O ranging from 1:2 to 1:10 dilution to ensure that the concentrations fell within the assay range. To each eppendorf, 125 μl of RC Reagent I was added, which was then vortexed and incubated at room temperature for 1 minute. Then 125 μl of RC Reagent II was added, vortexed and centrifuged at 15,000 xg for 5 minutes. The supernatant was discarded by inverting the eppendorfs onto an absorbent paper to ensure complete removal of liquid and only the precipitated protein pellet remained in the tube. Reagent S (20 μl) was added to Working Reagent A (1 ml) and 127 μl of this was added to each eppendorf and vortexed for 5 minutes or till all precipitate was dissolved. Reagent B (1 ml) was added to each eppendorf and incubated at room temperature for 15 minutes. 200 μl of all BSA standards and the samples were transferred into a 96 well plate in triplicates. A1 well was kept as blank by adding dH2O. All air bubbles were removed and the 96 well plate was read spectrophotometrically using a microtitre plate reader at a wavelength of 690 nm. The

absorbance values of the known proteins are plotted onto a standard curve and the values of the unknown protein are calculated from the line equation of that curve.

6.2.5.3 **One-dimensional gel electrophoresis**

Proteins were diluted in Western blot lysis buffer (Section 6.2.5.1) made up to 25 µl to obtain a protein concentration of 20 µg.

To one ml of Western blot lysis buffer, 50 µl of 2-Mercaptoethanol (#M-7522, Sigma Aldrich) was added to denature proteins.

The protein extracts were denatured in a thermo cycler by heating it at 95ºC for 5 minutes and subsequently transferred straight to ice to prevent reversal of protein denaturation. The proteins were then vortexed and centrifuged at 12,000 xg for 30 seconds. One or two (depending upon the number of samples), 12 % Precise Protein Gel (#25222, Thermo Scientific) were placed in a tank with a litre of Tris-HEPES-SDS running buffer (#28368). Molecular weight marker (10 µl) (Precision Plus Protein WesternC Standard, #161-0376, Bio-rad) was loaded onto the first well of the gel and 20 µl of each sample was loaded into the wells and empty wells filled with 20 µl of sample buffer. The gel was then electrophoresed at constant voltage of 140V for 40 – 90 minutes (**Figure 5.4**).

6.2.5.4 **Transfer to Nitrocellulose Membrane**

6.2.5.4.1 Wet transfer

Transfer buffer and distilled water ice block was stored at -80ºC for 30 minutes.

Transfer buffer contained:

- 200ml Methanol
- 800ml DH_2O
- 3.3g Trizma Base (#93304, Fluka)

- 14.4g Glycine (#G8898, Sigma Aldrich)

The gel containing the separated proteins was sandwiched with pre-soaked (in transfer buffer) sponge, filter paper and nitrocellulose membrane (#LC2001, Invitrogen) inside a cassette in the following order; black side of cassette, sponge, filter paper, gel, membrane, filter paper, sponge, transparent side of cassette. The cassettes and ice pack were placed in a tank containing a stirrer and 1 litre of transfer buffer was added. It was ensured that the black side of the cassette was facing the black section of the tank. Electrophoretic transfer was then carried out at a constant current of 400 mA at 4ºC for 1 hour.

6.2.5.4.2 Dry transfer using iBlot

Dry transfer of proteins from the gel to the nitrocellulose membrane was done using the iBlot transfer system (Invitrogen). The iBlot disposable gel transfer stacks (#IB3010-01, Invitrogen) were used. The Bottom Stack was placed on the iBlot machine and then the gels were placed on the nitrocellulose membrane contained in the Bottom Stack. If two gels were run, then both of them could be placed side by side on the same membrane. Pre-soaked filter paper (soaked in distilled water) was placed above the gels and air bubbles removed using a roller. The Top Stack was then put above the filter paper the electrode sponge placed on the lid. The lid was then secured and the iBlot machine ran for 7 minutes as per standard manufacturer's recommendations.

6.2.5.5 **Blocking of binding sites on nitrocellulose membrane**

Once the transfer of proteins occurred from the gel to the nitrocellulose membrane, the membrane was then placed in a Nalgene box and blocked with 20 mls of blocking solution (either 5 % low-fat Marvel milk powder diluted in TBS Tween-20 or 5% Bovine serum albumin). TBS was made up of 121 g Trizma base, 170 g Sodium chloride (#S3014, Sigma Aldrich), made to 1 litre with dH_2O and pH adjusted to 7.6 with concentrated HCL. TBS

Tween-20 was made up of 250 mls of TBS, 4750 mls of dH_2O and 2.5 mls Tween-20 (#P5927, Sigma Aldrich). The box containing the membrane was placed on an orbital shaker for 1 hour at room temperature or overnight at 4ºC at a rocking speed of 28 rpm.

6.2.5.6 **Immunoblotting**

The blocking solution was removed from the box containing the membrane and the membrane was incubated with 10 mls of primary antibody diluted in blocking solution on the orbital shaker. The membrane was then washed three times for five minutes each on the orbital shaker with 10 mls of TBS Tween-20 to remove unbound primary antibody. The membrane was then incubated with the relevant HRP conjugated secondary antibody depending upon the source of the primary antibody on the orbital shaker (see **Table 6.1** for list of antibodies).

6.2.5.7 **Protein detection**

The membrane was incubated with 8 mls of West Pico Stable Peroxide Solution and 8 mls of Supersignal West Pico Luminol Enhancer Solution from the Supersignal West Pico Chemiluminescent Substrate Kit (#34078, Thermo Scientific) and gently shaken manually for 5 minutes in the dark. The Supersignal West Pico Chemiluminescent Substrate Kit acts as a substrate for horseradish peroxidase enzyme present on the secondary antibodies and emits chemiluminescent signal that can be captured on a film. The membrane was then placed between clean plastic sheets. A CL-XPosure Film (#34090, Thermo Scientific) was placed on the plastic sheet containing the membrane and enclosed in a cassette for appropriate duration depending upon the antibody. The film was then developed in 250 ml of GBX Developer (#P7042, Sigma Aldrich) for approximately 1 minute, followed by 30 seconds incubation in 250 ml 5 % Acetic Acid, then fixed in 250 ml GBX Fixer (#P7167)

for 30 seconds and washed under running water for 30 seconds. The developed films were then air dried and analysed.

6.2.5.8 **Loading controls**

Membranes were also probed with loading controls such as GAPDH (ab9485, Abcam), Alpha Tubulin (ab7291, Abcam) and Beta Actin (ab8227, Abcam) to assess the accuracy of equal protein loading in each well. This allowed for fair comparison between samples in each well as the loading controls would normalise loading of samples and give semi-quantitative assessment of differential protein expression between samples such as drug treated *versus* control using densitometer (Section 2.2.5.10).

6.2.5.9 **Densitometer**

The density of the band in the film is directly proportional to the amount of protein present in the sample. This density was quantified and compared between samples. The film was scanned using the GS800 Calibrated Densitometer (Bio-Rad) and analysed using Quantity One software (Bio-rad). For each sample the test protein band was normalised against the respective loading control band. The relative densities of the bands of the proteins in question were calculated. Once the values of the relative densities were obtained they were compared with each other to assess the fold change between them.

6.2.6 **Immunohistochemistry**

Immunohistochemistry is a laboratory technique used to identify specific antigens in tissues or cells based on the principle of specific binding of an antibody to the antigen and its visualization by a light microscope (**Figure 5.6**).

6.2.6.1 **Tissue samples**

A series of 93 archival MPM tissue samples was available and had been previously characterised (O'Kane, Cawkwell et al. 2005). These were formalin-fixed paraffin-embedded tissue blocks of patients diagnosed with histologically proven MPM within the Hull and East Yorkshire Hospitals NHS Trust, UK from 1995 to 2000. Among the 93 tissue samples, 48 were epithelioid, 27 were biphasic and 18 were of sarcomatoid histology. Survival data was available for all the 93 patients. Local Research Ethics committee approval was obtained (see appendix C).

6.2.6.2 **Sample preparation**

Paraffin-embedded 4 µm thick sections of MPM archival tissue samples were cut using a microtome onto Super Frost Plus slides (Menzel-Glaser, Germany) and dried overnight at 37 ^{o}C in an incubator. Slides were dewaxed by submerging the slides in pre warmed Histoclear II (National Diagnostics, Hull, UK) for 10 minutes followed by dipping the slides in Histoclear II for 10 seconds each in 2 more pots of Histoclear II. The slides were then rehydrated with 100% ethanol for 10 seconds each in three pots. Endogenous peroxidase activity of RBC's was blocked using 30% Hydrogen peroxide (H_2O_2) in methanol for 20 minutes.

6.2.6.3 **Antigen retrieval**

Antigen retrieval was achieved by boiling slides in 1500 ml dH_2O containing 15 mls Antigen Unmasking Solution (H-3300, Vector Laboratories Inc., CA, USA), in a pressure cooker for 3 minutes at 15 psi. The Antigen unmasking solution is based on a citric acid formula and is effective in unmasking antigens at high temperatures.

6.2.6.4 **Blocking non specific sites**

Slides there then transferred to a pot containing TBS and assembled onto a sequenza system (Shandon, UK). The nonspecific staining was blocked by using either 100 µl of 1 X casein (SP-5020, Vector Laboratories Inc., CA, USA) diluted in TBS for 10 minutes or 100 µl pre diluted blocking serum (normal horse serum from the Vectastain Quick Kit (#PK-7800, Vector Laboratories Inc., CA, USA) for 10 minutes. Slides were then washed with TBS for 5 minutes each twice.

6.2.6.5 **Immunodetection**

Depending upon the blocking step the antibody was diluted in either 0.2 X casein in TBS (if blocked in casein) or 1.5% blocking serum (normal horse serum from the Vectastain Quick Kit) to achieve optimum dilution. One hundred microlitres of the diluted antibody was added to each slide and incubated at room temperature for 2 hrs (see **Table 6.1** for list of antibodies). A negative control with 100 µl of 0.2 X casein or 1.5% blocking serum was included (depending upon the blocking step) with each batch of slides. Antibody detection was carried out using either the StreptABComplex / HRP Duet Mouse/Rabbit kit (#K0492, Dako Ltd, Ely, UK) if blocked in casein or the biotin-streptavidin preformed complex technology using the R.T.U. Vectastain Quick Kit (#PK-7800, Vector Laboratories Inc., CA, USA).

6.2.6.5.1 StreptABComplex / HRP Duet Mouse/Rabbit kit

The slides were incubated with Reagent C (100 µl each slide) from the StreptABComplex / HRP Duet Mouse/Rabbit kit containing the biotinylated goat anti mouse/rabbit secondary antibody, diluted in TBS (1:100) for 30 minutes. The slides were washed with TBS for 5 minutes and removed from the sequenza system and stored in TBS.

6.2.6.5.2 R.T.U. Vectastain Quick Kit

The slides were incubated with the pre diluted biotinylated pan-specific universal secondary antibody (100 µl each slide) from the R.T.U. Vectastain Quick Kit for 20 minutes (the manufacturer's recommendation was 10 minutes, but this was found to be sub optimal). The slides were washed with TBS for 5 minutes and incubated with streptavidin / peroxidase complex reagent from the kit (100 µl) for 10 minutes. The slides were rewashed with TBS for 5 minutes and removed from the sequenza system and stored in TBS.

6.2.6.6 **Antibody Visualisation**

Slides were incubated with 0.02% DAB in TBS containing 0.0125% H_2O_2 for up to 30 minutes to allow for development of brown stain which could be seen under a light microscope. The staining was further enhanced by incubating the slides with Copper Sulphate (0.5% copper sulphate in 0.9% saline) for 5 minutes.

6.2.6.7 **Counterstaining, rehydration, clearing and mounting**

Slides were counterstained by prefiltered Harris Haematoxylin stain for 20 minutes. The slides were subsequently washed under running water to remove any excess haematoxylin. The counter stain was then differentiated in acid alcohol (1% HCL in 70% alcohol) for 10 seconds and rewashed under running water. Slides were rehydrated in three pots of 100% ethanol for 10 seconds each and cleared in three pots of Histoclear II for 10 seconds each.

Slides were mounted onto coverslips containing histomount (National Diagnostics, Hull, UK). Slides were then left to dry overnight and viewed under a light microscope.

6.2.6.8 **Histological scoring**

Scoring was done by a consultant histopathologist (Dr. Ann Campbell) who is the regional expert in MPM. Since different scoring methods were applied for different proteins, due to the nature of their expression, the scoring systems are discussed under the respective chapters (Section 7.2.2 and 8.2.2).

6.2.6.9 **Statistical analysis**

Statistical analysis was performed using the SPSS software version 17 and 18 (SPSS, Chicago, USA). Univariate survival 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 shown to be an independent prognostic variable in MPM.

6.2.7 **Flow cytometry**

6.2.7.1 **Sample preparation**

All cell lines (NCI-H2052, NCI-H2452, MSTO-211H, A549) were cultured in RPMI media, mildly trypsinised, centrifuged and counted in a Haemocytometer after Trypan blue staining. Two million cells for each cell line were taken, centrifuged at 400 x *g* for 3 minutes and resuspended in 200 µl of PBS/BSA/Sodium Azide solution (PBS 1000ml; BSA 2.5grams; Sodium Azide 6.24mls).

6.2.7.2 **Immunofluorescence staining of cell surface receptors**

Of the 200 µl cell suspension, 100 µl was incubated with 20 µl of EGFR antibody (BD Pharminogen; cat No 555997) and the other 100 µl was incubated with an isotype control (IgG2b,κ) to account for the non specific binding. The EGFR and the isotype control antibodies were conjugated with fluorochrome PE (R-phycoerythrin). The cell suspensions were then incubated in the dark for 30 minutes. Cells were then washed with one ml of PBS/BSA/Sodium Azide solution and centrifuged at 400 X *g* for 3 minutes (Thermoscientific IEC CL3IR multispeed centrifuge). The supernatant was discarded and the cells were resuspended in 500 µl of filtered PBS/BSA/Sodium Azide solution. The samples were immediately analysed on the BD FACSAria^{TM} II (BD Biosciences, Oxford, UK) using the FACSDiva software.

6.2.7.3 **FACS analysis**

The FACS was set up as per manufacturer's instructions, FACSDiva software opened and start up procedure was followed (**Figure 5.5**). The cell suspension was acquired and the forward scatter (FSC) and the side scatter (SSC) voltages were altered for the isotype control to view the cell population in a dot plot (**Figure 6.9**) (Section 5.5.3). The voltage for the fluorochrome PE was set such that the histogram peak was located to the first segment of the grid. The cell population was selected and labelled P1 (gated) and the data for 10,000 cells were recorded. A marker (P2) was placed on the isotype control histogram encompassing ≤1% of the P1 cells (**Figure 6.10**) which was then subtracted from the percentage of cells showing positivity for protein expression. The cell suspension incubated with the EGFR antibody was acquired and the location and peak of the histogram recorded.

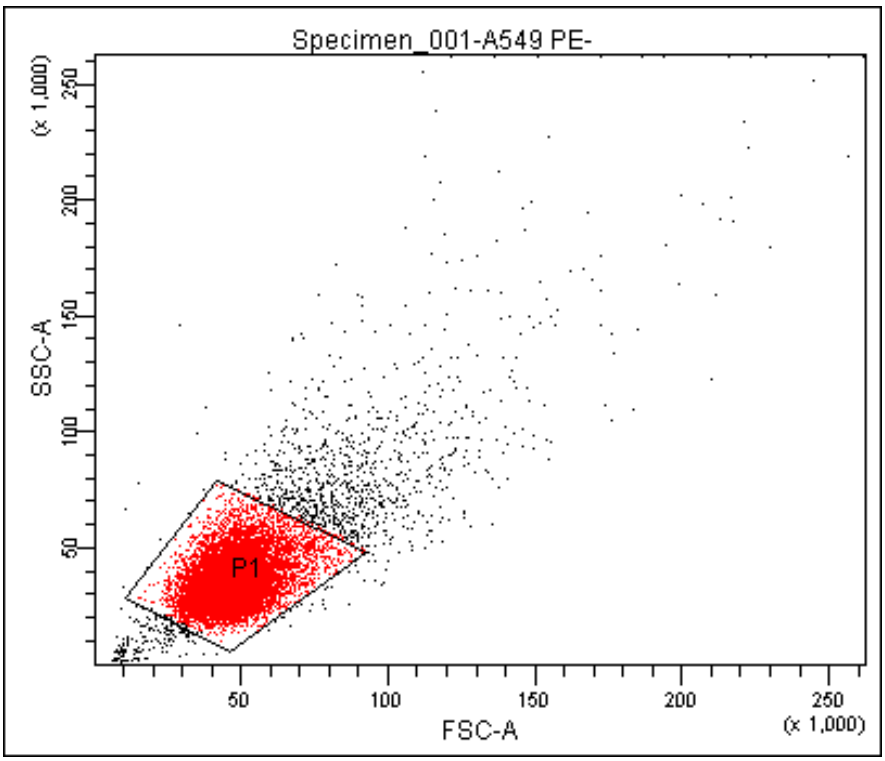

Figure 6.9 Cell acquisition by Flow cytometry. This dot plot demonstrates the A549 cell population labelled with the isotype control for EGFR antibody and acquired by flow cytometry. The voltage settings for the FSC and the SSC were altered to show the cell population. The cell population selected to determine the EGFR expression of the cell lines was selected (P1). The X-axis demonstrates the FSC and Y-Axis demonstrates the SSC. Each dot on the graph represents a cell.

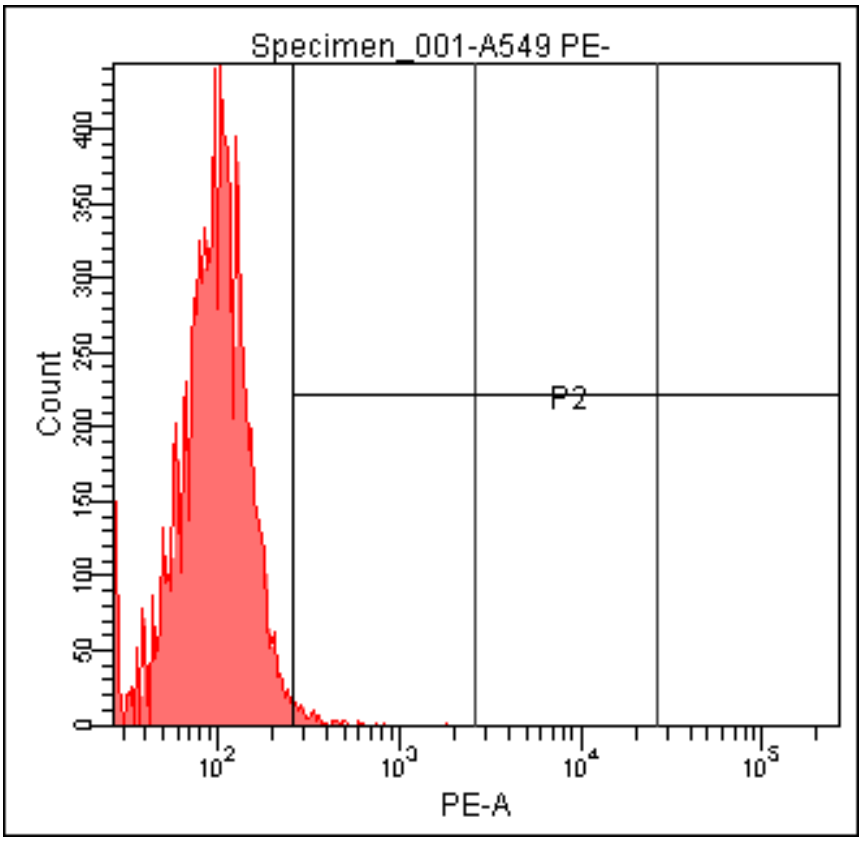

Figure 6.10 Histogram plot for isotype control. This figure demonstrates the histogram plot using P1 population of the isotype control of the A549 cell line (**Figure 6.9**). The voltage was altered so that the histogram peak was in the first segment of the grid. A marker (P2) was inserted (horizontal line on the X axis) to ensure ≤1% of the isotype control cells were encompassed by this marker. In a positive data set the histogram would fall within the P2 region (on the X axis) when the cell suspension incubated with the antibody is analysed. The X-axis demonstrates the relative fluorescence and the Y-Axis demonstrates the number of cells analysed.

6.2.8 **Ingenuity pathway analysis (IPA)**

All proteins identified by antibody microarray were analysed using IPA software. The gene name of all the proteins identified was checked against the International Protein Index (IPI) and National Centre for Biotechnology Information (NCBI) database and uploaded onto the IPA software. The filters and general settings for analysis were as follows:

- Reference set: Ingenuity Knowledge Base (Genes Only)
- Relationship to include: Direct
- Does not Include Endogenous Chemicals
- Genes were included
- Molecules per Network: 140
- Networks per Analysis: 25
- Data Sources: Select all
- Species: Human (Relaxed filter)
- Confidence: Experimentally Observed
- Tissues and Cell lines: Select all (Relaxed filter)

Once the genes were identified (ID's Mapped) analysis of the genes was commenced. Ingenuity Pathway Analysis maintains a database of genes and proteins along with their biological and chemical relationships, interactions, pathways, drug targets and biomarkers. This database is called Ingenuity pathways knowledge base (IPKB) and is regularly updated based on published data in scientific literature. Within this IPKB there are various types of information which are manually reviewed and updated such as Ingenuity® Expert Findings from experimentally-demonstrated information, Ingenuity® ExpertAssist Findings from recently published journal abstracts, Ingenuity® Expert Knowledge containing signalling and metabolic pathway information developed by Ingenuity experts

and Ingenuity® Supported Third Party Information selected from other databases like Entrez Gene, Gene Ontology and RefSeq. The relationship of genes and their products in the IPKB is abstracted into a large network called a Global Molecular network. The genes of the differentially expressed proteins identified which are also present in the Global Molecular network are called focus genes. The focused genes are overlaid into the Global Molecular network and the interactions of the focused genes amongst themselves along with various other connected genes within the Global Molecular network can be identified. Based on the focused genes, multiple networks may be identified and they are all given scores based upon the number of focused genes in relation to the total number of genes that could have been in the pathway. The higher the score, the increased number of focused genes in the network.

IPA also maintains a library of canonical pathways, which are metabolic and cell signalling pathways. Based on the dataset of the focused genes, various canonical pathways are identified. All the canonical pathways identified are relayed as a bar chart with the x axis showing the pathways and the y axis the ratio of the total number of focused genes in relation to the total number of genes that could have been in the pathway. Fisher's exact test was performed by IPA to determine the probability that the association between the focused genes and the canonical pathways identified was by chance and was displayed as a p-value.

Chapter 7. Immunohistochemical expression of PTEN Protein in MPM

Publications and Presentations

V Agarwal, A Campbell, K L Beaumont, L Cawkwell, M J Lind.

PTEN protein expression in malignant pleural mesothelioma.

Tumour Biology (*In Press*)

Agarwal V, Ranatunge D, Beaumont K, Campbell A, Lind MJ, Cawkwell L (2011) Putative biomarkers in malignant pleural mesothelioma. J Clin Oncol 29: e21008 (abstract).

Agarwal V, Beaumont KL, Campbell A, Lind MJ, Cawkwell L (2011) Expression of PTEN in malignant pleural mesothelioma. Lung Cancer 71: S19 (abstract). Poster - 9th Annual British Thoracic Oncology Group Conference, Dublin (January 2011)

Chapter aim

To identify the absence of PTEN protein expression and its prognostic relevance in MPM using immunohistochemistry in a series or archival MPM tissue samples.

7.1 **Introduction**

Phosphatase and Tensin Homolog (PTEN), also known as MMAC or TEP1, is a dual lipid and protein phosphatase encoded by the PTEN tumour suppressor gene on chromosome 10q23 (Li and Sun 1997; Li, Yen et al. 1997; Steck, Pershouse et al. 1997). The PTEN gene has been shown to undergo mutations or deletion in various malignancies such as endometrial, glioblastoma, prostrate, ovarian, breast, lung etc (Rhei, Kang et al. 1997; Risinger, Hayes et al. 1997; Steck, Pershouse et al. 1997; Tashiro, Blazes et al. 1997; Bostrom, Cobbers et al. 1998; Feilotter, Nagai et al. 1998; Kohno, Takahashi et al. 1998; Suzuki, Freije et al. 1998; Wang, Parsons et al. 1998; Ali, Schriml et al. 1999). Germline mutations of the PTEN gene are associated with Cowdens syndrome, which is autosomally transmitted and is associated with increased risk of breast and thyroid tumours (Liaw, Marsh et al. 1997; Nelen, van Staveren et al. 1997). When cells are stimulated by growth factors, PI3K converts Phosphatidylinositol (4,5)-biphosphate (PIP2) into Phosphatidylinositol (3,4,5)-triphosphate (PIP3), which phosphorylates AKT and its downstream signalling cascade resulting in cell survival and proliferation. The PTEN protein negatively regulates phosphorylation of AKT by dephosphorylating PIP3 into PIP2 (Maehama and Dixon 1998). Loss of PTEN protein may result in increased accumulation of PIP3 and constitutive activation of AKT resulting in increased cell survival (Section 3.1).

In MPM, various small studies have shown that PTEN protein is rarely lost, but a recent large immunohistochemical study of 341 MPM samples demonstrated that PTEN protein was lost in 62% and its loss was associated with poor survival (Opitz, Soltermann et al. 2008) (**Table 3.1**). Since there has been only one large study evaluating the expression of PTEN protein in MPM, this needs further validation as it may have a significant impact on

our understanding of the pathophysiology of MPM and our choice to targeted treatment options. In this study we aimed to analyse PTEN protein expression by immunohistochemistry in a previously described cohort of 86 samples (O'Kane, Cawkwell et al. 2005).

7.2 **Materials and Methods**

7.2.1 **Tissue sample collection**

Eighty six archival tissue samples of patients diagnosed with MPM at Hull Royal Infirmary, Hull, UK from 1992 to 2000 were obtained. Clinicopathological data for all patients were available. Immunohistochemical analysis was performed in all of the 86 MPM tissue samples (46 epithelial, 24 biphasic, 16 sarcomatoid) to determine the expression of PTEN protein. Normal pleura tissue sample was included in the cohort of the slides stained to act as a reference slide.

7.2.2 **Immunohistochemistry**

Immunohistochemistry was performed using R.T.U. Vectastain Quick Kit (#PK-7800, Vector Laboratories Inc., CA, USA) as detailed in section 6.2.6 with anti- PTEN primary antibody (ab32199; Abcam) at a dilution of 1:50.

All slides were scored by a consultant histopathologist (Dr. Anne Campbell), as detailed in section 6.2.6.8. Colorectal tissue samples stained with antibody were used as a positive control and slide with antibody omitted as a negative control.

7.2.3 **Statistical Analysis**

Statistical analysis was performed using the SPSS software version PASW statistics 18 (SPSS, Chicago, USA). Univariate survival analysis for histological subtype and PTEN

protein expression was performed using Kaplan Meier curves with log rank analysis. Multivariate analysis was calculated using Cox regression analysis.

7.3 **Results**

Mesothelial cells in normal pleura demonstrated positive staining for PTEN protein and served as a positive reference (**Figure 7.1A**). Fibroblasts and endothelial cells were also stained with anti-PTEN antibody and served as an internal control (**Figure 7.1B**). In slides where internal control was present, it was taken as a reference along with normal pleura and in slides where internal control was not seen normal pleura only was taken as a positive reference.

The staining was recorded as (**Figure 7.2**)

- 0 - negative (no staining seen)
- 1 - weak (intensity less than that of normal pleura positive reference slide)
- 2 - strong (intensity equal to or greater than that of normal pleura)

Staining for PTEN protein in the MPM cells was seen in the cytoplasm as well as the nucleus. Cytoplasmic staining was used to calculate the PTEN protein staining score. PTEN protein staining score was 0 in 27% (23/86), 1 in 27% (23/86) and 2 in 46% (40/86) (**Table 7.1**).

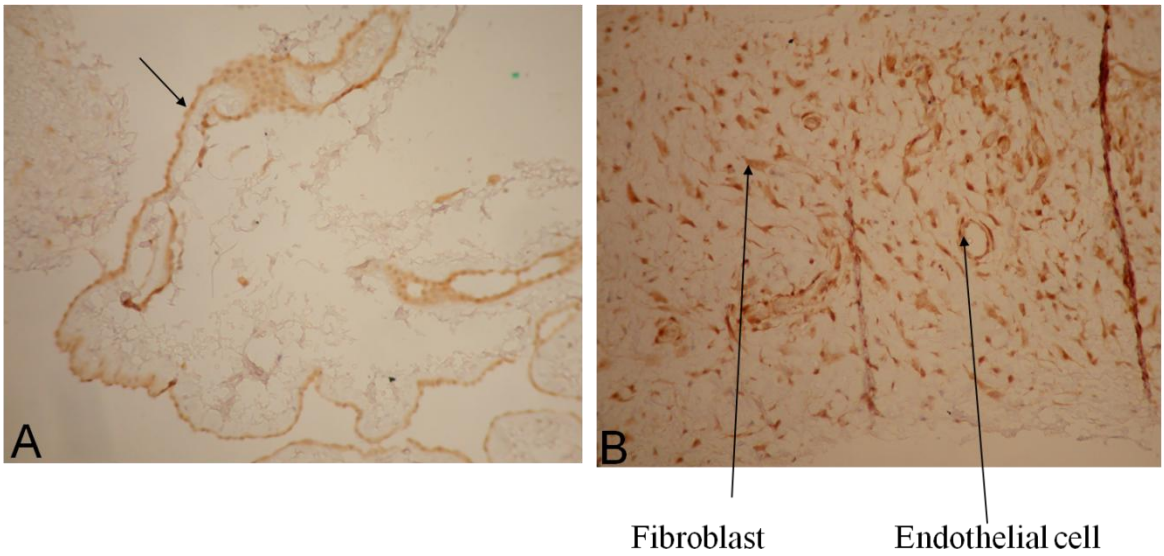

Figure 7.1 Microphotograph of normal pleura and internal control. (A) Normal pleura demonstrating positive staining for PTEN protein expression (arrow; score 2). (B) Fibroblasts and endothelial cells stained positive and acted as internal control.

Table 7.1 Frequency of PTEN protein expression in all tumour samples along with their histological subtypes. PTEN0= protein absent; PTEN1= intensity < normal pleura; PTEN2= intensity ≥ normal pleura).

Samples	**Total**	**PTEN staining Score**		
		PTEN 0	PTEN 1	PTEN 2
All Samples	86	23 (26.7%)	23 (26.7%)	40 (46.5%)
Epithelial	46	10 (22%)	11 (24%)	25 (54%)
Biphasic	24	7 (29%)	6 (25%)	11 (46%)
Sarcomatoid	16	6 (37.5%)	6 (37.5%)	4 (25%)

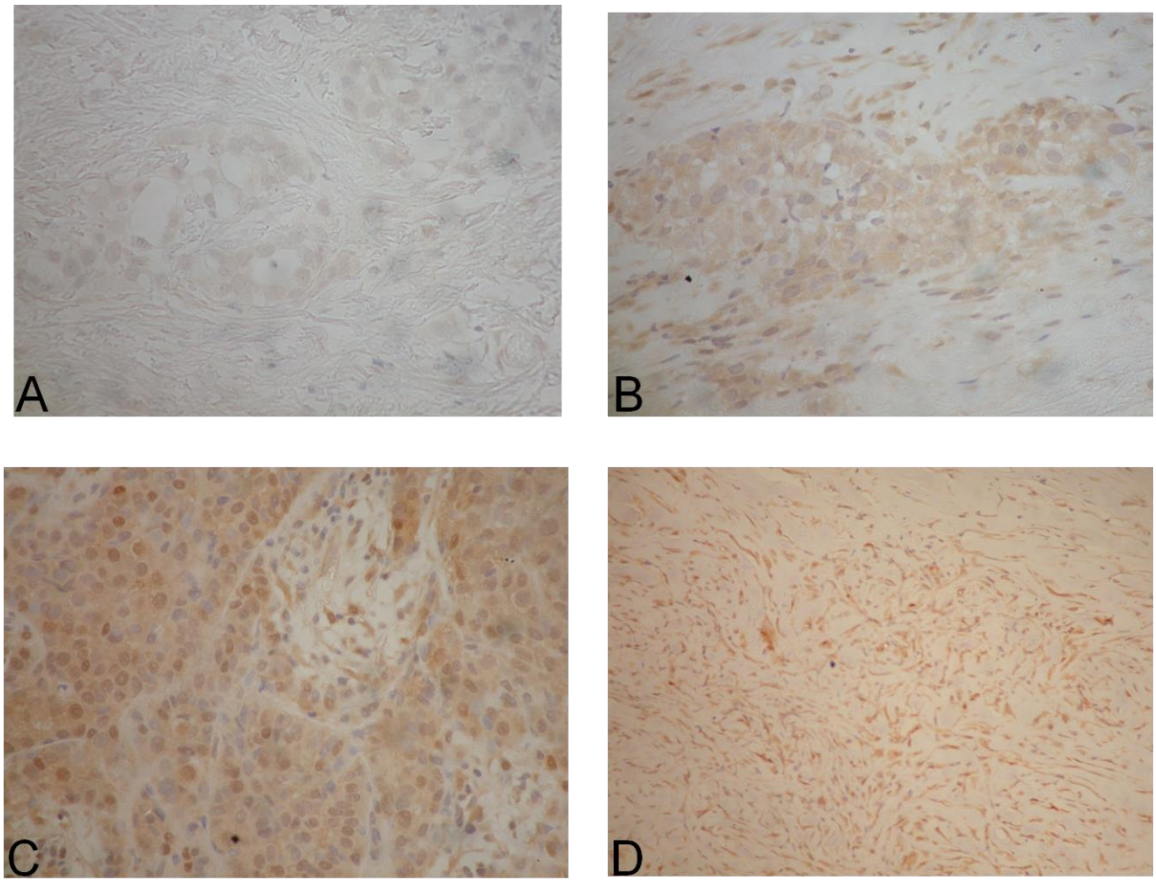

Figure 7.2 PTEN protein staining of MPM tissue samples. **Figure 7.2A** demonstrates negative staining in MPM (PTEN score 0). **Figure 7.2**B demonstrates positive staining, but with intensity less than that of the normal pleura (PTEN score 1). **Figure 7.2**C demonstrates strong staining in epithelioid histology with intensity stronger than that of the normal pleura (PTEN score 2). **Figure 7.2**D demonstrates strong staining in sarcomatoid histology with intensity stronger than that of the normal pleura (PTEN score 2).

Survival data was available for all 86 patients. Univariate analysis using Kaplan Meier survival curves demonstrated that epithelioid histological subtype was associated with better prognosis ($p<0.001$) (**Figure 7.3A**). The median survival for epithelioid histology was 10.3 months (95% CI 12.5 – 21.5 months), biphasic was 5.5 months (95% CI 4.8 – 9.1 months) and sarcomatoid was 3.4 months (95% CI 2.9 – 6.1 months). Reduced expression

of PTEN protein was not associated with poor survival in univariate analysis using Kaplan Meier survival curves (p=0.223) (**Figure 7.3B**). When histological subtype was taken into consideration, multivariate Cox regression analysis demonstrated that absence of PTEN protein expression was not an independent prognostic variable (HR 0.79; 95% CI 0.61 – 1.01; p=0.06) (**Table 7.2**).

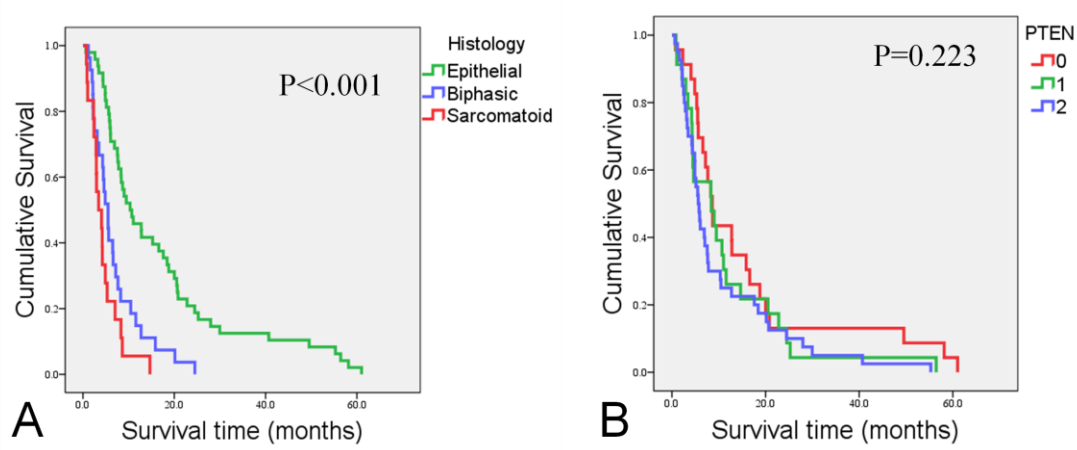

Figure 7.3 Univariate analysis of PTEN expression. Kaplan Meier survival curves demonstrating improved survival with epithelial histological subtype (p<0.001) (A). No association between survival and PTEN protein expression was seen (p=0.223) (B).

Table 7.2 This table demonstrates multivariate analysis for PTEN expression using histological subtype as a confounding factor.

	HR	95%CI (lower)	95%CI (upper)	P value
PTEN	1.79	0.61	1.01	0.06
Histological subtype				<0.001
Biphasic	0.22	0.12	0.4	<0.001
Sarcomatoid	0.58	0.3	1.1	0.09
Epithelial	1.000			

7.4 **Discussion**

We demonstrated that PTEN protein expression was absent in 27% and reduced in a further 27% of MPM tissue samples. However, absence of PTEN protein expression was not found to be of prognostic significance in univariate analysis.

A large tissue microarray based study using immunohistochemistry had previously reported that PTEN protein expression was absent in 62% (n=341) of MPM tumour samples (Opitz, Soltermann et al. 2008) and the absence of PTEN protein expression was associated with poor prognosis (n=129; p=0.003), which was independent of histological subtype. This difference in the frequency of PTEN protein expression, when compared to our results, could be because of different scoring methods. In the study by Opitz *et al* (2008), the scoring was done in tissue microarrays using mouse monoclonal anti-PTEN antibody (Novocastra, Sweden) and was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) and the staining of the normal pleura was not done. In our study, the scoring was done in immunohistochemical slides using rabbit monoclonal antibody (ab32199; Abcam),

in reference to normal pleura where, 0 represented negative expression; 1 represented weak expression (intensity less than that of normal pleura) and 2 represented strong expression (intensity equal to or greater than that of normal pleura). This variation in the technique, antibody used and the scoring methodology may account for the difference seen in frequency of PTEN protein expression between the two studies. It is important to use expression shown in the normal pleura as a comparative reference guide, because the absence of PTEN protein cannot be demonstrated unless its presence is initially confirmed in normal pleura.

In our study, we demonstrated PTEN protein expression in both the cytoplasm as well as the nucleus. Earlier studies had shown that PTEN protein was localised exclusively to the cytoplasm (Li and Sun 1997; Whang, Wu et al. 1998). However, subsequent studies have shown that PTEN protein is present in the cytoplasm as well as the nucleus (Salmena, Carracedo et al. 2008). Nuclear PTEN protein has important physiological functions independent of AKT activation, such as p53 acetylation, induction of apoptosis and cell cycle progression (Salmena, Carracedo et al. 2008).

Absence of PTEN protein may result due to mutations of the PTEN gene on chromosome 10, promoter methylation, transcription repression, posttranslational modification or delocalisation of the PTEN protein (Salmena, Carracedo et al. 2008). Although absence of PTEN protein may demonstrate a state of homozygous loss of the PTEN gene, the weak staining may demonstrate a heterozygous loss of the PTEN gene which may play an important role in cancer initiation and progression (Salmena, Carracedo et al. 2008). In mouse models, a heterozygous PTEN gene status in addition to loss of p27 has been shown to induce prostate cancers (Di Cristofano, De Acetis et al. 2001). In another study, inactivation of one PTEN allele resulted in the occurrence of prostrate epithelial

hyperplasia in mice, although homozygous deletion of the PTEN gene was required for the induction of advanced prostatic cancer (Trotman, Niki et al. 2003). Gray *et al* (1998) evaluated 37 primary prostate cancers for PTEN allele deletion and single allele deletion was seen in 26/37 (70%) of the tumours (Gray, Stewart et al. 1998). Whang *et al* (1998) created ten xenografts from prostate cancer tissues implanted in immune deficient mice. On evaluation of the PTEN gene, only one of the ten xenografts was found to have a homozygous deletion. Five of the remaining nine xenografts had reduced or absent PTEN mRNA or protein expression, but intact PTEN gene suggesting that PTEN inactivation may occur at the transcription level (Whang, Wu et al. 1998). This suggests that the reduction of PTEN protein expression, either due to loss of a single PTEN allele or due to abnormal transcription, may play a role in cancer initiation and progression in addition to the homozygous PTEN gene deletion.

Since the loss of PTEN protein results in activation of AKT and increased cell survival, the PTEN protein can be therapeutically manipulated by increasing the expression of PTEN protein. To assess the effect of over expression of PTEN protein in MPM, a study transfected two MPM cell lines (REN and I-45) with adenoviral vectors AdPTEN and studied their effects (Mohiuddin, Cao et al. 2002). PTEN gene mutation status of both the cell lines was unknown and a basal level of PTEN protein was present, which on transfection with AdPTEN was significantly increased. Forced over expression of PTEN protein resulted in decreased phosphorylation of AKT and increased apoptotic cell death of the cells. In another study, wild type as well as mutant PTEN was introduced in M43 cell line using adenovirus. M43 is a MPM cell line which showed homozygous deletion of the PTEN gene and elevated pAKT activity. Cells with wild type PTEN gene reduced AKT phosphorylation and decreased cell cycle progression (Altomare, You et al. 2005).

In this study we have demonstrated that PTEN protein was either absent or reduced in a significant number of MPM tumours when compared with its expression in normal pleura. This was however, not associated with survival. Absence or reduction of PTEN protein expression may play a significant role in its oncogenic phenotype by activating AKT and be an important therapeutic target. Targeting proteins further downstream of the PTEN protein such as AKT and MTOR may provide further therapeutic benefit in MPM. In Chapter 10 we evaluated the PTEN protein expression and the cytotoxic effect of MTOR inhibition in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines.

Chapter 8. Expression of Lipoxygenase in malignant pleural mesothelioma

Publications and Presentations

Agarwal V, Ranatunge D, Campbell A, Lind MJ, Cawkwell L (2012) Expression of Lipoxygenase in malignant pleural mesothelioma. Manuscript submitted (Journal of Cancer Research and Clinical Oncology).

Agarwal V, Ranatunge D, Beaumont K, Campbell A, Lind MJ, Cawkwell L (2011) Putative biomarkers in malignant pleural mesothelioma. J Clin Oncol 29: e21008 (abstract).

Agarwal V, Ranatunge D, Campbell A, Lind MJ, Cawkwell L (2011) Expression of 5 & 12 Lipoxygenase in malignant pleural mesothelioma. Lung Cancer 71: S19 (abstract). Poster - 9th Annual British Thoracic Oncology Group Conference, Dublin (January 2011)

Chapter aim

To demonstrate the expression and prognostic relevance of 5LOX and 12LOX proteins in MPM using immunohistochemistry in a series of archival MPM tissue samples.

8.1 **Introduction**

Arachidonic acid is metabolised by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes resulting in the production of prostanoids, leukotrienes (LT), hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs), which have been implicated in inflammation and carcinogenesis (Chapter 4). Our laboratory and others (**Table 4.1**) have previously shown using immunohistochemistry (IHC) that COX-2 is over expressed in MPM (O'Kane, Cawkwell et al. 2005) and that the specific COX-2 inhibitor DuP-697 can potentiate the cytotoxic effects of Pemetrexed in MPM cell lines (O'Kane, Eagle et al. 2010).

Three isoenzymes exist for lipoxygenase, 5LOX, 12LOX and 15LOX among which 5LOX and 12LOX have been implicated in carcinogenesis (Section 4.2.2). The expression of 5LOX and 12LOX has recently been associated with carcinogenesis in various solid tumours. The 5LOX and 12LOX proteins are generally absent in normal epithelia, but are expressed in various epithelial cancers including colon (Ohd, Nielsen et al. 2003; Barresi, Grosso et al. 2007), oesophageal (Chen, Wang et al. 2004; Hoque, Lippman et al. 2005), prostate (Gupta, Srivastava et al. 2001; Matsuyama, Yoshimura et al. 2004), bladder (Yoshimura, Matsuyama et al. 2003), oral (Li, Sood et al. 2005), melanoma (Winer, Normolle et al. 2002), renal (Yoshimura, Inoue et al. 2004), pancreatic (Hennig, Ding et al. 2002) and breast (Natarajan, Esworthy et al. 1997; Jiang, Douglas-Jones et al. 2003). To our knowledge, this is the first study to investigate the expression of the 5LOX and 12LOX proteins using IHC in archival MPM samples.

8.2 **Materials and methods**

8.2.1 **Samples**

Local Research Ethics Committee approval was granted for the study (ref 11/00/212). Ninety-three archival tissue samples of patients diagnosed with MPM at Hull Royal Infirmary from 1992 to 2000 were obtained, of which 86/93 (92%) patients were male. Clinicopathological details for all samples were available. There were 48/93 (51%) epithelial, 27/93 (29%) biphasic and 18/93 (19%) sarcomatoid histological subtypes.

8.2.2 **Immunohistochemistry**

All 93 MPM archival tissue samples were analysed by immunohistochemistry as previously described in section 6.2.6 to determine 5LOX and 12LOX protein expression. Briefly, following antigen retrieval for 3 minutes in a pressure cooker at 15psi containing 1:100 Antigen Unmasking Solution (#H-3300, Vector Laboratories Inc, CA, USA) non specific staining was blocked by using 1x casein (#SP-5020, Vector Laboratories Inc, CA, USA). The anti-5LOX antibody (#ab39347, Abcam) was applied at a final concentration of 1:50 and the anti-12LOX antibody (#ab23678 Abcam) was applied at 1:100. A negative control (antibody omitted) and a positive control section (colorectal cancer) were included with each batch of slides. Following incubation for 2 hours, antibody detection was carried out using the StreptABComplex / HRP Duet Mouse/Rabbit kit (#K0492, Dako Ltd, Ely, UK). Positive staining was visualised using DAB as chromogen. All slides were independently reviewed by three independent reviewers as discussed in section 6.2.6.8. Positive staining was recorded if there was moderately strong staining in at least 25% of malignant cells. Negative staining was recorded if less than 25% of malignant cells showed moderately strong staining, or if only weak/no staining could be seen.

8.2.3 **Statistical analysis**

Statistical analysis was performed using SPSS software version 17.0 (SPSS, Chicago, USA). Univariate survival analysis for 5LOX and 12LOX 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 has been shown to be an independent prognostic variable in MPM. Co-expression of proteins was analysed using Fishers exact test.

8.3 **Results**

8.3.1 **5LOX protein expression**

Of the 93 tissue samples, 88 were adequately stained by immunohistochemistry to detect 5LOX protein expression, with identifiable tumour present. Positive staining of 5LOX protein was found predominantly in the nucleus of the malignant cells, with varying intensity (**Figure 8.1A**). Infiltrating lymphocytes also demonstrated positive staining and served as an internal positive control. Positive 5LOX protein expression was seen in 65/88 (73%) of MPM tissue samples (**Table 8.1**). In univariate analysis, the positive expression of 5LOX was associated with improved survival (median survival 8.3 months *versus* 4.6 months in 5LOX negative; p=0.006; **Figure 8.2**). However, when histological subtype was taken into consideration, multivariate Cox regression analysis demonstrated that 5LOX expression was not an independent prognostic variable (HR 1.58; 95% CI 0.96 – 2.64; p=0.074) (**Table 8.2**).

8.3.2 **12LOX expression**

Eighty-three tissue samples were adequately stained using IHC to detect 12LOX protein expression. Positive staining of 12LOX protein was found predominantly in the nucleus

and cytoplasm of the malignant cells, with varying intensity (**Figure 8.1B**). Positive 12LOX protein expression was seen in 69/83 (83%) of MPM tissue samples (**Table 8.1**). The expression of 12LOX was not associated with survival (p=0.455; **Figure 8.3**). Co-expression of 5LOX and 12LOX was seen in 55/82 samples and this was statistically significant (p=0.006, Fishers exact, **Table 8.3B**).

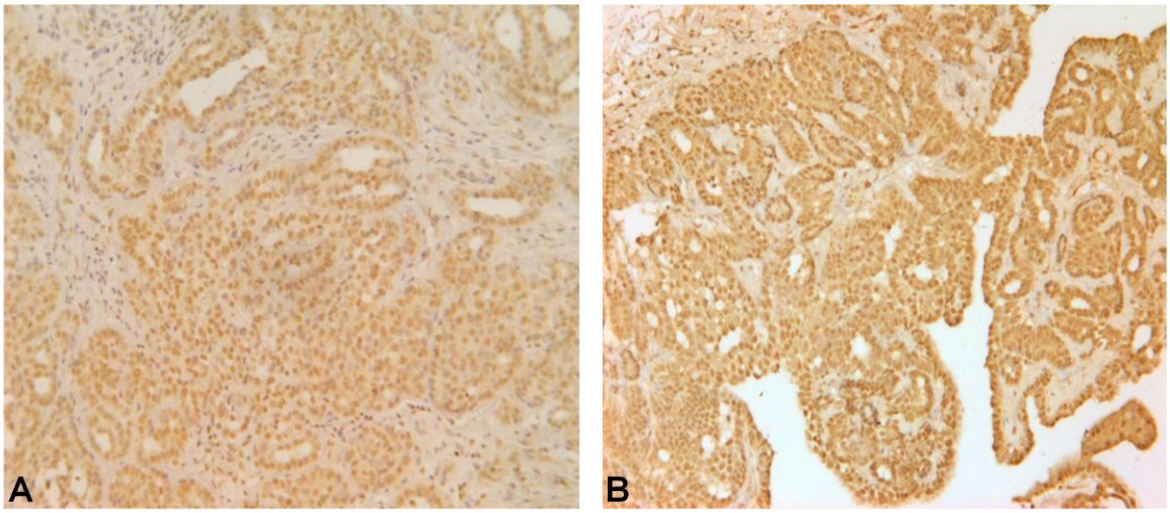

Figure 8.1 5LOX and 12LOX protein staining of MPM tissue samples. This figure demonstrates MPM tissue samples demonstrating positive staining with (A) anti-5LOX antibody (ab39347, Abcam) and (B) anti-12LOX antibody (ab23678, Abcam). Magnification X 200.

Table 8.1 Immunohistochemical analysis of 5LOX and 12LOX protein expression categorised by histological subtype.

	Total	Epithelial	Biphasic	Sarcomatoid
5LOX positive	65/88 (73%)	39/45 (86%)	17/25 (68%)	9/18 (50%)
12LOX positive	69/83 (83%)	38/44(86%)	21/25 (84%)	10/14 (71%)

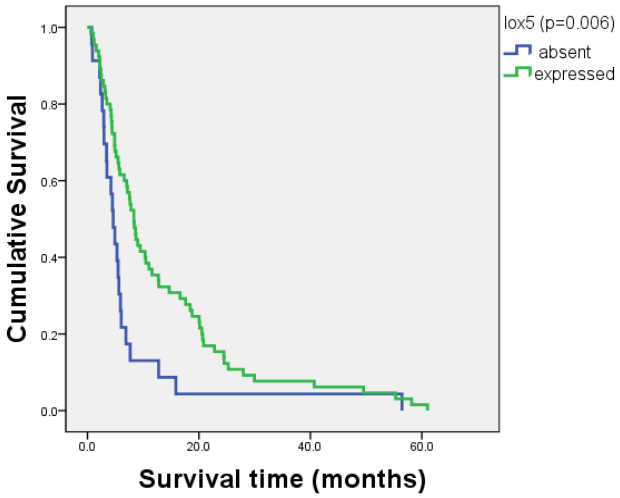

Figure 8.2 Survival analysis for 5LOX protein expression. Kaplan Meier plot showing univariate analysis of 5LOX expression (p=0.006, log rank). The median survival was 8.3 months in 5LOX-positive cases (green line) *versus* 4.6 months for 5LOX-negative cases (blue line)

Table 8.2 This table demonstrates multivariate analysis for 5LOX expression using histological subtype as a confounding factor.

	HR	95%CI (lower)	95%CI (upper)	P value
5LOX	1.59	0.96	2.64	0.074
Histological subtype				<0.001
biphasic	0.23	0.12	0.42	<0.001
sarcomatoid	0.57	0.3	1.06	0.075
epithelial	1.000			

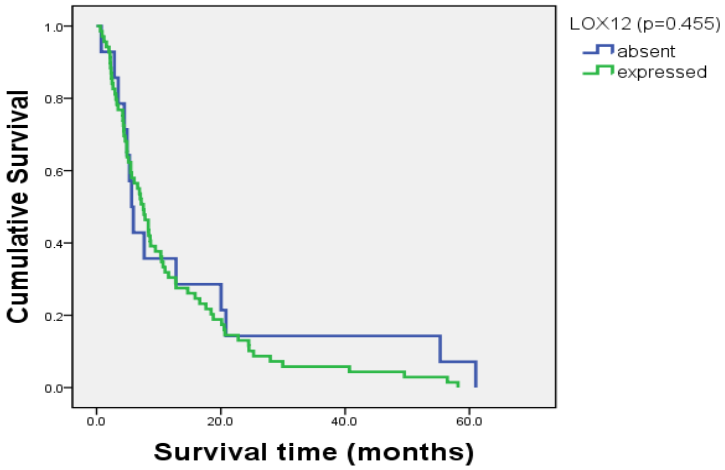

Figure 8.3 Survival analysis for 12LOX protein expression. Kaplan Meier plot showing univariate analysis of 12LOX expression (p=0.455, log rank). The expression of 12LOX was not associated with survival.

8.3.3 **Correlations between 5LOX, 12LOX and COX-2 expression**

Using updated survival data, we then correlated previously published COX-2 protein expression results from this laboratory (O'Kane, Cawkwell et al. 2005) with the 5LOX and 12LOX results in the same cohort of 93 patients. Positive expression of the COX-2 protein was previously demonstrated in 58/93 (62%) of the samples (O'Kane, Cawkwell et al. 2005). There was no significant correlation between COX-2 and 12LOX expression (**Table 8.3C**). However positive 5LOX expression was significantly more likely in the presence of positive COX-2 expression (p=0.002, Fisher's exact, **Table 8.3A**). Of the 88 samples which were successfully analysed for both 5LOX and COX-2, 47/88 (53%) expressed both proteins. In univariate survival analysis the co-expression of 5LOX and COX-2 (n=47) was significantly associated with improved prognosis when compared with samples which did not express either 5LOX or COX-2 (n=15) with median survival times of 8.6 months *versus* 3.4 months (p<0.001, log rank; **Figure 8.4**). This finding was independent of histological

subtype, as demonstrated by multivariate Cox regression analysis ($p<0.001$). However when taken separately in Cox regression analysis, COX-2 status and histological subtype, but not 5LOX status, were significantly contributing to survival outcome. Previous findings had also revealed COX-2 to be an independent prognostic factor (O'Kane, Cawkwell et al. 2005).

Table 8.3 This table demonstrates the correlations between COX-2 / 5LOX expression (A), 12LOX / 5LOX expression (B) and 12LOX / COX-2 expression (C). P values were calculated based on the Fisher's exact test.

A. 5LOX/COX-2 cross tabulation

Fisher's Exact Test (2 sided) = 0.002		COX-2		
		absent	expressed	Total
5LOX	absent	15	8	23
	expressed	18	47	65
Total		33	55	88

B. 12LOX/5LOX cross tabulation

Fisher's Exact Test (2 sided) = 0.006		5LOX		
		absent	expressed	Total
12LOX	absent	8	6	14
	expressed	13	55	68
Total		21	61	82

C.12LOX/COX-2 cross tabulation

Fisher's Exact Test (2 sided) = 0.227		COX-2		
		absent	expressed	Total
12LOX	absent	7	7	14
	expressed	22	47	69
Total		29	54	83

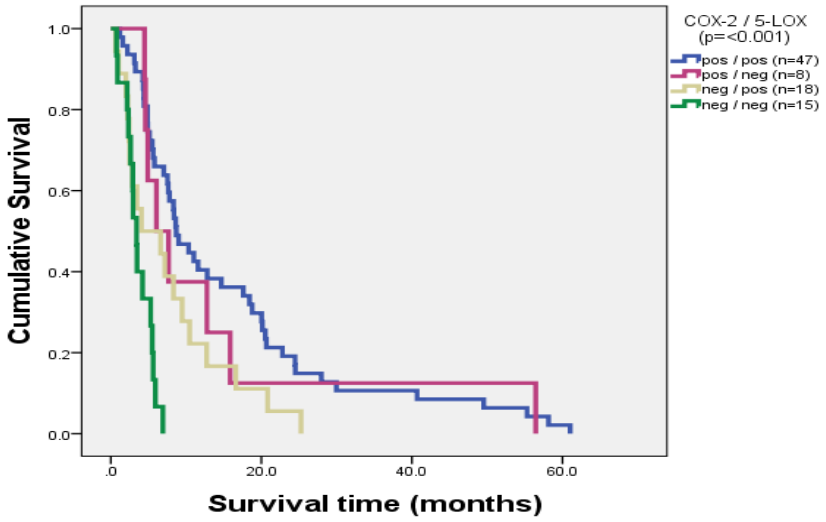

Figure 8.4 Survival analysis for combined COX-2/5LOX protein expression. Kaplan Meier plot showing univariate analysis of combined COX-2/5LOX expression status ($p<0.001$, log rank). The median survival was 8.6 months in COX-2 positive/5LOX positive cases (blue line), 6 months in COX-2 positive/5LOX negative cases (pink line), 4 months in COX-2 negative/5LOX positive cases (yellow line) and 3.4 months in COX-2 negative/5LOX negative cases (green line).

8.4 **Discussion**

In this study both 5LOX and 12LOX proteins demonstrated a significant frequency of over-expression in MPM and may therefore be considered as possible therapeutic targets. This is a similar finding to other reports in various epithelial tumour types such as colon, rectum, oesophagus, pancreas, oral, bladder, prostrate, breast, renal and melanoma. We have previously shown that COX-2 is over-expressed in MPM and that COX-2 inhibitors may have a role to play in MPM therapy (O'Kane, Cawkwell et al. 2005) and we have now

demonstrated a positive correlation for 5LOX and COX-2 co-expression. This has previously been reported in colorectal cancer where a positive correlation between 5LOX and COX-2 expression was demonstrated in a series of 50 adenocarcinomas (Barresi, Grosso et al. 2007). This suggests that both the 5LOX and COX-2 metabolic pathways of Arachidonic acid are activated in the majority of MPM samples. Individually, the *in vitro* cytotoxic effects of both LOX and COX-2 inhibitors have been demonstrated previously in mesothelioma cell lines (Marrogi, Pass et al. 2000; Romano, Catalano et al. 2001; O'Kane, Eagle et al. 2010). Inhibiting both the 5LOX and COX-2 pathways simultaneously may result in an enhanced therapeutic effect compared to the inhibition of either pathway independently. This will need further evaluation in MPM cell lines.

In this study we also investigated the prognostic significance of 5LOX, 12LOX and COX-2 protein expression. Neither 5LOX nor 12LOX were found to be independent prognostic variables. This is in keeping with the observations made in 61 colorectal tissue samples, where 5LOX expression was also not associated with survival (Ohd, Nielsen et al. 2003). To date there have not been any other reports on the prognostic value of 5LOX or 12LOX in MPM.

Chapter 9. Inhibition of Epidermal Growth Factor Receptor in malignant pleural mesothelioma cells

Publications and presentations

Agarwal V, Lind MJ, Cawkwell L. Inhibition of EGFR and MTOR pathway in malignant pleural mesothelioma. Manuscript in preparation

Agarwal V, Lind MJ, Cawkwell L. Inhibition of Epidermal Growth Factor Receptor in malignant pleural mesothelioma. European Journal of Cancer 2011; 47(Suppl 1):S141. (Poster presentation at The 16th ECCO, 36th ESMO, 30th ESTRO European Multidisciplinary Cancer Congress. Sept 2011, Sweden)

Chapter aim

To evaluate the expression of EGFR and the presence of EGFR, KRAS and BRAF mutation in MPM cell lines (NCI-H2052, NCI-H2452, MSTO-211H) and NSCLC cell line (A549).

To evaluate the effect of EGFR inhibition using Cetuximab and Gefitinib in MPM cell lines and A549 cell line using the MTS assay.

9.1 **Introduction**

EGFR has been associated with an oncogenic phenotype in many cancers and anti-EGFR therapies are now widely used in NSCLC, colon cancers and Head and neck cancers (Ciardiello and Tortora 2008). Activation of EGFR by ligand stimulation leads to stimulation of a host of intracellular signal transduction pathways resulting in cell proliferation, failure of apoptosis, invasion and metastasis (Bogdan and Klambt 2001; Hynes and Lane 2005; Citri and Yarden 2006). The two major pathways stimulated by EGFR activation are the PI3K/AKT/MTOR pathway and the RAS/RAF/MAPK pathways (**Figure 2.2**).

Two groups of anti-EGFR therapies have been developed; monoclonal antibodies against the extracellular domain of EGFR, such as Cetuximab and Panitumumab; small molecule tyrosine kinase inhibitors (TKI) against the intracellular tyrosine kinase domain of EGFR, such as Gefitinib and Erlotinib (**Figure 9.1**).

Immunohistochemical studies have shown that EGFR is over-expressed in 44 to 97% of MPM tissue samples (**Table 2.2**) (Dazzi, Hasleton et al. 1990; Govindan, Kratzke et al. 2005; Destro, Ceresoli et al. 2006; Edwards, Swinson et al. 2006; Garland, Rankin et al. 2007). Two Phase II clinical trials have shown that EGFR TKIs (Gefitinib and Erlotinib) are not effective in MPM (Section 2.2.6). Biomarkers that induce resistance or sensitivity to anti-EGFR therapies such as EGFR, KRAS, BRAF and PIK3CA mutations are not commonly seen in MPM (Sections 2.2.7, 2.2.8, 2.2.9 and 2.2.10). Currently there are no published studies evaluating the EGFR, KRAS and BRAF mutation status of NCI-H2052, NCI-H2452 and MSTO-211H cell lines. The MSTO-211H, NCI-H2052, NCI-H2452 and A549 cells have previously demonstrated to harbour wild type PIK3CA gene (Janmaat, Rodriguez et al. 2006; Yutaro Suzuki, Hideki Murakami et al. 2009). The presence of

EGFR activating mutations may have significant anti tumour response to EGFR TKIs and the presence of EGFR resistant mutations, KRAS or BRAF mutations may induce resistance to anti-EGFR therapy (Section 2.2).

In this study we aimed to evaluate the expression of EGFR in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines and the effect of anti-EGFR inhibitors (Gefitinib and Cetuximab) in these cell lines using MTS assays. All cell lines were also evaluated for EGFR TK, KRAS and BRAF mutation status.

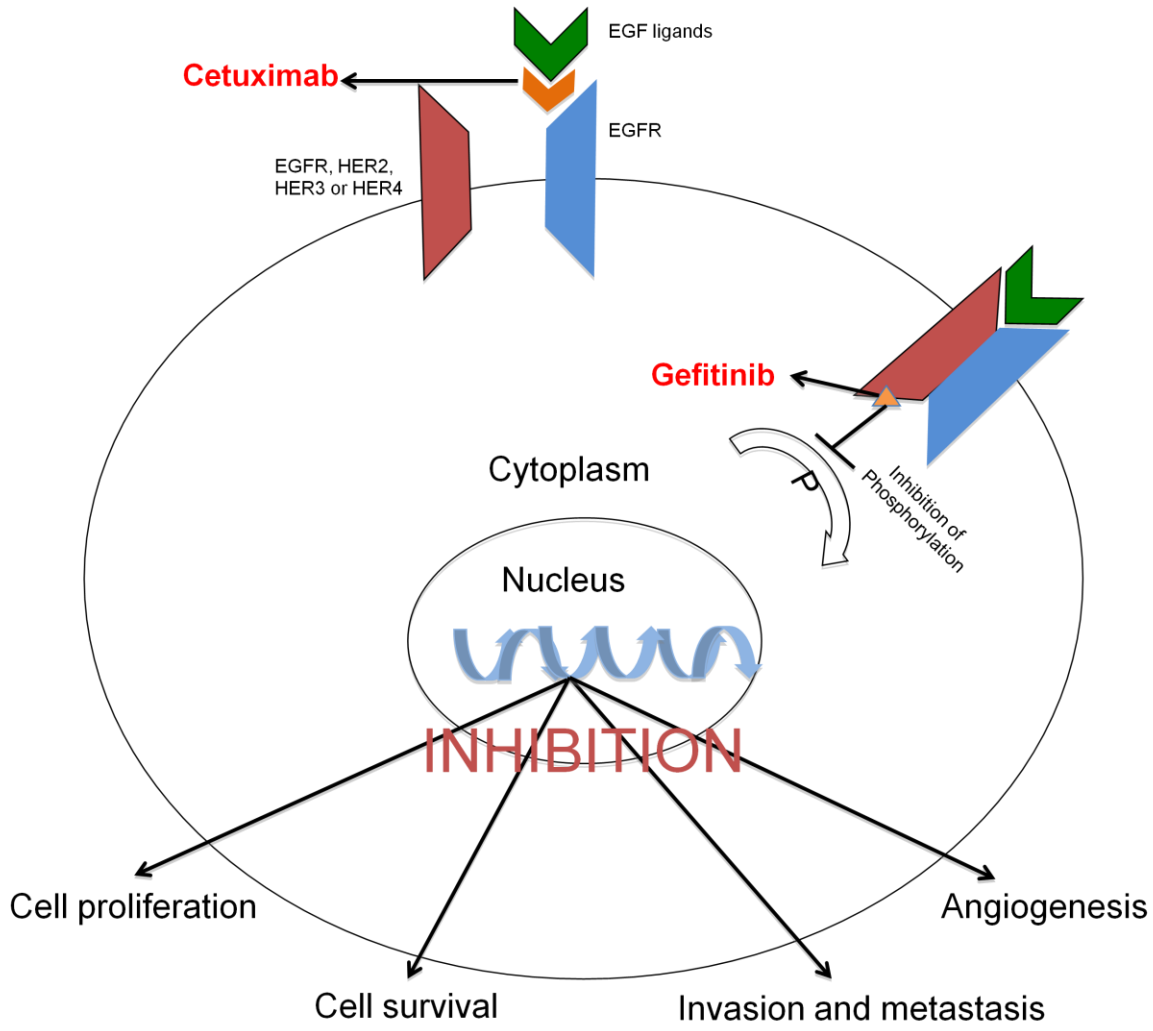

Figure 9.1 The mechanism of action of Cetuximab and Gefitinib. Cetuximab targets the extracellular domain of the EGFR and competes with the EGF ligand for binding, thus preventing EGFR dimerisation and tyrosine kinase phosphorylation. Gefitinib acts on the intracellular component of the EGFR and competes with the ATP at the tyrosine kinase domain and inhibit EGFR auto-phosphorylation.

9.2 **Materials and Methods**

Mesothelioma cell lines MSTO-211H, NCI-H2052 and NCIH2452 were obtained from the American Type Culture Collection (ATCC) and the lung cancer cell line (A549) was obtained from the European Collection of Cell Cultures (ECACC). All cell lines were maintained in RPMI 1640 medium as detailed in Section 6.1.1.

Anti-EGFR antibody was obtained from Abcam (cat no ab2430) for western blotting and from BD Pharminogen (cat No 555997) for flow cytometry (**Table 6.1**)

Cetuximab was obtained from Merck KGaA, Germany and Gefitinib (Cat. No. 3000) was purchased from Tocris Bioscience (Sections 6.1.3.1 and 6.1.3.5). MTS reagent was obtained from Promega (CellTiter 96® AQueous One Solution Cell Proliferation Assay; G3582; Promega, Madison, WI).

9.2.1 **Western blot**

Western blotting was performed to analyse the status of the EGFR using anti-EGFR antibody (ab2430, Abcam) as described in section 6.2.5.

9.2.2 **Flow cytometry**

Flow cytometry was used to identify EGFR expression in the cell lines as detailed in section 6.2.7. One million cells of each cell line was incubated with EGFR antibody (BD Pharminogen; cat No 555997) along with an isotype control and analysed on the BD FACSAria^{TM} II using the FACSDiva software.

9.2.3 **Mutational analysis**

MSTO-211H, H2052, H2452 and A549 cell lines were analysed for EGFR, KRAS and BRAF mutations, as lack of EGFR activating mutations or the presence of EGFR resistant mutation, KRAS mutation or BRAF mutation may induce resistance to anti-EGFR

therapies. All mutational analysis was performed by the Department of Cellular Pathology, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK.

9.2.3.1 **EGFR mutation**

EGFR mutational screening was performed using the Amplification Refractory Mutation System (ARMS) and Scorpions techniques to identify common mutations using real time Polymerase chain reaction (PCR). The EGFR exons 18 to 21 was also sequenced using Sanger sequencing (Section 2.2.1.1).

9.2.3.1.1 Arms-Scorpion PCR

EGFR mutational screening analysis was performed in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines using the Therascreen® EGFR RGQ PCR Kit (Qiagen, Germany, catalogue number 870111). Among the common mutations identified in the EGFR domain (Section 2.2.1.1) the kit enables identification of 29 of the commonest mutations in the EGFR gene, including the T790M mutation known to induce resistance to EGFR TKIs (**Figure 9.2**).

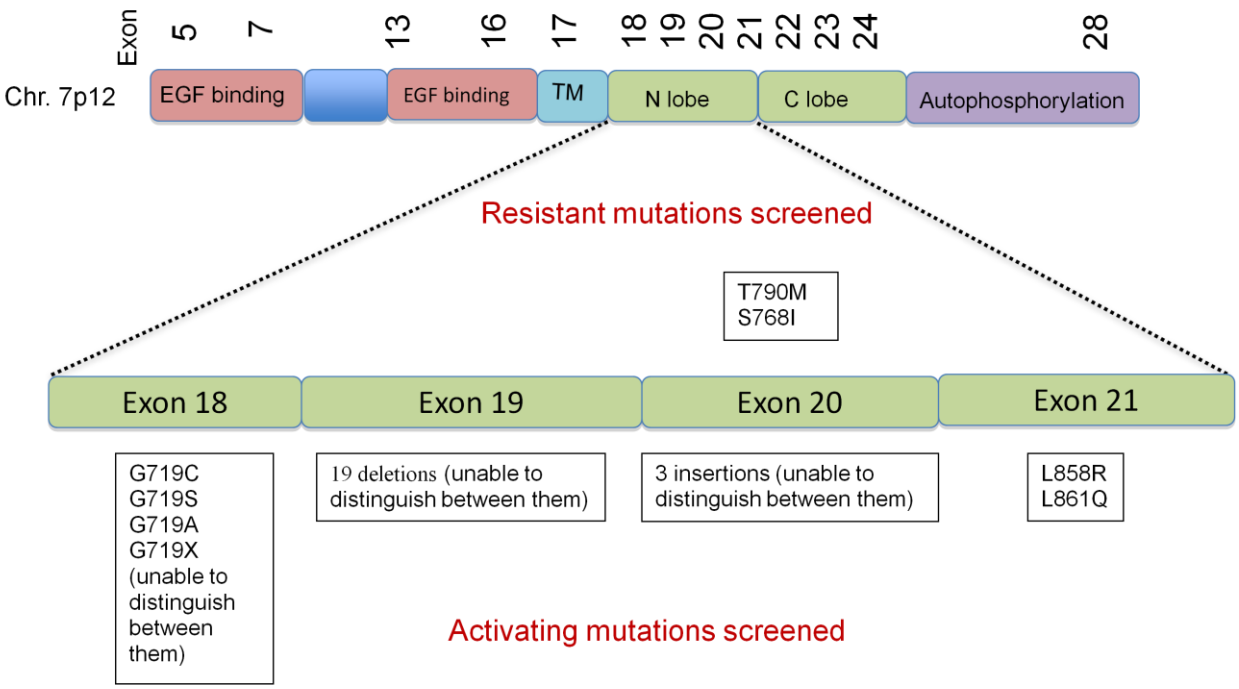

Figure 9.2 EGFR mutation analysis. This figure demonstrates all the EGFR mutations analysed by the Qiagen Therascreen EGFR RGQ PCR Kit. The presence of activating mutations is associated with good response to EGFR TKIs and the presence of resistant mutations is associated with resistance to EGFR TKIs. (Adapted and modified from (Sharma, Bell et al. 2007).

9.2.3.1.2 Sanger Sequencing

EGFR exons 18 to 21 was sequenced using bidirectional Sanger sequencing with primers designed using Primer-3 software.

9.2.3.2 **KRAS mutation**

KRAS mutational screening analysis was performed in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines using the Therascreen® KRAS Pyro Kit (Qiagen, Germany, catalogue number 971460). The Therascreen® KRAS Pyro Kit is a nucleic acid sequence-

based detection test based on pyrosequencing technique for the quantitative analysis of mutations in codon 12, 13 and 61 of the human KRAS gene (**Figure 9.3**). The kit consists of two assays: one for analysis of codon 12 (GGT) and 13 (GGC) which were sequenced in the forward direction and the second for codon 61 (CAA) which was sequenced in the reverse direction (Section 2.2.1.2 for mutation details).

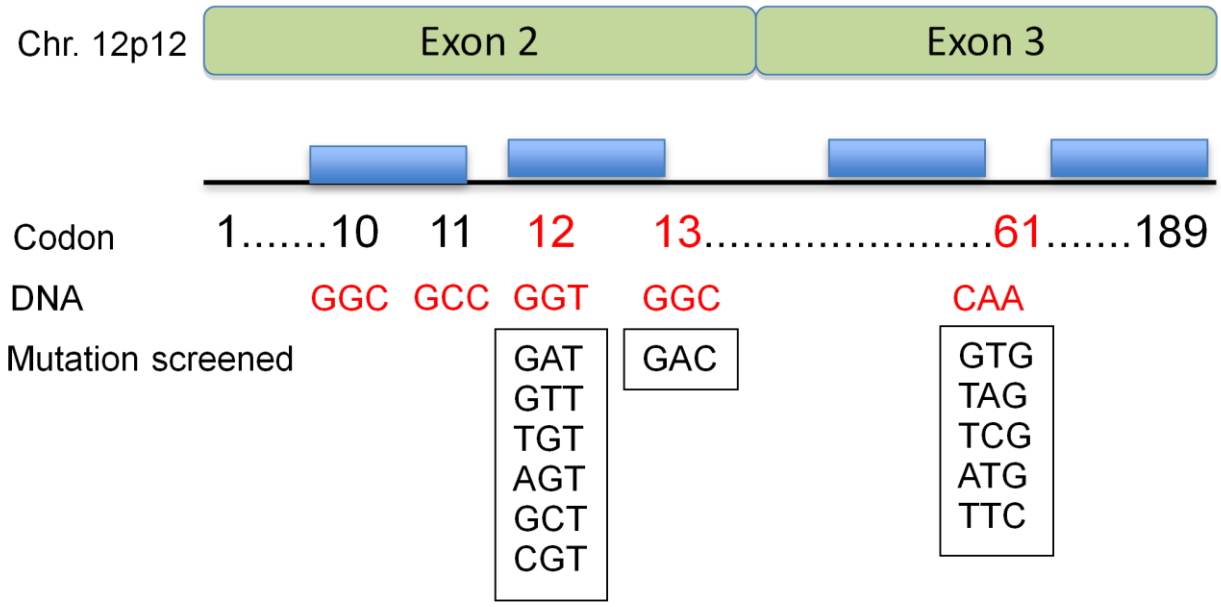

Figure 9.3 KRAS mutation analysis. KRAS mutations identified using the Therascreen KRAS Pyro Kit. The presence of KRAS mutations may induce resistance to anti-EGFR therapy.

9.2.3.3 **BRAF mutation**

BRAF mutational screening analysis was performed in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines using the Therascreen® BRAF Pyro Kit (Qiagen, Germany, catalogue number 971470). Therascreen® BRAF Pyro Kit, similar to the KRAS kit, is also a nucleic acid sequence-based detection test based on pyrosequencing technique for the

quantitative analysis of mutations in codon 600 (GTG) of the human BRAF gene and is sequenced in reverse direction (Section 2.2.4).

9.2.4 **MTS Assay**

All cell lines were cultured as detailed in section 6.2.1 and cells counted as detailed in section 6.2.2. The optimum number of cells for each cell line (MSTO-211H - 5000 cells per well, NCI-H2052 – 1000 cells per well, NCI-H2452 – 1000 cells per well and A549 – 1000 cells per well) was determined (see appendix A) and seeded onto a 96 well plate as detailed in section 6.2.3. MTS assay was performed as detailed in section 6.2.4. In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated 3 times. Cells were then exposed to varying concentrations of Cetuximab and Gefitinib from day 2 onwards for 72 hours. The drugs and media were replaced daily. Media only was used as a control for Cetuximab and 0.01% DMSO diluted in media was used as a control for Gefitinib (Section 6.2.4.1.1). The growth of treated cells as a percentage of control cells was calculated (see appendix A). The average of the three experiments was taken, plotted onto a graph and 50% cell growth inhibition was calculated for each drug. The drug concentration of Cetuximab taken for the assay ranged from 100 nM to 1.75μM. At the FDA approved dosing level of Cetuximab, the mean steady state peak concentration ranges from 168 to 235 μg/ml (Mukohara, Engelman et al. 2005). Based on the upper limit of 235 μg/ml, the molar concentration calculated for Cetuximab was 1.6 μM hence 1.75 μM was arbitrarily taken as the maximum concentration in our study. The drug concentration of Gefitinib (20nM to 20 μM) was optimised. The concentration of Gefitinib calculated based on the mean steady state plasma concentrations (615 ng/ml) at the FDA approved dose was 1.4 μM (Herbst, Maddox et al. 2002; Mukohara, Engelman et al. 2005). A study by Nutt *et al* (2009) had demonstrated 50% cell growth inhibition in

MSTO-211H cells to Gefitinib at a dose of 18.3 0.7 μM and therefore we extended our dose range of Gefitinib to a maximum of 20 μM (Nutt, O'Toole et al. 2009). Drug inhibition was considered significant if at least 50% cell growth inhibition was seen compared to control.

9.3 **Results**

9.3.1 **EGFR expression by Western blot**

Despite using the EGFR antibody in various dilutions (ranging from 1:1000 to 1:200) with various incubation periods (2 hours at room temperature and 16 hours at 4 ^{0}C) we were unable to optimise the antibody for use in the cell lines. A549 cells have previously been shown to express EGFR by western blotting and hence were used as an external control (Tracy, Mukohara et al. 2004).

9.3.2 **EGFR Expression by Flow Cytometry**

All cell lines demonstrated positivity for EGF receptor. Among the 10,000 cells assessed for each cell line, 98.6% of A549; 98.4% of MSTO-211H; 98.2% of NCI-H2052 and 98.2% of NCI-H2452 cells showed positive expression for EGFR (**Figure 9.4**).

9.3.3 **Mutation analysis**

9.3.3.1 **EGFR mutation analysis**

There were no mutations identified in the EGFR gene in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines using the Arms-Scorpion PCR technique. The whole Exon sequenced using the Sanger sequencing did not reveal any known or unknown novel mutations in any of the cell lines.

9.3.3.2 **KRAS mutation analysis**

A single missense mutation (codon 12 GGT>AGT; protein.Gly12Ser) was identified at codon 12 of the KRAS gene in A549 cell line (**Figure 9.5**). The MSTO-211H, NCI-H2052 and NCI-H2452 cell lines did not harbour any KRAS mutations.

9.3.3.3 **BRAF mutation analysis**

The MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines did not harbour any BRAF mutations.

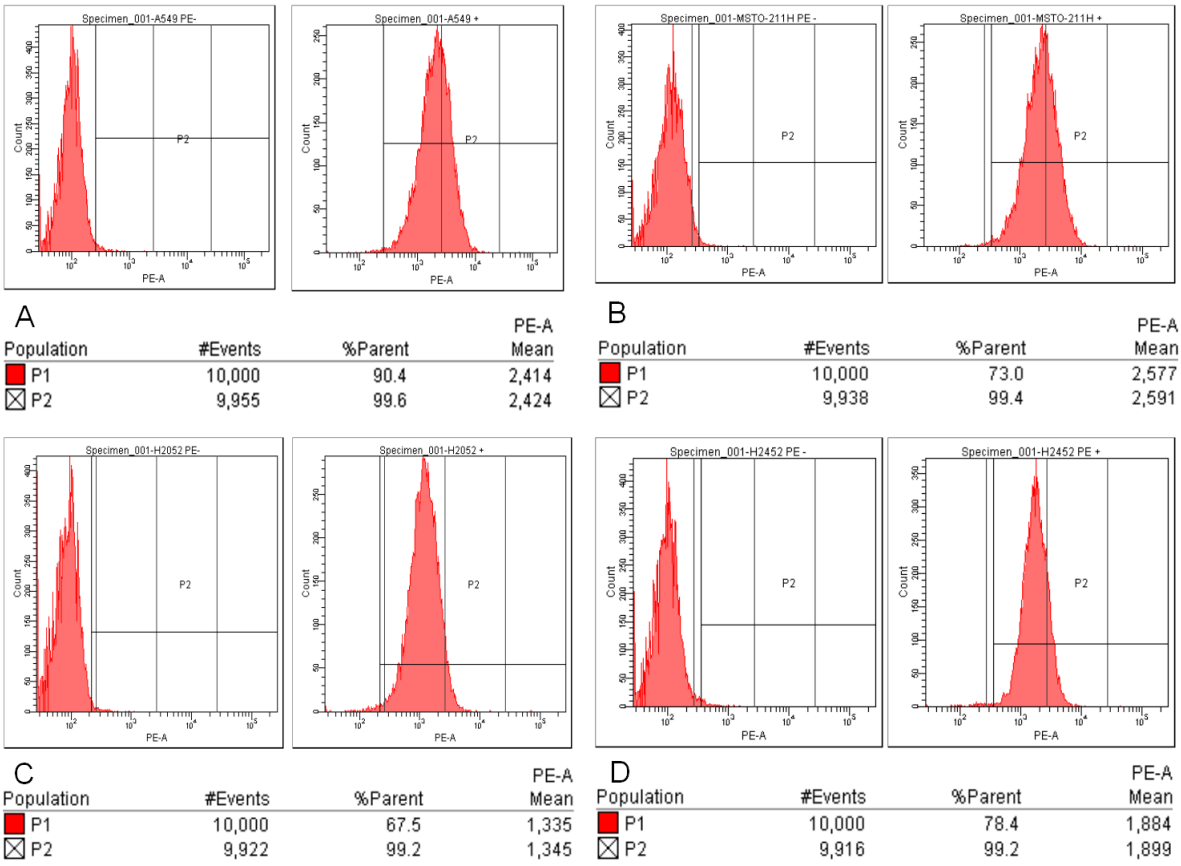

Figure 9.4 EGFR expression in MPM cell lines. This is a histogram plot demonstrating positive EGFR expression in (A) A549; (B) MSTO-211H; (C) NCI-H2052 and (D) NCI-H2452 cell lines. Each histogram consists of two regions in the X axis. The region on the left is where the cells negative for EGFR expression (including the isotype control) are

represented and the region on the right (marked as P2) is where the cells positive for EGFR expression are represented. For each pair of histograms, the graph on the left is a PE conjugated isotype control with P2 marker set to contain ≤1% of cells. The histogram on the right is PE conjugated antibody against EGFR showing cells within the P2 region expressing EGFR. The X-axis demonstrates the relative fluorescence and the Y-Axis demonstrates the number of cells analysed.

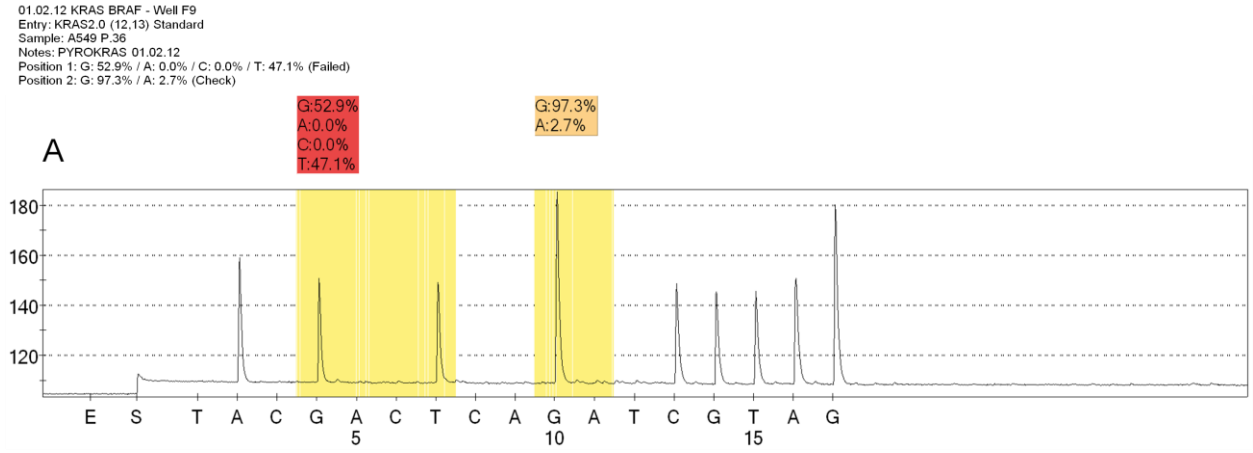

Figure 9.5 A549 cell line demonstrating the presence of missense KRAS mutation at codon 12. This Figure demonstrates the first peak at A followed by G and then T which represents the mutated sequence AGT at codon 12 (wild type sequence GGT).

9.3.4 **MTS Assay**

All cell lines showed 50% cell growth inhibition by Gefitinib at varying concentrations (**Table 9.1**), but at doses higher than 1.4 μM, which was the concentration of Gefitinib calculated based on the mean steady state plasma concentrations at the FDA approved dose (**Figure 9.6**). At higher concentration of Gefitinib (>5 μM) the NCI-H2052, NCI-H2452 and MSTO-211H cells demonstrated growth inhibition values less than 0%. This would

indicate that the number of cells was less than that of the control at the starting point, thereby demonstrating a cytotoxic effect. Cetuximab showed no significant growth inhibition in NCI-H2052, NCI-H2452 and A549 cell lines (**Table 9.1**), but MSTO-211H cells were sensitive to Cetuximab and 50% cell growth inhibition was seen at a concentration of 1.6 µM, which was within the clinically relevant dose range (**Figure 9.7**).

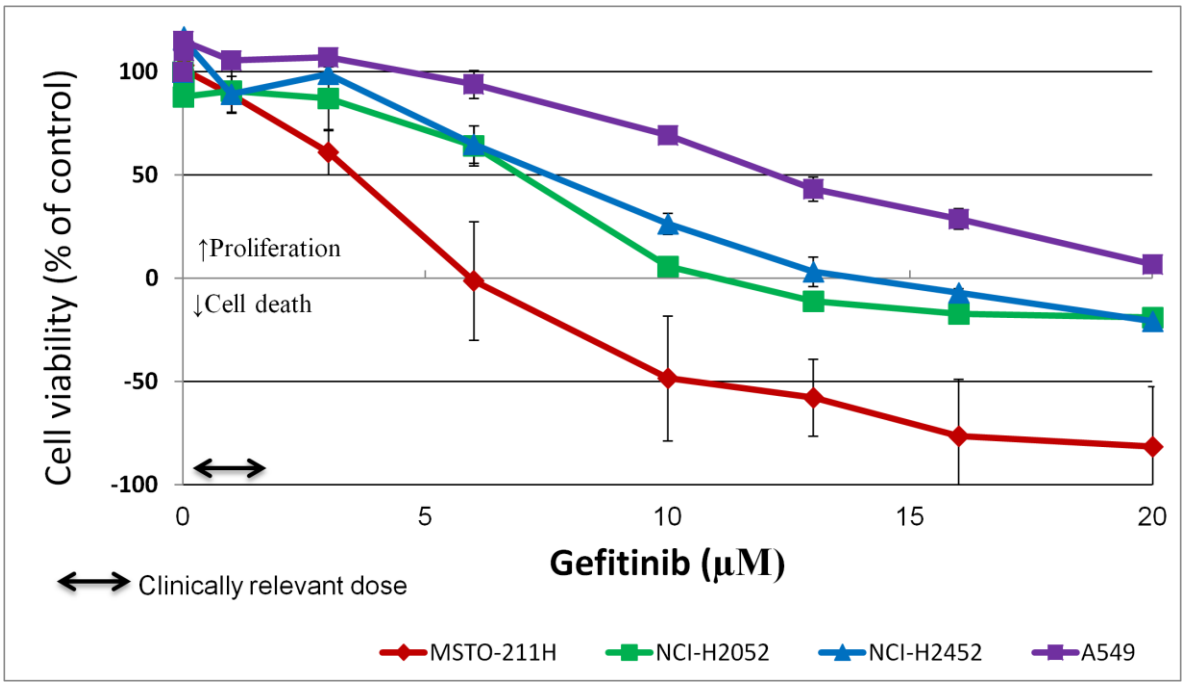

Figure 9.6 The antiproliferative activity of Gefitinib. The data presented is a mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control (0.01% DMSO) and the error bars indicate standard error of the mean.

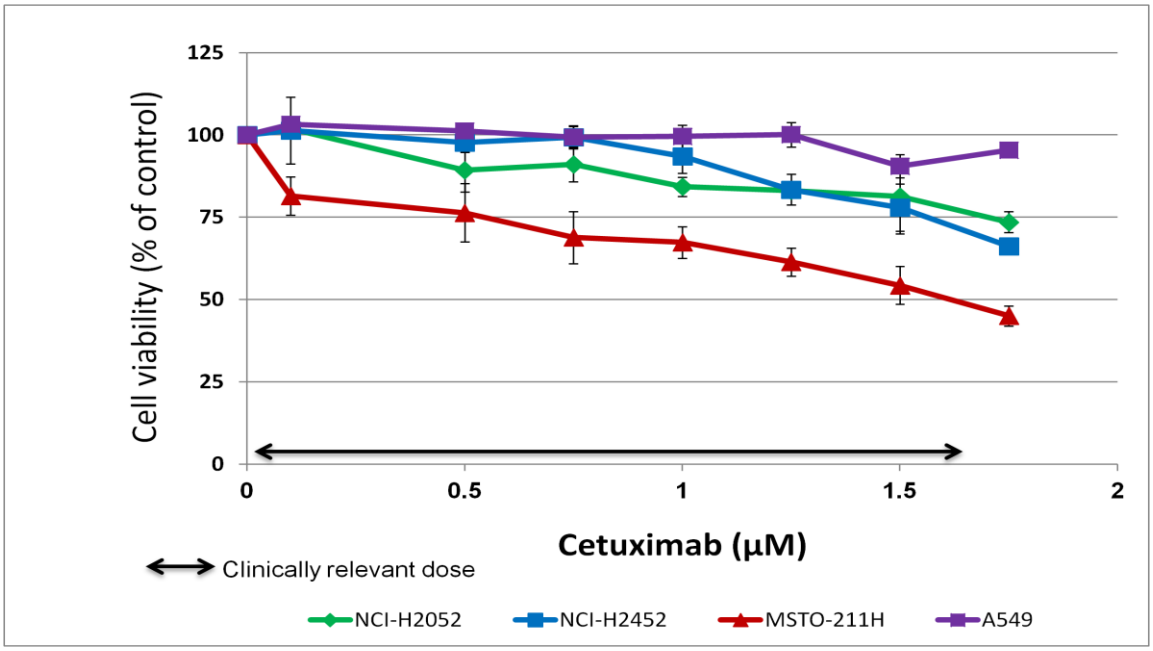

Figure 9.7 The antiproliferative activity of Cetuximab. The data presented is a mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control (media only) and the error bars indicate standard error of the mean.

Table 9.1 Growth inhibition due to Gefitinib and Cetuximab. This table demonstrates the 50% cell growth inhibition values achieved in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines on incubation with Gefitinib and Cetuximab, for 72 hours and assessed by MTS assay. Value in bold is within the clinically relevant dose range. EGFR protein expression of the cell lines is also shown. (NA; Not achieved)

	Gefitinib	**Cetuximab**	**EGFR protein**
NCI-H2052	6 µM	NA	Expressed
NCI-H2452	3.7 µM	NA	Expressed
MSTO-211H	1.5 µM	**1.63 µM**	Expressed
A549	13 µM	NA	Expressed

9.4 **Discussion**

In this study, flow cytometry analysis demonstrated more than 98% EGFR expression in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines. Western blotting was unsuccessful in demonstrating the expression of EGFR in the cells lines, including the control A549 cell line. The western blot technique is well established in the laboratory. Since the flow cytometry demonstrated near universal expression of EGFR in all cell lines (including control) the lack of expression in western blotting likely to be due to an unreliable antibody. Other techniques that could have been used to demonstrate expression of EGFR proteins are immunocytochemistry and ELISA (Section 5.5). In addition EGFR gene amplification and polysomy can be analysed using techniques such as Polymerase Chain Reaction and fluorescent *in situ* hybridization (FISH). A549 has previously been shown to express EGFR when analysed by flow cytometry (Janmaat, Kruyt et al. 2003) as well as by western blot (Tracy, Mukohara et al. 2004). The NCI-H2052, NCI-H2452 and MSTO-211H cell lines have also been shown to express EGFR when analysed by flow cytometry (Nayak, Garmestani et al.). Various other authors have also demonstrated positive expression of EGFR in A549 and MSTO-2111H cell lines by western blot (Janne, Taffaro et al. 2002; Nutt, O'Toole et al. 2009). Western blot analysis of MPM cells (H2461 and H226) has demonstrated that the treatment of cells with EGF results in phosphorylation of EGFR (p-EGFR) and its downstream signalling proteins such as AKT (p-AKT) and ERK1/2 (p-ERK) (Janne, Taffaro et al. 2002; Nutt, O'Toole et al. 2009).

In our study there were no EGFR, KRAS or BRAF mutations identified in NCI-H2052, NCI-H2452 and MSTO-211H cell lines. A single KRAS mutation (codon 12 GGT>AGT; protein.Gly12Ser) was identified in the A549 NSCLC cell line. Other studies have previously identified the same KRAS mutation in the A549 cell line (Krypuy, Newnham et

al. 2006). This KRAS mutation may result in constitutive activation of the RAS/RAF/MAPK pathway thereby inducing resistance to EGFR inhibition by Cetuximab (Sections 2.2.1.2 and 2.2.3). A549 cell line have also shown to have a wild type EGFR TKI domain with no activating mutations thereby demonstrating resistance to TKIs (Tracy, Mukohara et al. 2004; Krypuy, Newnham et al. 2006). Currently there are no published studies evaluating the EGFR, KRAS and BRAF mutation status of NCI-H2052, NCI-H2452 and MSTO-211H cell lines.

In our study, 50% cell growth inhibition was induced by Cetuximab in the MSTO-211H cell line at the dose of 1.6 μM, which was the upper limit of the mean steady state peak concentration achieved in Phase I trials at FDA approved dosing levels (Mukohara, Engelman et al. 2005). At the maximum dose of 1.75 μM, NCI-H2052, NCI-H2452 and A549 cell lines did not demonstrate any significant antiproliferative effects. Gefitinib demonstrated antiproliferative effects in all cell lines, but at doses significantly higher than that achieved based on the mean steady state plasma concentrations at FDA approved dose levels (Herbst, Maddox et al. 2002; Mukohara, Engelman et al. 2005). To date, there are no published studies investigating the anti-proliferative effect of Cetuximab in MPM cell lines. Antibodies such as Cetuximab, have various modes of action *in vivio*, such as receptor blockade, down regulation of receptors due to internalization and degradation or by activating the host immune system resulting in antibody-dependent cell mediated cytotoxicity (Kurai, Chikumi et al. 2007; Chames, Van Regenmortel et al. 2009). Hence, *in vitro* assessments, by means of MTS assays in cell line models for example, may not accurately assess the effectiveness of antibody treatments and any interpretation of the effectiveness of antibodies may need further confirmation using in vivo studies.

The A549 cell line has been show to be resistant to the antineoplastic effects of Cetuximab and Gefitinib using the MTT assay (Janmaat, Kruyt et al. 2003). In another study, the A549 cell line was found to be resistant to the effects of Cetuximab and Gefitinib when analysed using the MTS assay (Mukohara, Engelman et al. 2005). Tracy *et al* (2004) also demonstrated lack of effect of Gefitinib in A549 cell line using the MTS assay (Tracy, Mukohara et al. 2004).

In the study by Mukohara *et al* (2005) the dose of Gefitinib required for antiproliferative effect on NSCLC cell lines (HC3255, DFCILU-011 and PC-9) with EGFR TKI activating mutation present when analysed using MTS assay, ranged from 10 to 63 nM which was significantly less than that seen in our study with any cell line (Mukohara, Engelman et al. 2005). The A549 cell line with wild type EGFR was resistant to the anti proliferative effects of Gefitinib. In our study Gefitinib resulted in 50% growth inhibition only at significantly higher doses compared to clinically relevant dose level. This may suggest that our MPM cell lines may be resistant to the anti-proliferative effects of Gefitinib, which may be due to the presence of wild type EGFR TKI domain or the presence of mutations downstream of the EGFR signalling pathway (**Figure 2.6**).

There is a paucity of information available in the literature regarding the effect of Gefitinib in MPM cell lines (MSTO-211H, NCI-H2052, NCI-H2452). Giovannetti *et al* (2011) studied the effect of Gefitinib and Erlotinib among other drugs in MSHO-211H, NCI-H2052, NCI-H2452 and NCI-H28 cell lines using the MTT assay. The dose of Gefitinib required to inhibit 50% of cells was; MSTO-211H – 4.91 μM; NCI-H2052 – 5.22 μM, NCI-H2452 – 4.83 μM and NCI-H28 – 3.99 μM (Giovannetti, Zucali et al. 2011). This was roughly similar to dose of Gefitinib required to inhibit 50% of cells our study in NCI-H2052 (6 μM) and NCI-H2452 (3.7 μM), but not in MSTO-211H (1.5 μM) cells when

analysed using MTS assays. Another study by Janne *et al* (2002) demonstrated no effect of Gefitinib on either A549 or MSTO-211H cell lines with Gefitinib dose up to 10 μM when analysed by MTS assay (Janne, Taffaro et al. 2002). In our study the dose of Gefitinib required to inhibit 50% of the A549 cells was 13 μM. The study published by Nutt *et al* (2009) demonstrated the effect of Gefitinib among other drugs in MPM cell lines (MSTO-211H, NCI-H28 and NCI-H226) using a SulphurRhodamine cell proliferation assay. Growth inhibition was seen in all cell lines, but the dose required for 50% growth inhibition was significantly high (MSTO-211H – 18.3 μM, NCI-H28 – 5.7 μM and NCI-H226 – 20.5 μM) in comparison to the clinically relevant dose level or to that in our study in MSTO-211H cells (Nutt, O'Toole et al. 2009).

9.5 **Conclusion**

In this study, we demonstrated for the first time that there are no EGFR, KRAS or BRAF mutations identified in MSTO-211H, NCI-H2052 and NCI-H2452 cell lines. A single KRAS (codon 12 GGT>AGT; protein.Gly12Ser) mutation was seen in the A549 cell line. The role of Cetuximab in MPM had not been previously evaluated. In our study, Cetuximab demonstrated an anti-proliferative effect in the MSTO-211H cell line. All other cell lines were resistant to the effect of Cetuximab. The resistance to Cetuximab in A549 cells may be due to the presence of KRAS mutation. Gefitinib did demonstrate cytotoxic effect in all of the cell lines, but at dose levels that were significantly higher in comparison to the clinically relevant dose level. This lack of cytotoxic effect of Gefitinib may be as a result of absence of activating EGFR mutations. The resistance to anti-EGFR therapy may be overcome by multi targeted therapy. In Chapter 12 we aimed to overcome anti-EGFR therapy resistance by combining Cetuximab and Gefitinib in turn with MTOR inhibitors (Rapamycin and Ku0063794) and COX-2 inhibitor (Celecoxib).

Chapter 10. Inhibition of Mammalian Target of Rapamycin in malignant pleural mesothelioma cells

Publications and presentations

Aqarwal V, Lind MJ, Cawkwell L (2011) Growth Inhibition of Mammalian Target of Rapamycin (MTOR) in Malignant Pleural Mesothelioma. European journal of cancer (Oxford, England : 1990) 47: S140-S141(abstract). Poster - ECCO 17 - 36th ESMO Multidisciplinary Congress, Stockholm (September 2011)

Chapter aim

To evaluate the expression of PTEN protein in MPM cell lines (NCI-H2052, NCI-H2452, MSTO-211H) and NSCLC cell line (A549).

To evaluate the effect of MTORC1 and MTORC2 inhibition using Rapamycin (MTORC1 inhibitor) and Ku0063794 (MTORC1 and MTORC2 inhibitor) in MPM cell lines using the MTS assay.

10.1 **Introduction**

Activation of the PI3K/AKT/MTOR pathway plays an important role in many tumour types including MPM. Immunohistochemical analysis of MPM tissue samples has demonstrated that the pAKT and pMTOR are over expressed and the tumour suppressor gene, PTEN, is lost in a significant proportion of cases (**Table 3.1**). MTOR can be assembled into two different complexes (MTORC1 and MTORC2). MTORC1 is sensitive to the inhibitory effects of Rapamycin, whereas MTORC2 is Rapamycin insensitive.

In this study we aimed to analyse the cytotoxic effect of MTORC1 inhibition using Rapamycin and the effect of combined MTORC1 and MTORC2 inhibition using Ku0063794 in MPM cell lines using the MTS cell proliferation assay (**Figure 10.1**). Since loss of PTEN results in activation of the MTOR pathway and has been shown to be a poor prognostic biomarker, we also assessed the status of PTEN protein in our cell lines using Western blotting. To further confirm the subsequent activation of the MTOR pathway as a result of loss of PTEN, p70S6K status of the cell lines was also assessed as a surrogate marker for MTORC1 activation.

10.2 **Materials and methods**

The MPM cell lines MSTO-211H, NCI-H2052 and NCI-H2452 were obtained from the American Type Culture Collection and the lung cancer cell line (A549) were obtained from the European Collection of Cell Cultures. All cell lines were maintained in RPMI 1640 medium as detailed in section 6.1.1.

The MTORC1 inhibitor Rapamycin (cat no 1292), and the combined MTORC1/MTORC2 inhibitor Ku0063794 (cat no 3725) were purchased from Tocris Biosciences (Sections 6.1.3.7 and 6.1.3.8).

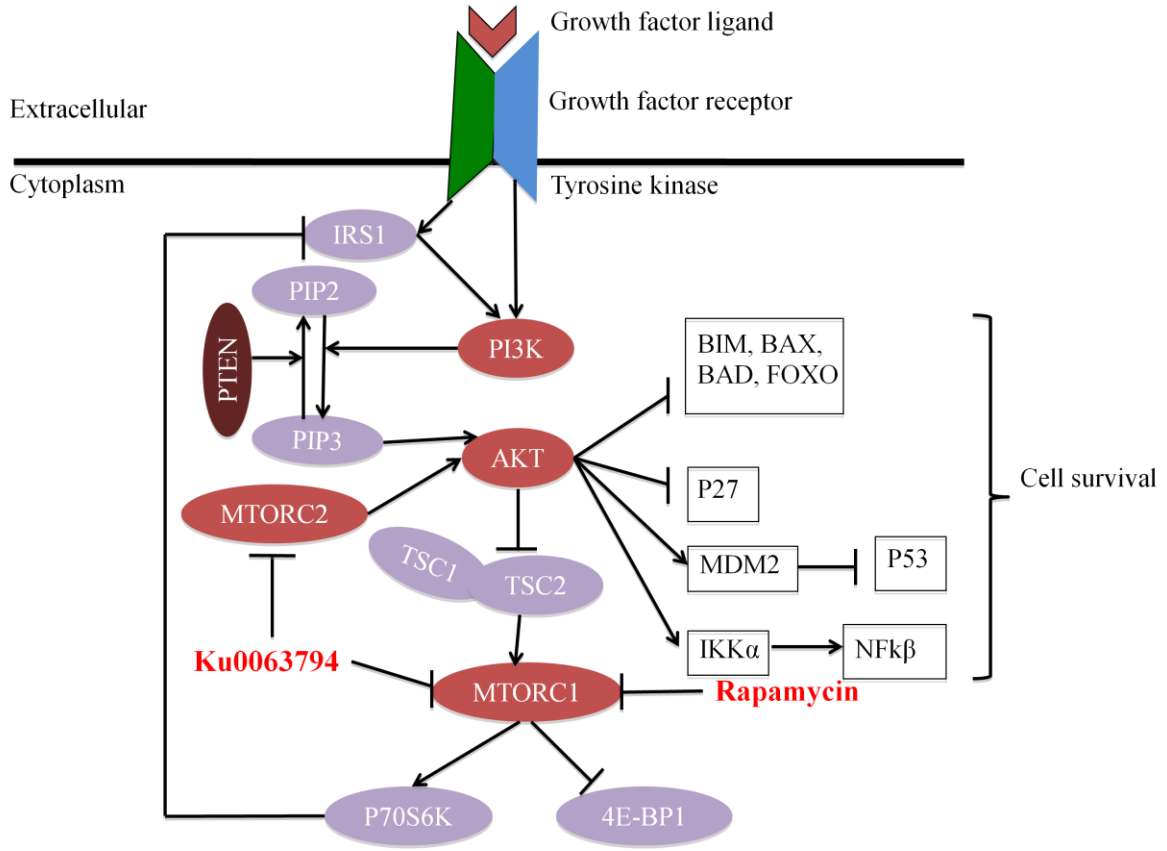

Figure 10.1 Mechanism of action of Rapamycin and Ku0063794. Rapamycin inhibits MTORC1 where as Ku0063794 is a kinase inhibitor and inhibits both MTORC1 and MTORC2.

10.2.1 **Western Blot**

Western blotting was performed to analyse the status of the PTEN protein and subsequent p70S6K activation as a result of loss of PTEN in the cell lines as detailed in section 6.2.5. Protein lysate (20µg) 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 (phospho T389) antibody (ab32359, Abcam) (**Table 6.1**)

10.2.2 **MTS Assay**

All cell lines were cultured and counted as detailed in section 6.2.1 and section 6.2.2. The optimum number of cells (Appendix A) for each cell line (MSTO-211H - 5000 cells per well, NCI-H2052 – 1000 cells per well, NCI-H2452 – 1000 cells per well and A549 – 1000 cells per well) was seeded onto a 96 well plate as detailed in section 6.2.3 and MTS assay was performed as detailed in section 6.2.4. In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated 3 times. The drug concentration of Rapamycin and Ku0063794 taken for the assay ranged from 100 pM to 1μM. The concentration of Rapamycin calculated based on the mean steady state plasma concentrations of 12.7 ng/ml at the maximum tolerated dose of 6 mg per day was 13.9 nM (Jimeno, Rudek et al. 2008). The clinically relevant dose range of Ku0063794 is currently not known. Control used was 0.01% DMSO. Drug inhibition was considered significant if at least 50% cell growth inhibition was seen in the drug treated cells compared to the drug carrier control containing 0.01% DMSO.

10.3 **Results**

10.3.1 **Western Blot**

PTEN protein was absent in the MSTO-211H cell line, weakly expressed in the A549 cell line and strongly expressed in NCI-H2052 and NCI-H2452 cells. The p70S6K protein was expressed in MSTO-211H and A549 cells, but not in NCI-H2052 and NCI-H2452 cells (**Figure 10.2**).

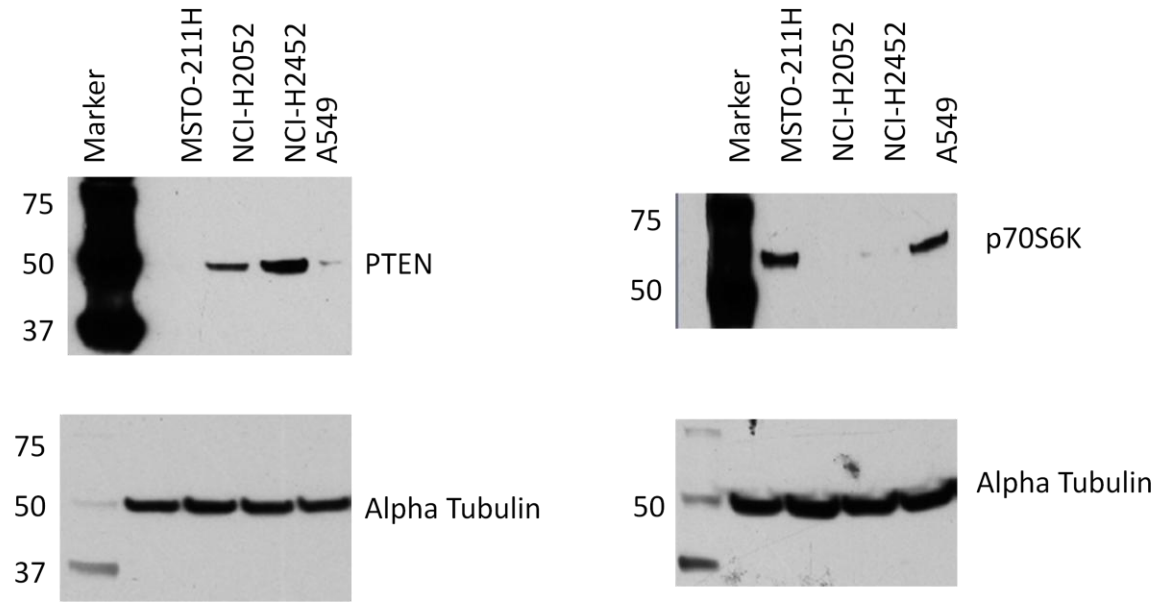

Figure 10.2 Western blot demonstration of the status of PTEN protein and p70S6K protein. Western blot demonstrating absence of PTEN protein expression in the MSTO-211H cells, weak expression in the A549 cells and strong expression in NCI-H2052 and NCI-H2452 cells. The p70S6K protein was present MSTO-211H and A549 cells and absent in NCI-H2051 and NCI-H2452 cells. The image shows results with anti-PTEN antibody (ab32199, Abcam) (1:400 dilution over 2 hours at room temperature) at 47 kDa and anti-p70S6K antibody (ab32359, Abcam) (1:1000 dilution over 16 hours at 4 ^{0}C) at 70 kDa. Alpha tubulin (ab7291, Abcam) was used as a loading control at 50 kDa indicating equal loading.

10.3.2 **MTS Assay**

Rapamycin (**Figure 10.3**) and Ku0063794 (**Figure 10.4**) demonstrated 50% cell growth inhibition in the NCI-H2052, NCIH2452 and A549 cells, but not in the MSTO-211H cells (**Table 10.1**). At concentration of Ku0063794 >800 nM, the NCI-H2052, cells demonstrated growth inhibition values of less than 0%. This would indicate that the number of cells was less than that of the control at the starting point, thereby demonstrating a cytotoxic effect.

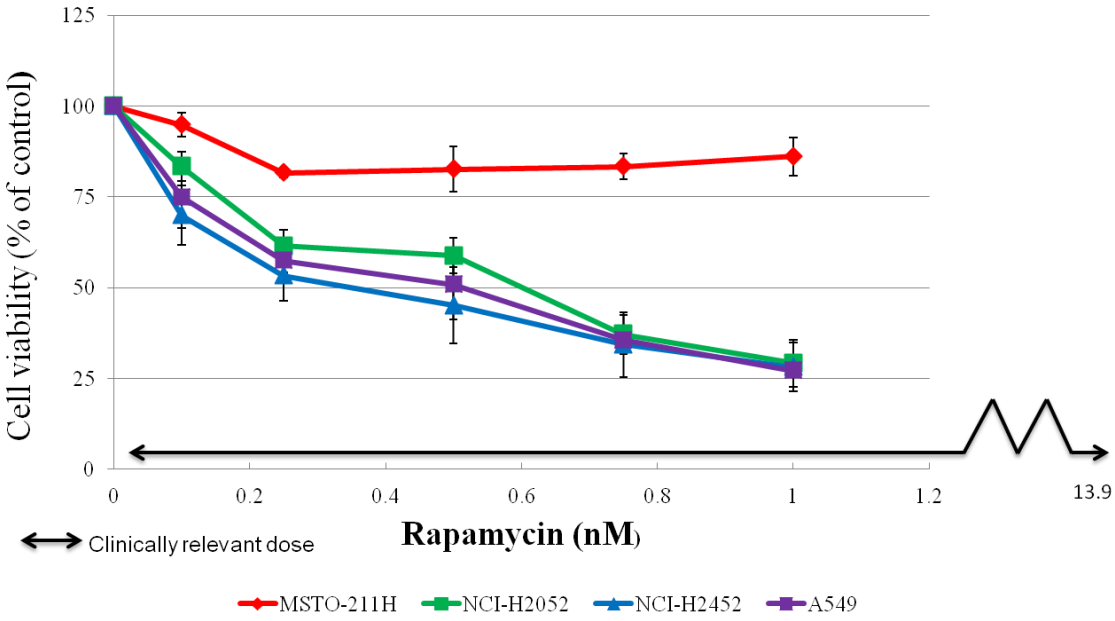

Figure 10.3 The antiproliferative activity of Rapamycin. The data presented is the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. Despite the dose used for Rapamycin ranged from 100 pM to 1μM, the data shown here is up to 1 nM as the maximum effect obtained was at this dose level. MSTO-211H did not show additional anti proliferative activity up to 1 μM. The maximum clinically relevant dose calculated was 13.9 nM.

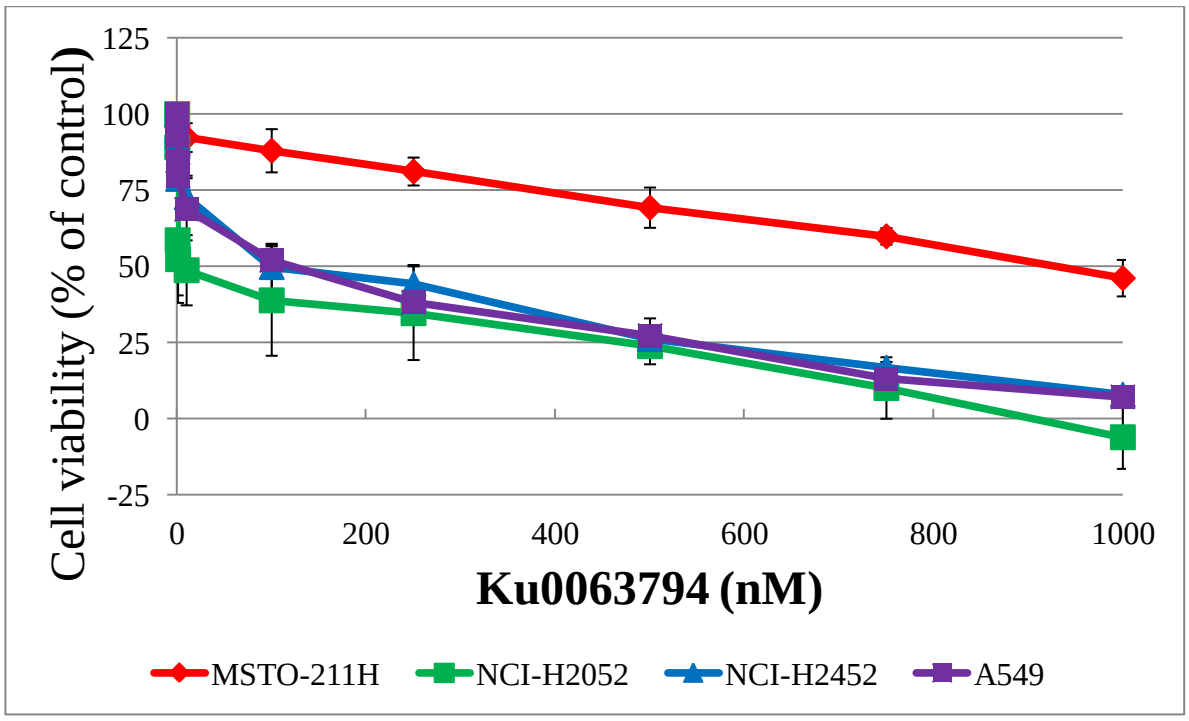

Figure 10.4 The antiproliferative activity of Ku0063794. The data presented the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean. Clinically relevant dose for Ku0063794 is not known.

Table 10.1 Growth inhibition due to Rapamycin and Ku0063794. This table demonstrates the 50% cell growth inhibition values achieved in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines on incubation with Rapamycin and Ku0063794, for 72 hours and assessed by MTS assay. Values in bold were within the clinically relevant dose range for Rapamycin. Clinically relevant dose range for Ku0063794 is currently not known. PTEN protein and p70S6K protein expression of the cell lines is also shown. (NA; Not achieved)

Cell lines	**Rapamycin (pM)**	**Ku0063794 (nM)**	**PTEN protein**	**P70S6K protein**
NCI-H2052	**610**	195	expressed	Absent
NCI-H2452	**517**	358	expressed	Absent
MSTO-211H	NA	NA	Absent	Expressed
A549	**553**	337	weakly expressed	Expressed

10.4 **Discussion**

In this study western blot demonstrated that PTEN protein was not expressed in MSTO-211H cells and weakly expressed in A549 cells resulting in activation of the MTORC1 pathway as demonstrated by expression of p70S6K in the MSTO-211H and A549 cell lines. The absence of PTEN protein expression in MSTO-211H cells may suggest that PTEN/AKT/MTOR pathway may be activated in these cells and may therefore respond to MTOR inhibitors.

Significant inhibition was however seen in the NCI-H2052, NCI-H2452 and A549 cells, but not in MSTO-211H cells with Rapamycin. The concentration of Rapamycin calculated based on the mean steady state plasma concentrations of 12.7 ng/ml at the maximum tolerated dose of 6 mg per day was 13.9 nM (Jimeno, Rudek et al. 2008). In our study, 50%

cell growth inhibition in NCI-H2052, NCI-H2452 and A549 cells was seen at approximately 500 - 600 pM range, which was significantly below the achievable plasma steady state concentration. In a study by Hartman *et al* (2010), of the six MPM cell lines (MS257, H2052, MS589, MS248, MS924 and JMN1B) studied, three MPM cell lines (MS248, MS924 and JMN1B) responded to 100 nM of Rapamycin when analysed using MTS assay (Hartman, Esposito et al. 2010). Despite NCI-H2052 cells not having shown any response in this study, our study demonstrated significant response in NCI-H2052 cells to Rapamycin. Rapamycin has also shown to inhibit a MPM cell line (M43) as well as enhance the apoptotic effect of various apoptotic regimes in MPM tumour fragment spheroids (Section 3.1.2.2).

Combined inhibition of MTORC1 and MTORC2 in MPM has previously not been assessed. In our study, 50% cell growth inhibition using Ku0063794 was seen at approximately 200- 350 nM range. Ku0063794 inhibits both MTORC1 and MTORC2 in Human embryonic kidney cells (HEK-293) with an IC50 of approximately 10 nM (Garcia-Martinez, Moran et al. 2009). In another study, Ku0063794 blocked the MTORC1 activity at 10 nM as evidenced by dephosphorylation of p70S6K and the MTORC2 activity at 100 nM as evidenced by dephosphorylation at AKT (Serine 473) in Human vein endothelial cells (HUVEC) (Dormond-Meuwly, Roulin et al. 2011).

Since the absence of PTEN protein expression and the expression of p70S6K protein was seen in the MSTO-211H cells we may have expected a response to MTOR inhibition. However, we found that the MSTO-211H cell line was not responsive to either MTORC1 alone or combined MTORC1 and MTORC2 inhibition. This may be because its oncogenic phenotype may be mediated via the AKT pathway further upstream of MTOR in MPM. The activation of MTORC1 results in the activation of p70S6K protein which inhibits IRS1

and reduces the activation of PI3K and AKT (**Figure 10.1**) (Shi, Yan et al. 2005). Inhibiting the MTORC1 protein may result in activation of AKT by inhibiting the p70S6K/IRS1/PI3K negative feedback loop. MPM tumour spheroids on treatment with Rapamycin have demonstrated an increase in AKT phosphorylation (Barbone, Yang et al. 2008). Loss of PTEN protein expression and the inhibition of negative feedback loop may have resulted in increased activation of AKT and contributed to the resistance of MSTO-211H cells to MTOR inhibition. However, this hypothesis will need to be validated in subsequent studies.

Currently there are two early phase clinical trials evaluating the benefit of Rapamycin analogues in MPM (Section 3.1.3). There are a few early phase clinical trials evaluating the effect of MTOR kinase inhibitors in advanced solid tumours, but none are MPM specific. MTOR kinase inhibitors currently undergoing early phase clinical trials are OSI-027 (OSI), Phase I; AZD-8055 (AstraZeneca), Phase I/II; AZD-2014 (AstraZeneca), Phase I and INK128 (Intellikine), Phase I (Zaytseva, Valentino et al. 2012).

Combined inhibition of EGFR and MTOR may be an important therapeutic strategy in overcoming resistance to either target alone. In epithelioid sarcoma cell lines (VAESBJ and Epi544), the combined effect of Erlotinib and Rapamycin was more pronounced compared to either drug alone demonstrating strong synergy when analysed using MTS assays as well as VAESBJ xenografts grown in mice (Xie, Ghadimi et al. 2011). It is currently not known if this combination of anti-EGFR therapy and anti-MTOR therapy may provide additive effect compared to either agent alone in MPM. In Chapter 12 we aimed to combine EGFR inhibitors (Gefitinib and Cetuximab) with MTOR inhibitors (Rapamycin and Ku0063794) to evaluate the additive effect of combined inhibition.

Chapter 11. Inhibition of Cyclooxygenase enzyme 2 in malignant pleural mesothelioma cells

Chapter aim

To evaluate the effect of COX-2 inhibition using Celecoxib in MPM cell lines using the MTS assay.

11.1 **Introduction**

COX-2 is an inducible enzyme which catalyses the conversion of Arachidonic acid to PG in response to proinflammatory or mitogenic signals. It is over-expressed in many solid tumours and is a potential target for therapeutic intervention (Gasparini, Longo et al. 2003; Hull 2005; Rao and Knaus 2008; Ghosh, Chaki et al. 2010). Inhibition of COX-2 has been shown to have a significant anti-tumour effect in many solid tumours (Ghosh, Chaki et al. 2010; Menter, Schilsky et al. 2010). Various studies have demonstrated that COX-2 is expressed in MPM tissue samples (**Table 4.1**). COX-2 inhibitors have significant anti-neoplastic effect in MPM cell lines as well as mouse models (**Table 4.2**).

Previous work at our laboratory has shown that specific COX-2 inhibitor DuP-697 has significant anti-proliferative effects when incubated with the mesothelioma cell lines (MSTO-211H and NCI-H2052) and the lung cancer cell line (A549) for 72 hours as assessed by the MTT assay (O'Kane, Eagle et al. 2010). DuP-697 was also shown to potentiate the cytotoxic effect of Pemetrexed by at least 4-fold in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines. Celecoxib is a specific COX-2 inhibitor that is commonly used in clinical practice and is derived from DuP-697 (**Figure 11.1**) (Blobaum and Marnett 2007). In this study we aimed to study the anti-proliferative activity of Celecoxib in mesothelioma cell lines.

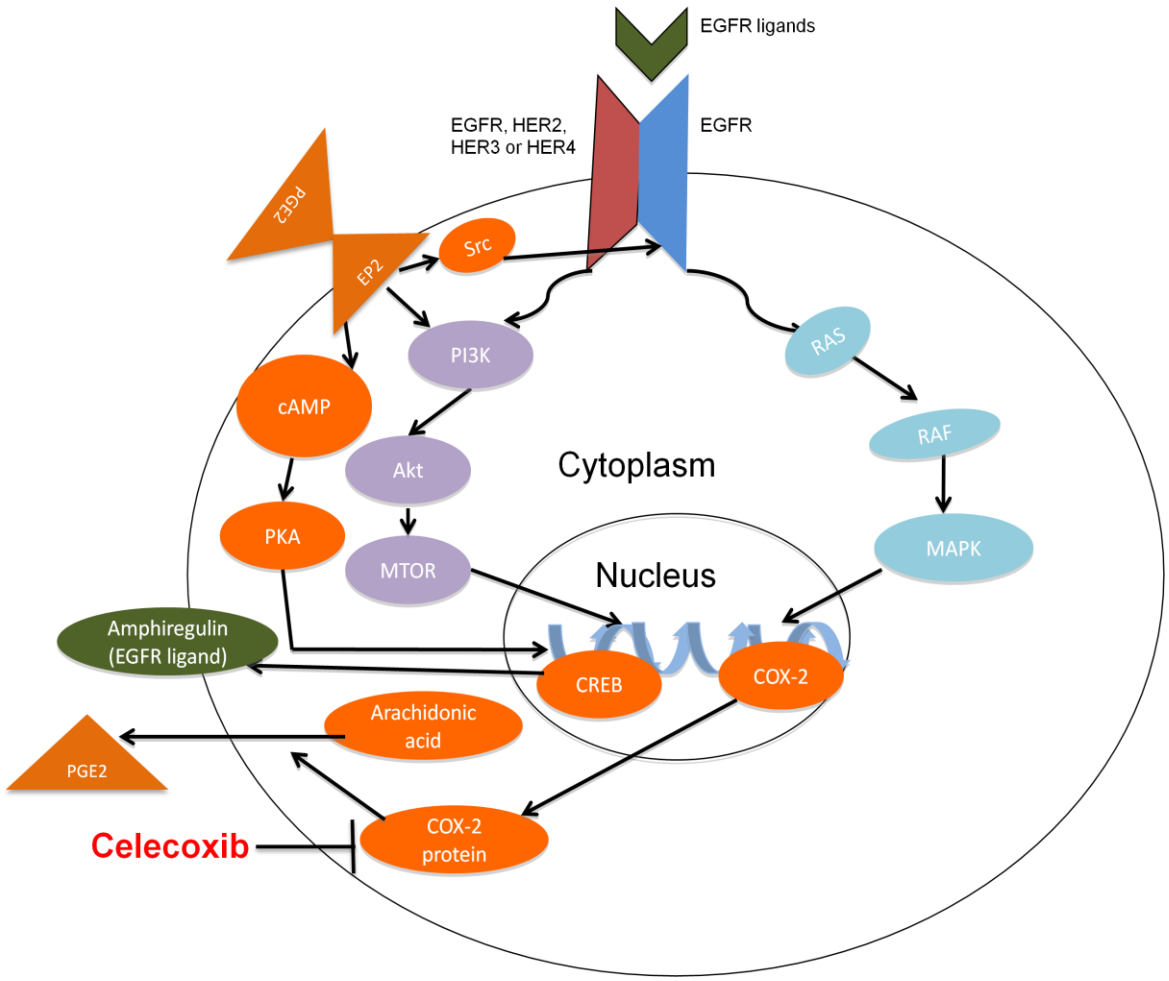

Figure 11.1 COX-2 and EGFR cross talk. This figure demonstrates the complex interaction between COX-2 and EGFR with the mechanism of action of Celecoxib. The EGFR on activation by its ligand activates the RAS/RAF/MAPK pathway resulting in increased production of COX-2 protein which converts Arachidonic acid to PGE2. PGE2 acts on the EP receptor and activates EGFR via Src, PI3K protein and increases the production of Amphiregulin via PKA. Amphiregulin is an EGFR ligand which activates EGFR thereby resulting in a positive feedback loop. Celecoxib acts by inhibiting COX-2 thereby reducing the production of PGE2 (Adapted and modified from (Dannenberg, Lippman et al. 2005).

11.2 **Materials and methods**

The MPM cell lines MSTO-211H, NCI-H2052 and NCI-H2452 were obtained from the American Type Culture Collection and the lung cancer cell line (A549) were obtained from the European Collection of Cell Cultures. All cell lines were maintained in RPMI 1640 medium as detailed in section 6.1.1. All cell lines have been previously shown to express the COX-2 protein (O'Kane, Eagle et al. 2010). Celecoxib was obtained from Pfizer (Section 6.1.3.9).

11.2.1 **MTS Assay**

All cell lines were cultured and counted as detailed in section 6.2.1 and section 6.2.2. The optimum number (see appendix A) of cells for each cell line (MSTO-211H - 5000 cells per well, NCI-H2052 – 1000 cells per well, NCI-H2452 – 1000 cells per well and A549 – 1000 cells per well) was seeded onto a 96 well plate as detailed in section 6.2.3 and MTS assay was performed as detailed in section 6.2.4. In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated 3 times. The drug concentration of Celecoxib ranged from 1 μM to 100 μM. Control used was 0.01% DMSO diluted in media. The concentration of Celecoxib calculated based on the steady state plasma concentrations of 1 μg/ml at the dose of 400 mg per day was 2.6 μM (Grossman, Olson et al. 2008). Drug inhibition was considered significant if at least 50% cell growth inhibition was seen compared to the drug carrier control containing 0.01% DMSO.

11.3 **Results**

Celecoxib (**Figure 11.2)** demonstrated 50% cell growth inhibition in the NCI-H2052, NCIH2452, MSTO-211H and A549 cell lines, but at doses higher than the maximum clinically relevant dose of 2.6 μM (**Table 11.1**). At higher concentration of Celecoxib (>40

μM) all cells demonstrated growth inhibition values less than 0%. This would indicate that the number of cells was less than that of the control at the starting point, thereby demonstrating a cytotoxic effect.

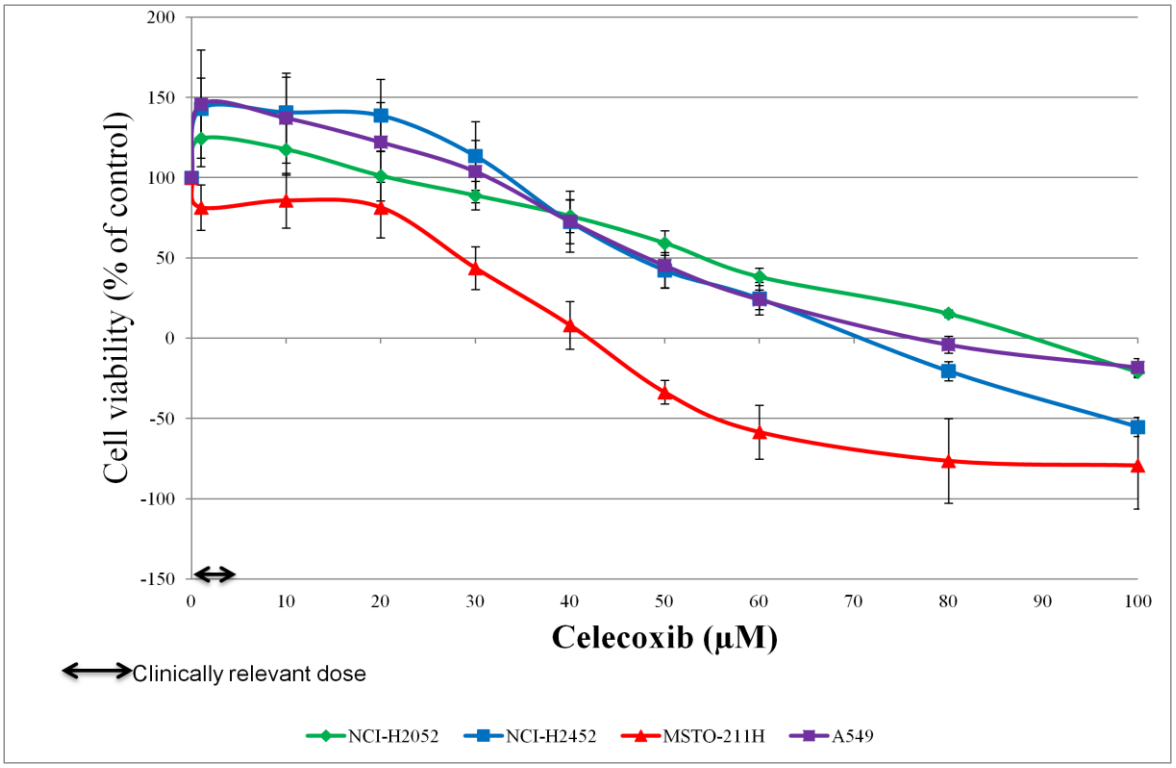

Figure 11.2 The antiproliferative activity of Celecoxib. The data presented is the mean of three independent experiments. Each data point is expressed as a percentage of cell growth relative to the control and the error bars indicate standard error of the mean.

Table 11.1 Growth inhibition due to Celecoxib. This table demonstrates the concentration of Celecoxib at which 50% cell inhibition was seen compared to control. Clinically relevant maximum dose for Celecoxib is 2.6 μM. COX-2 protein expression of the cell lines is also shown (O'Kane, Eagle et al. 2010).

Cell lines	Celecoxib (μM)	COX-2 protein
NCI-H2052	54	expressed
NCI-H2452	49	expressed
MSTO-211H	22	expressed
A549	53	expressed

11.4 **Discussion**

Celecoxib showed significant growth inhibition in all cell lines at a concentration of approximately 50 μM in NCI-H2052, NCI-H2452 and A549 cell lines and at 22 μM in MSTO-211H cell line which where all significantly higher than the clinically relevant dose range. Celecoxib has previously shown to inhibit mesothelioma cell lines by Catalano *et al* (2004) (Catalano, Graciotti et al. 2004). The mesothelioma cell lines MPP89, H-Meso and Ist-Mes1 and normal mesothelium cells were incubated with Celecoxib for 48 hours and cell growth inhibition was assessed using the MTS assay. The study demonstrated significant growth inhibition in the mesothelioma cell lines with the mean IC50 value of Celecoxib being 35+/- 12 μM. Normal mesothelium did not show any growth inhibition (Catalano, Graciotti et al. 2004). Celecoxib demonstrated anti-proliferative effects in A549 cells (24 hour incubation) at an IC50 value of 73 μM when evaluated using the MTS assay (Backhus, Petasis et al. 2005).

Schroeder *et al* (2004) evaluated the effect of Celecoxib in head and neck cancer cell lines expressing COX-2 protein (UMSCC-11A/11B, UMSCC-14A, UMSCC-17B, UMSCC-22B) and those not expressing COX-2 protein (UMSCC-14B, UMSCC-17A, UMSCC-22A) and was analysed using the MTT assay (Schroeder, Yang et al. 2004). The dose of Celecoxib required to inhibit PGE2 production by 89% in COX-2 positive cells (UMSCC-14A) was 0.1 μM. Celecoxib failed to inhibit PGE2 production in COX-2 negative cells (UMSCC-14B). Despite the dose of Celecoxib required to inhibit PGE2 production was 0.1 μM, the dose required to cause 50% growth inhibition ranged from 25 to 75 μM in all the cell lines irrespective of the COX-2 status of the cell lines. This suggests that Celecoxib may act as a cytotoxic agent in a manner independent of COX-2 / PGE2 inhibition.

Studies done at our laboratory have previously demonstrated that COX-2 inhibitors such as DuP-697 have significant cytotoxic effect in MPM cells and A549 cells. Celecoxib is derived from DuP-697 and is FDA approved for use in clinical practice and has a good safety profile. This study demonstrates that Celecoxib, similar to DuP-697, is also effective in MPM cells, but at doses higher than clinically achievable. The clinically relevant dose range for DuP-697 is currently not known.

Significant cross talk exists between the COX-2 and the EGFR pathways. Combined inhibition of EGFR and COX-2 may therefore enhance the therapeutic effect in comparison to single target inhibition. In a single study, five MPM cell lines (NCI-2452, MPP89, Ist-Mes-1, Ist-Mes-2 and MSTO-211) were incubated with Gefitinib and Rofecoxib to assess for synergy. The Ist-Mes-2 cells, but not the other cells, demonstrated synergistic effect to the combination when compared to either drug alone (Stoppoloni, Canino et al. 2010). It is yet unknown whether the combination of EGFR inhibitors (Monoclonal antibodies and

TKIs) and Celecoxib would enhance the cytotoxic effects of either agent when used alone in MPM cells. In Chapter 12 we aimed to combine EGFR inhibitors (Gefitinib and Cetuximab) with Celecoxib to evaluate the additive effect of combined inhibition.

Chapter 12. Overcoming resistance to EGFR inhibitors in malignant pleural mesothelioma

Publications and presentations

Agarwal V, Lind MJ, Cawkwell L (2012) Inhibition of EGFR, MTOR and COX pathways in mesothelioma cells. Lung Cancer 75: S22 (abstract). Poster - 10th Annual British Thoracic Oncology Group Conference, Dublin (January 2012)

Chapter aim

To identify if resistance to anti EGFR inhibition can be overcome by combining Cetuximab and Gefitinib individually with Rapamycin, Ku0063794 and Celecoxib in MPM cell lines using the MTS assay.

12.1 **Introduction**

EGFR, MTOR and COX-2 proteins are frequently up regulated in malignant pleural mesothelioma (**Table 2.2**, **Table 3.1** and **Table 4.1**). The results of the relevant protein expression and mutation of NCI-H2052, NCI-H2452, MSTO-211H and A549 cells obtained in our study are summarised in **Table 12.1**. The effectiveness of single agent Cetuximab, Gefitinib, Celecoxib, Rapamycin and Ku0063794 at clinically relevant dose in the cell lines obtained in our study are summarised in **Table 12.2**. Inhibition of EGFR using Cetuximab and Gefitinib has not demonstrated any significant effect in growth inhibition of MPM cells (Sections 9.3.4, 10.3.2 and 11.3). Resistance to Gefitinib may be due to lack of activating EGFR TK mutations in the MPM cells. Both the KRAS and the BRAF mutations were also not seen in MPM cell lines. Due to the absence of KRAS and BRAF mutations, we expected some anti-proliferative activity from Cetuximab, which was seen only in MSTO-211H cells, but not in NCI-H2052 and NCI-H2452 cells. The A549 cells harboured a single KRAS mutation, which may be one of the reasons for Cetuximab resistance in A549 cells. This suggests that resistance to Cetuximab may be mediated by downstream pathways other than the RAS/RAF/MAPK pathway. The PI3K/AKT/MTOR pathway is another downstream signalling pathway of the EGFR and may induce resistance to anti-EGFR therapy by being activated by other proteins such as PGE2, loss of PTEN or other growth factor receptors such as IGF-R and PDGFR. Significant cross talk exists between the EGFR, PI3K/AKT/MTOR and the COX-2/PGE2 pathways (**Figure 12.1**). Anti-EGFR therapy resistance may be induced by COX-2/PGE2 or by the activation of the PI3K/AKT/MTOR pathway. In this study we aimed to overcome EGFR resistance by combining EGFR inhibitors (Cetuximab and Gefitinib) with MTOR inhibitors (Rapamycin and Ku0063794) and COX-2 inhibitor (Celecoxib).

Table 12.1 Protein expression and mutations status of cell lines. This table demonstrates the protein expression and the mutation status of the NCI-H2052, NCI-H2452, MSTO-211H and A549 cells. The 'Section' refers to the paragraph in the text or reference where the full details can be obtained from.

	NCI-H2052	NCI-H2452	MSTO-211H	A549	Section
EGFR protein	Expressed	Expressed	Expressed	Expressed	9.3.2
PTEN protein	Expressed	Expressed	Absent	Weakly expressed	10.3.1
P70S6K protein	Absent	Absent	Expressed	Expressed	10.3.1
COX-2 protein	Expressed	Expressed	Expressed	Expressed	O'Kane (2010)
EGFR TK mutation	Absent	Absent	Absent	Absent	9.3.3.1
KRAS mutation	Absent	Absent	Absent	Present	9.3.3.2
BRAF mutation	Absent	Absent	Absent	Absent	9.3.3.3
PIK3CA	Absent	Absent	Absent	Absent	Janmaat (2006); Suzuki (2009).

Table 12.2 Single agent inhibition in cell lines. This table demonstrates the effectiveness of single agent Cetuximab, Gefitinib, Celecoxib, Rapamycin and Ku0063794 in NCI-H2052, NCI-H2452, MSTO-211H and A549 cells at clinically relevant dose. The clinically relevant dose of Ku0063794 is currently not known (?). The 'Section' refers to the paragraph in the text where the full details can be obtained from.

	NCI-H2052	NCI-H2452	MSTO-211H	A549	Section
Cetuximab	Not effective	Not effective	Effective	Not effective	9.3.4
Gefitinib	Not effective	Not effective	Not effective	Not effective	9.3.4
Celecoxib	Not effective	Not effective	Not effective	Not effective	11.3
Rapamycin	Effective	Effective	Not effective	Effective	10.3.2
Ku0063794	?Effective	?Effective	?Not effective	?Effective	10.3.2

12.2 **Materials and Methods**

12.2.1 **MTS assay**

The EGFR and COX-2 positive cell lines MSTO-211H, NCI-H2052, NCI-H2452 (mesothelioma) and A549 (lung cancer) were utilised. All cell lines were cultured and counted as detailed in section 6.2.1 and section 6.2.2. The optimum number (see appendix A) of cells for each cell line (MSTO-211H - 5000 cells per well, NCI-H2052 – 1000 cells per well, NCI-H2452 – 1000 cells per well and A549 – 1000 cells per well) was seeded onto a 96 well plate as detailed in section 6.2.3 and MTS assay was performed as detailed in section 6.2.4. In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated twice. Cells were incubated with either single agent Cetuximab or Gefitinib, and Cetuximab and Gefitinib in turn was combined with

Celecoxib, Rapamycin or Ku0063794. A fixed dose of Celecoxib, Rapamycin or Ku0063794 was added to a range of concentration of Cetuximab and Gefitinib to see if 50% growth inhibition could be achieved by the combination at clinically relevant dose of Cetuximab (1.6 µM) and Gefitinib (1.4 µM) (Section 9.2.4).

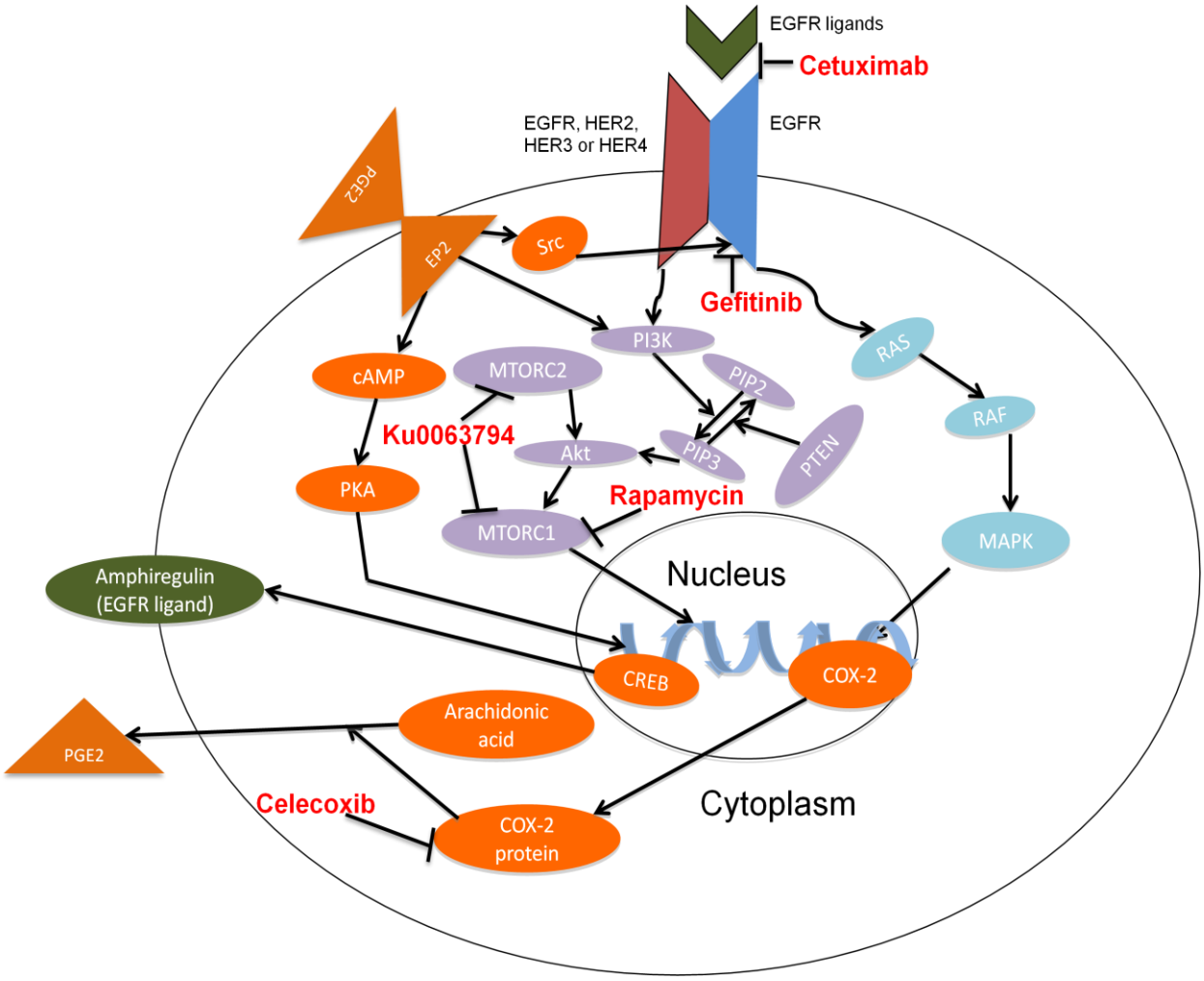

Figure 12.1 EGFR pathway interactions. This figure demonstrates the interaction between the EGFR, PI3K/AKT/MTOR and the COX-2/PGE2 pathway. The target proteins inhibited by Cetuximab, Gefitinib, Rapamycin, Ku0063794 and Celecoxib are also shown.

The concentration of Cetuximab used ranged from 100 nM to 1.75 µM and Gefitinib from 100nM to 10 µM. The maximum concentration for Gefitinib was reduced to 10 µM compared to 20 µM used in single agent studies (Section 9.2.4) since the maximum concentration of Gefitinib which resulted in 50% cell growth inhibition was 13 µM in A549 cells (**Table 9.1**). In MPM cells the dose of Gefitinib required for 50% cell growth inhibition was below or equal to 6 µM. To enhance the therapeutic value of Gefitinib by the combination, we expected the concentration of Gefitinib to fall below 10 µM. Cetuximab and Gefitinib were then combined with fixed doses of Celecoxib, Rapamycin and Ku0063794. The fixed concentrations for Celecoxib, Rapamycin and Ku0063794 were calculated based on their half maximal inhibitory concentration of the individual drugs in each cell line when used as single agents in Chapter 10 and 11.

12.2.2 **Statistics**

The half maximal inhibitory concentration of Celecoxib, Rapamycin and Ku0063794 for each cell line was calculated using GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, California) and 50% growth inhibition with the drug combinations were analysed as as detailed in section 6.2.4.2.1.

12.3 **Results**

The half maximal inhibitory concentrations of Celecoxib, Rapamycin and Ku0063794 calculated for MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines individually based on their anti-proliferative activity as single agents as described in Chapters 10 and 11 are detailed in **Table 12.3**.

Table 12.3 Inhibitory concentrations of drugs. This table demonstrates the half maximal inhibitory concentrations of Celecoxib, Rapamycin and Ku0063794 in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cell lines using GraphPad Prism 5 software.

	NCI-H2052	NCI-H2452	MSTO-211H	A549
Celecoxib	50 μM	50 μM	38 μM	42 μM
Rapamycin	541 pM	228 pM	2.6 nM	278 pM
Ku0063794	8 nM	40 nM	335 nM	31 nM

To evaluate the clinical implication of EGFR inhibition with Cetuximab and Gefitinib, and combination of Cetuximab and Gefitinib in turn with Celecoxib, Rapamycin and Ku0063794, we calculated the concentration of the combinations at which 50% cell growth inhibition was seen using XY scatter graph using Microsoft excel 2007 as detailed in section 6.2.4.2.1. The aim was to potentiate the cytotoxic effect of Cetuximab and Gefitinib by reducing the concentration required for 50% cell growth inhibition to less than the clinically relevant dose. The maximum clinically relevant concentration of Cetuximab calculated was 1.6 μM and Gefitinib was 1.4 μM (Section 9.2.4). In this study MSTO-211H cells did not respond to single agent Cetuximab. This was surprising as we had previously demonstrated in section 9.3.4 that single agent Cetuximab had anti-proliferative effect on MSTO-211H cells and the dose of Cetuximab required for 50% cell growth inhibition was 1.63 μM, which was at the upper limit of the clinically achievable dose range. As we were unable to replicate the response data we concluded that single agent Cetuximab was not effective in MSTO-211H cells. We found that the combination of Celecoxib and Cetuximab reduced the dose of Cetuximab required for 50% cell inhibition to less than the maximum clinically relevant dose of 1.6 μM in NCI-H2052 (1.5 μM) , NCI-H2452 (0.4

μM) and A549 (1.1 μM) cells, but not in MSTO-211H cells (**Figure 12.2 and Table 12.4**). The combination of Ku0063794 and Cetuximab reduced the dose of Cetuximab required for 50% cell inhibition to less than 1.6 μM in MSTO-211H (1.3 μM) cells but not in NCI-H2052, NCI-H2452 and A549 cells. No significant growth inhibition to clinically relevant dose was seen with Rapamycin and Cetuximab combination. The combination of Gefitinib and Celecoxib demonstrated enhanced cytotoxicity in all the cell lines, but the dose of Gefitinib required for 50% cell growth inhibition was above the maximum clinically relevant dose range of 1.4 μM (**Figure 12.3** and **Table 12.4**). No significant growth inhibition to clinically relevant dose was seen with either Rapamycin and Gefitinib combination or Ku0063794 and Gefitinib combination.

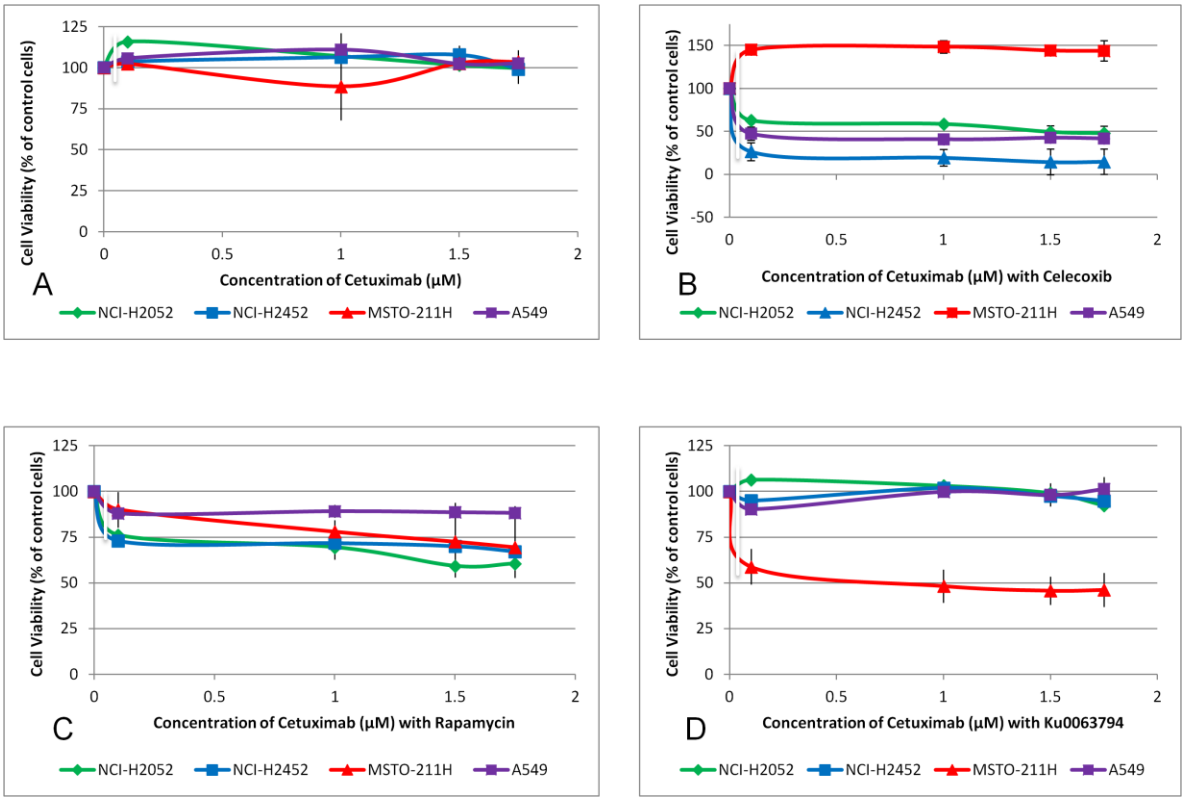

Figure 12.2 The anti-proliferative activity of Cetuximab combinations. The XY scatter graphs represent the anti-proliferative effect of Cetuximab (A), Cetuximab and Celecoxib

(B), Cetuximab and Rapamycin (C) and Cetuximab and Ku0063794 (D). The concentration of Celecoxib, Rapamycin and Ku0063794 used were as detailed in **Table 12.3**. The control used contained media only, hence the control values are disconjugated from the treated values.

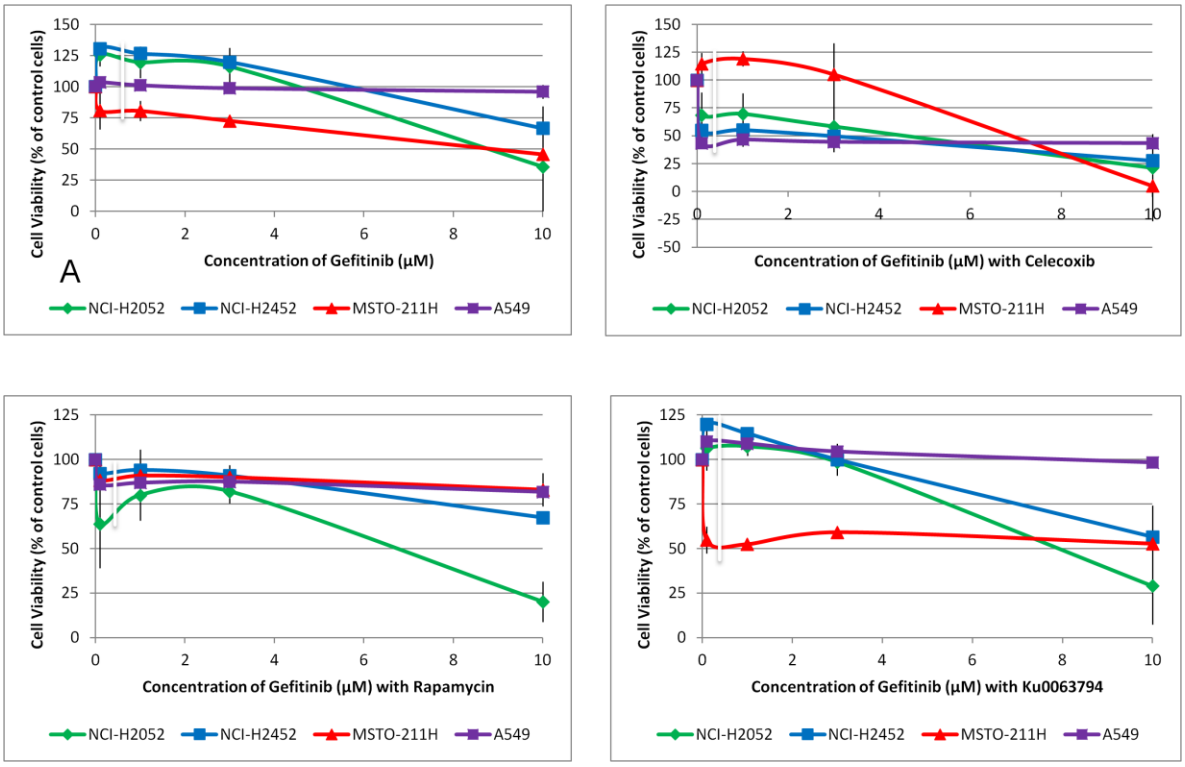

Figure 12.3 The anti-proliferative activity of Gefitinib combinations. The XY scatter graphs represent the anti-proliferative effect of Gefitinib (A), Gefitinib and Celecoxib (B), Gefitinib and Rapamycin (C) and Gefitinib and Ku0063794 (D). The concentration of Celecoxib, Rapamycin and Ku0063794 used were as detailed in **Table 12.3**. The error bars in graph A and D are wide at high Gefitinib doses of 10 µM. This could be as a result of having only 2 replicates in the experiments. The control used contained media only, hence the control values are disconjugated from the treated values.

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Table 12.4 Growth inhibition due to Cetuximab and Gefitinib combinations. This table demonstrates the concentration of Cetuximab and Gefitinib required for 50% cell growth inhibition as single agents and when combined in turn with Celecoxib, Rapamycin and Ku0063794. The values in bold are the concentration of Cetuximab below the clinically relevant dose of 1.6 μM. The maximum clinically relevant concentration of Gefitinib was 1.4 μM.

	NCI-H2052	NCI-H2452	MSTO-211H	A549
Cetuximab alone	Not achieved	Not achieved	Not achieved	Not achieved
Cetuximab+ Celecoxib	**1.5 μM**	**0.4 μM**	Not achieved	**1.1 μM**
Cetuximab+ Rapamycin	Not achieved	Not achieved	Not achieved	Not achieved
Cetuximab+ Ku0063794	Not achieved	Not achieved	**1.3 μM**	Not achieved
Gefitinib alone	9 μM	Not achieved	8.8 μM	Not achieved
Gefitinib + Celecoxib	5 μM	4.5 μM	6.4 μM	5.1 μM
Gefitinib + Rapamycin	5.9 μM	Not achieved	Not achieved	Not achieved
Gefitinib + Ku0063794	7.8 μM	Not achieved	9.9 μM	Not achieved

12.4 **Discussion**

In this study we demonstrated that single agent Cetuximab was not effective in MSTO-211H cells lines in contradiction to our findings in section 9.3.4. We demonstrated that the addition of Celecoxib to Cetuximab reduced the concentration of Cetuximab required for 50% cell growth inhibition to less than 1.6 μM, which was the maximum clinically relevant dose was taken in NCI-H2052 (1.5 μM) , NCI-H2452 (0.4 μM) and A549 (1.1 μM) cells. An enhanced growth effect was seen in the Cetuximab and Celecoxib combination when compared to single agent Cetuximab in one cell line (MSTO-211H) (**Figure 12.2**). This is likely to be an artifact as we could not explain this effect and it was not seen in any other cell lines. The combination of Ku0063794 and Cetuximab also reduced the concentration of Cetuximab required for 50% cell growth inhibition to less than 1.6 μM in MSTO-211H (1.3 μM) cells (**Figure 12.2**). When Rapamycin and Cetuximab was combined the concentration of Cetuximab required to achieve 50% cell growth inhibition was not reached at the maximum concentration of 1.75 μM.

Gefitinib when combined with Celecoxib, Rapamycin or Ku0063794 was unable to produce 50% cell growth inhibition within the clinically relevant dose in any of the cell lines.

The study by Stoppoloni *et al* (2010), in MPM cell lines (MPP89, 1st-Mes-1 and 1st-Mes-2) analysed the cytotoxic effect of Gefitinib and Rofecoxib as single agents and in combination (Stoppoloni, Canino et al. 2010). Gefitinib and Rofecoxib reduced the cell growth of all MPM cells when the cells were incubated with either Gefitinib or Rofecoxib as single agents and analysed using cell proliferation assays. However, when Gefitinib and Rofecoxib were combined, synergistic effects were seen only in 1st-Mes-2 cells. The combination of Gefitinib and Rofecoxib in MPP89 and 1st-Mes-1 cells was found to be

antagonistic in nature. In our study the combination of Celecoxib to Cetuximab enhanced the cytotoxic effect of Cetuximab in NCI-H2052, NCI-H2452 and A549 cells, but in MSTO-211H cells, resulted in a stimulatory effect. The exact reason for this variable effect with anti-EGFR therapy and anti-COX-2 therapy combination in MPM cells is currently not known and may need further evaluation.

There are no published studies evaluating the cytotoxic effects of Cetuximab and Celecoxib combination in MPM. A phase II clinical trial evaluating the effect of combined EGFR and COX-2 blockade using Cetuximab and Celecoxib in chemotherapy refractory metastatic colorectal cancers was terminated early due to lack of significant benefit of the combination compared to previously published Cetuximab monotherapy data (Chan, Lafleur et al. 2011). Chen *et al* (2008) evaluated the combined effect of Gefitinib and Celecoxib in three NSCLC cell lines (A549, GLC82 and SW1573) and demonstrated that the combination has additive effect in comparison to either drug alone when analysed using MTT assays (Chen, He et al. 2008). Similar additive effect was seen with Celecoxib and Gefitinib combination in five head and neck cancer cell lines (Tu177, Tu212, 212LN, 686LN and 886LN) when compared to either agent alone (Chen, Zhang et al. 2004). This is in keeping with our finding of significant enhanced cytotoxic effect when Celecoxib was combined with Gefitinib in A549 cells compared to single agent Gefitinib. Clinical studies in NSCLC have however failed to demonstrate the additive benefit of Celecoxib and Gefitinib combination compared to single agent Gefitinib (Gadgeel, Ruckdeschel et al. 2007; Agarwala, Fisher et al. 2008). One of the reasons for lack of anti-proliferative effect of Celecoxib and Gefitinib combination at clinically relevant doses in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cells may be due to lack of EGFR TK activating mutations (**Table 12.1**). Gadgeel *et al* (2007) treated EGFR TK mutation positive lung cancer cell lines (H3255 and H1650)

and wild type EGFR TK lung cancer cell line (H1781) with Gefitinib, Erlotinib or Celecoxib alone and the combination of Gefitinib or Erlotinib with Celecoxib and analysed the outcome using MTT assays (Gadgeel, Ali et al. 2007). The study demonstrated that Celecoxib significantly enhanced the cytotoxic effect of Gefitinib and Erlotinib in the mutation positive cell lines, but not in the cells with wild type EGFR.

Buck *et al* (2006) evaluated the synergistic effect of EGFR inhibitor Erlotinib and Rapamycin in 22 cell lines of a wide variety of cancers (NSCLC, pancreatic, colon, and breast cancer) (Buck, Eyzaguirre et al. 2006). Of the 22 cell lines 12 of them demonstrated significant synergism with the Rapamycin and Erlotinib combination. Among the 22 cell lines, five cell lines harboured PI3K or PTEN mutations and all the cell lines harbouring the PI3K or PTEN mutations demonstrated synergism to the Rapamycin and Erlotinib combination. In our study the combination of Cetuximab and Ku0063794 resulted in significant growth inhibition and the addition of Ku0063794 to Cetuximab reduced the dose of Cetuximab required for 50% cell growth inhibition to within clinically relevant dose in MSTO-211H cells. Single agent Ku0063794 has failed to demonstrate any significant activity in MSTO-211H cells (**Table 10.1**). MSTO-211H cells have demonstrated loss of PTEN protein expression and expression of p70S6K protein by western blot (**Table 12.1**). The inhibition of MTORC1 protein may result in feedback stimulation of AKT by inhibiting the p70S6K/IRS1/PI3K negative feedback loop (**Figure 10.1**) thereby inducing resistance to single agent Ku0063794 (Shi, Yan et al. 2005). Complete inhibition of AKT may be required by inhibiting the p70S6K/IRS1/PI3K negative feedback loop. The addition of Cetuximab to Ku0063794 may inhibit this feedback loop by inhibiting PI3K resulting in complete blockade to AKT. Other studies have demonstrated that the addition of Erlotinib can suppress Rapamycin induced

activation of AKT (Wang, Hawk et al. 2008). The molecular mechanism hypothesised will need to be validated in subsequent studies as no significant growth inhibition was seen with Cetuximab and Rapamycin combination or Gefitinib and Rapamycin / Ku0063794 combinations.

Chapter 13. Proteomic exploration of the molecular mechanism of action of the specific COX-2 inhibitor DuP-697

Acknowledgment

This part of the study was kindly supported by Yorkshire Cancer Research.

Contributions

Dr. Gina Eagle (PhD) - cell treatment and protein extraction

Dr. Victoria Hodgkinson (PhD) - antibody microarray analysis

Dr. Vijay Agarwal – Data mining and validation

Publications and presentation

Agarwal V, Hodgkinson VC, Eagle GL, Scaife L, Lind MJ, Cawkwell L. Proteomic (antibody microarray) exploration of the molecular mechanism of action of the specific COX-2 inhibitor DuP 697. International Journal of Oncology. (*In Press*)

Agarwal V, Hodgkinson VC, Eagle GL, Scaife L, Lind MJ, Cawkwell L (2010) Proteomic analysis of the mechanism of action of DuP-697 in mesothelioma cells. Oral presentation - The 10th International Conference of the International Mesothelioma Interest Group, Kyoto, Japan (August 2010).

Chapter aim

To study the mechanism of action of COX-2 inhibitor DuP-697 using antibody microarray, IPA, and western blot. This will also be an initial pilot study to see if antibody microarray can be used to identify mechanism of action of drugs.

13.1 **Introduction**

Previous work in our laboratory had demonstrated that COX-2 is over expressed in 59% (51/86) of archival malignant pleural mesothelioma tissue samples, a finding supported by similar studies (Marrogi, Pass et al. 2000; Edwards, Faux et al. 2002; Baldi, Santini et al. 2004; Cardillo, Spugnini et al. 2005; O'Kane, Cawkwell et al. 2005). The cytotoxic effect of COX-2 inhibitors has been demonstrated in mesothelioma cell lines (Marrogi, Pass et al. 2000; Catalano, Graciotti et al. 2004) and recently we reported that specific COX-2 inhibitors, including DuP-697, induce anti-proliferative effects in mesothelioma cell lines that the cytotoxic effect of Pemetrexed chemotherapy can be enhanced by the addition of DuP-697 (O'Kane, Eagle et al. 2010). Several COX-2 inhibitors which are currently used in clinical practice, including Celecoxib and Rofecoxib, are derived from DuP-697 (Blobaum and Marnett 2007).

DuP-697 is therefore worthy of further clinical investigation. However the molecular mechanism of action of DuP-697 has not been widely studied. In normal proliferating human umbilical vein endothelial cells (HUVECs) expressing low levels of COX-2, DuP-697 was shown to induce apoptosis and this was associated with the upregulation of caspase 3, 8 and 9 (Churchman, Baydoun et al. 2007). In the K562 chronic myeloid leukaemia cell line, DuP-697 induced G1-S cell cycle arrest and apoptosis with upregulation of caspase 8 (Peng, Zhang et al. 2008). These hypothesis-driven studies suggest that the mechanism of cytotoxic action of DuP-697 may be via induction of apoptosis. We aimed to explore, using a novel proteomic platform, the molecular mechanism of action of this compound using cell lines derived from solid tumours. This

was a preliminary pilot study to see if antibody microarray technique can be used to identify mechanism of action of drugs.

13.2 **Materials and Methods**

13.2.1 **Cell line treatments and protein extraction**

The cell treatment and protein extraction was performed by Dr. Gina Eagle (PhD). DuP-697 was previously demonstrated to have a cytotoxic effect in the COX-2 positive mesothelioma cell line MSTO-211H and in the lung cancer cell line A549, which was originally selected as a COX-2 positive control cell line (O'Kane, Eagle et al. 2010). In order to induce a visible cytotoxic effect in DuP-697 treated cells (50% reduction in cell numbers compared to untreated control), MSTO-211H and A549 cells were treated with 31.7 µM and 50 µM DuP-697 (#1430, Tocris Bioscience) respectively, for 72 hours. Drug carrier (dimethyl sulfoxide; DMSO) only was added to control (untreated) cells. Protein extraction from control cells and cells treated with DuP-697 was performed for subsequent antibody microarray and western blot analysis. This was achieved by suspending cells in 1 ml of 'Buffer A', which is a lysis buffer provided in the antibody microarray kit, incubating on an end-over-end rotator for 5 minutes at 4 °C, and subsequent centrifugation, to yield a minimum of 1 mg protein in a total volume of 1 ml. Protein concentration was determined by the Bradford Assay (#B6916, Sigma Aldrich). For western blot, protein extraction was performed as described in section 6.2.5.1.

13.2.2 **Antibody microarray analysis**

The antibody microarray analysis was performed by Dr. Victoria Hodgkinson (PhD). The Panorama XPRESS Profiler725 antibody microarray Kit (Sigma Aldrich #XP725) was used for antibody microarray which consists of 725 antibodies, spotted in duplicate, onto a

nitrocellulose-coated glass microscope slide (see appendix E for list of antibodies). The expression of a wide variety of proteins was analysed, including those involved in apoptosis, cell cycle, signalling, proliferation and cell adhesion. Protein labelling, protein binding and image analysis were carried out in low-light conditions, using the method previously described (Smith, Watson et al. 2006; Hodgkinson, Elfadl et al. 2011).

13.2.2.1 **Protein Labelling**

Proteins were labelled with Cy3 (PA23001, GE Healthcare) [control] and Cy5 (PA25001, GE Healthcare) [treated] fluorescent dyes, according to manufacturer's instructions.

13.2.2.2 **Protein Binding**

Prior to protein binding, dye-to-protein molar ratios were determined for each sample to ensure successful labeling of proteins. These were calculated as specified in the antibody microarray kit protocol, and as recommended, samples were only used if the ratio was >2. Equal amounts of protein from each sample (50-150 µg) were mixed with array incubation buffer (supplied) then incubated with the slide for 45 minutes, protected from the light.

13.2.2.3 **Image Acquisition and Analysis**

The slide was scanned (GenePix Personal 4100A Microarray Scanner, Axon Instruments) and analysed using GenePix Pro (Axon Instruments) and Acuity software (Axon Intruments). Normalisation, based on the Lowess method, was performed within Acuity software to identify differentially expressed proteins (DEPs). For optimum quality control, spot criteria were applied to only include spots with <3 % saturated pixels, those which were not flagged as absent and those that had 'relatively uniform intensity' and were detectable above the background. Experiments which showed 'percentage substance matched' values ≥ 90 % were carried forward, to ensure only slides of good quality were

accepted for further analysis. Differentially expressed proteins were considered significant with a fold change ≥ 1.8, whilst fold changes ≥ 1.5 were also recorded for each experiment for use as supporting data (**Figure 13.1**) (Hodgkinson, Elfadl et al. 2011).

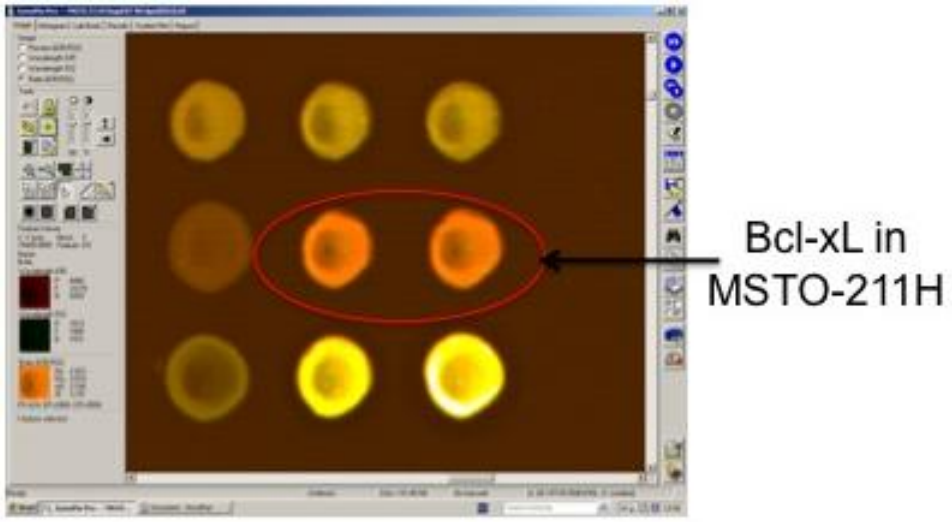


Figure 13.1 Antibody microarray slide demonstrating upregulation (red) of Bcl-xL in duplicate spots in MSTO-211H cells. Downregulated proteins would have been demonstrated as a green spot in duplicate and equal expression demonstrated as yellow spots in duplicate.

13.2.3 **Ingenuity Pathway Analysis**

Gene identifiers which corresponded to the DEPs were identified from the Ingenuity® Knowledge Base and the dataset was analysed through the use of Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) as detailed in section 6.2.8. The dataset containing gene identifiers of the DEPs was uploaded into the application and each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. Canonical Pathways Analysis was used to identify pathways from the IPA library that were most significant to the dataset.

13.2.4 **Western blotting**

Validatory dye-swap experiments were not performed during the antibody microarray analysis due to cost and sample availability. Therefore western blotting was used to determine and confirm the direction of protein expression change. Western blotting was carried out as detailed in section 6.2.5. In brief, proteins were extracted in Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 1% protease inhibitor mix and 0.00125% bromophenol blue) and 20 ug was electrophoresed on a 12% Precise gel (#25222, Pierce) at a constant voltage of 140 V for 40 minutes. Proteins were transferred using the iBlot dry transfer system (#IB3010-01, Invitrogen) onto nitrocellulose membrane. The membrane was blocked in 5% non fat dry milk dissolved in tris buffered saline containing 0.05% Tween 20. A primary antibody against BCL2L1 (Bcl-xL; #B9429, Sigma Aldrich) was applied at 1:5000 for 2 hours (**Table 6.1**). A primary antibody against BID (#ab32060, Abcam) was applied at 1:300 for 16 hours. As loading control, a primary antibody against alpha tubulin (#ab7291, Abcam) was applied at 1:2500 for 2 hours. The relevant secondary antibody (#SC-2030 or #SC-2031, Santa Cruz Biotechnology) was applied at 1:1000 for 1 hour and bands were detected using the Supersignal West Pico Chemiluminscent Substrate Kit (#34078, Pierce). Films were scanned using a GS800 calibrated densitometer (Bio-Rad) with Quantity One software (Bio-Rad). Following data normalisation against the loading control, differential expression between samples was calculated.

13.3 **Results**

Antibody microarray analysis identified 32 unique proteins, which demonstrated ≥ 1.8-fold difference in expression in at least one cell line, when comparing DuP-697 treated *versus* control (drug carrier only) cells (**Table 13.1**).

Of these, 20 DEPs demonstrated ≥ 1.8-fold difference in 2/2 cell lines. The dataset of 32 DEPs was submitted to IPA and the top relevant canonical pathway was "Apoptosis Signalling", which involved 5 DEPs: BCL2L1 (Bcl-xL), BID, CHUK (IKK), FASLG and RAF1 (**Figure 13.2**).

Table 13.1 List of DEPs identified by antibody microarray. A total of 32 unique DEPs identified using antibody microarray analysis following DuP-697 treatment of MSTO-211H mesothelioma cells and A549 lung cancer cells. Significant expression fold change (≥ 1.8) is indicated in bold. For proteins which show ≥ 1.8-fold change in expression in one cell line, supporting data from the second cell line is shown upward of 1.5-fold and non significant values (below 1.5-fold) are indicated "---". Highlighted protein targets were selected for western blot analysis. Downregulated proteins are demonstrated as minus values.

Ab # **(Sigma Aldrich)**	Protein target	Gene identifier	A549	MSTO-211H
P0084	Pinin	PNN	**7.67**	**7.32**
Z0377	Zxyin	ZYX	**-4.39**	**-4.74**
C1862	Coilin	COIL	**-4.6**	**-3.46**
A5968	AP-1	JUN	**2.49**	**2.97**
B3183	tBID	BID	**2.42**	**2.54**
C7736	Centrin	CETN1	**2.51**	**2.04**
S5446	SUMO-1	SUMO1	**2.44**	**2.09**
C6219	Connexin-43	GJA1	**2.44**	**2.01**
M0445	MDMX	MDM4	**2.13**	**2.39**
A0844	AP-2a	TFAP2A	**2.37**	**2.28**
A7107	AP2		**-2.06**	**-2.04**
I6139	IKKa	CHUK	**2.37**	**1.99**

E8526	E2F4	E2F4	**2.37**	**1.96**
S1190	SLIPR / MAGI-3	MAGI3	**2.15**	**1.92**
B9429	Bcl-xL	BCL2L1	**2.13**	**2.24**
S9809	Sp1	SP1	**2.13**	**1.95**
F3648	Fibronectin	FN1	**1.99**	**2.12**
F2051	Fas Ligand	FASLG	**1.81**	**2.14**
V7881	Vitronectin	VTN	**1.99**	**1.82**
H9912	Hsnf5 / INI1	SMARCB1	**1.96**	**1.84**
A7833	ATF-1	ATF1	**1.83**	**1.84**
R8274	RIP Receptor Interacting Protein	RIPK1	**2.11**	*1.74*
T5942	14-3-3 theta/tau	YWHAQ	*-1.64*	**-2.22**
T1075	Tal	LRSAM1	**2.05**	*1.72*
C3470	Connexin-32	GJB1	**2.04**	*1.76*
R1151	c-Raf pSer621	RAF1	*1.78*	**1.99**
R4904	Reelin	RELN	**1.95**	*1.67*
S3934	Smad4 (DPC4)	SMAD4	**1.87**	*1.61*
R3529	Rnase L	RNASEL	*-1.59*	**-1.82**
A5044	alpha Actinin	ACTN1	**1.82**	*1.66*
E8767	c-erbB-3	ERBB3	**1.82**	---
C3956	c-Myc	MYC	**1.81**	---
L1538	LIN-7	LIN7A	**1.8**	*1.57*

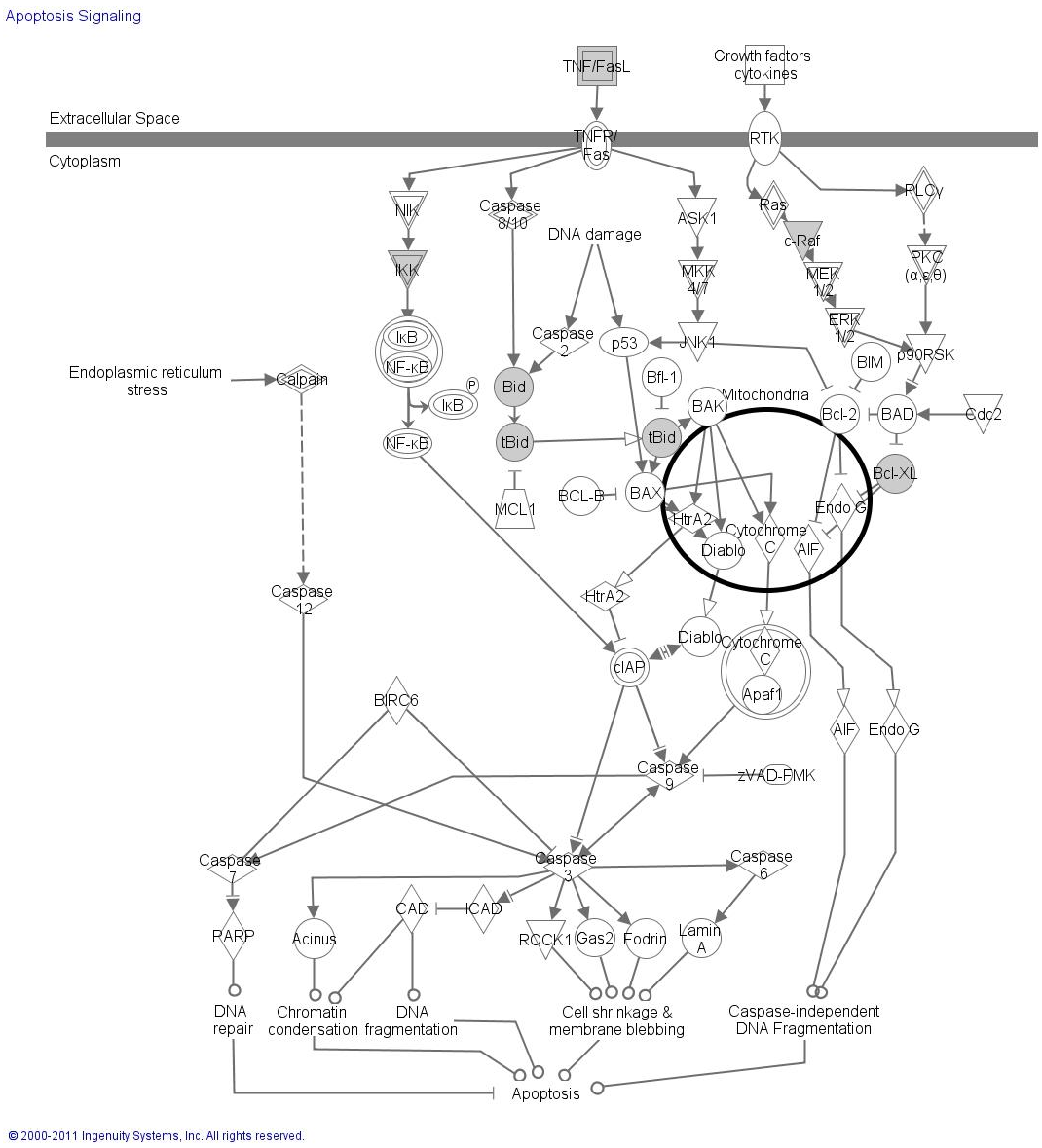
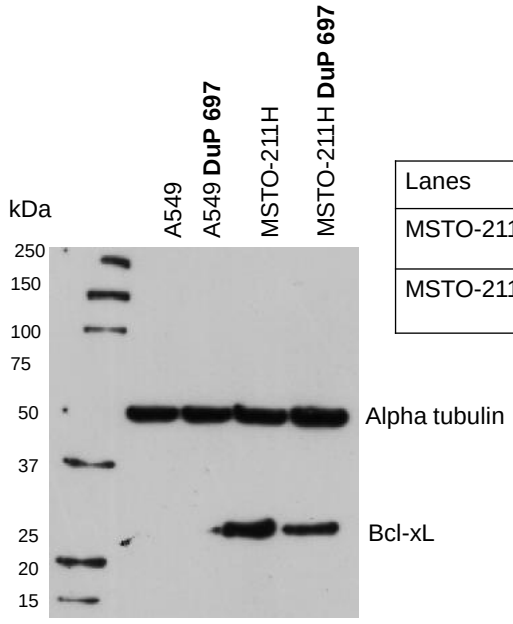


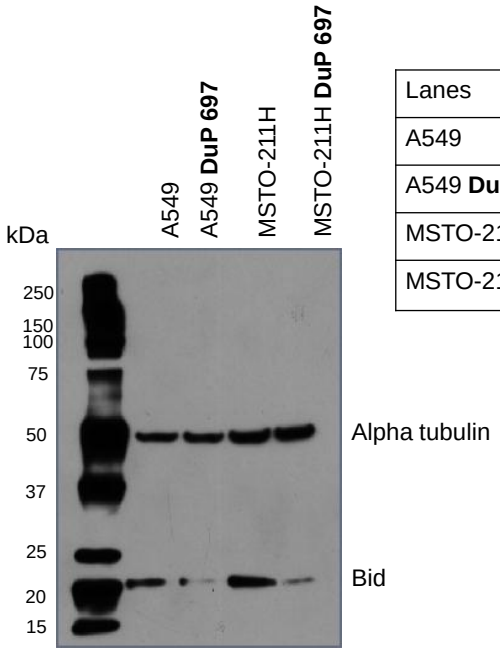
Figure 13.2 Apoptosis signalling pathway. Apoptosis Signalling canonical pathway from IPA (Ingenuity® Systems, www.ingenuity.com) showing BCL2L1 (Bcl-xL), BID, CHUK (IKK), FASLG and RAF1 which were identified by antibody microarray analysis.

The BCL2L1 (Bcl-xL) and BID proteins were selected for further analysis using western blotting. The anti-apoptotic BCL2L1 (Bcl-xL) protein was down regulated by 2.48 fold in the MSTO-211H cell line when treated with DuP-697 (**Figure 13.3**). The anti-tBID antibody (B3183), which was present on the antibody microarray, proved to be unreliable in the western blot application. However full length BID was found to be down regulated in both the MSTO-211H and A549 and the cell lines by a fold change of 10.16 and 14.52 respectively, following treatment with DuP-697 (**Figure 13.4**).



Lanes	Normalised	Ratio
MSTO-211H	93.7	2.48
MSTO-211H **DuP 697**	37.7	

Figure 13.3 Western blotting image demonstrating expression of BCL2L1.Western blotting demonstrated a significant decrease (2.48-fold) in expression of BCL2L1 (Bcl-xL) in the MSTO-211H cells following treatment with DuP-697. The BCL2L1 (Bcl-xL) protein could not be detected in the A549 cell line. The primary antibody against BCL2L1 (Bcl-xL) used here (#B9429, Sigma Aldrich) is expected to detect a band size of approximately 27 kD. Alpha tubulin (#ab7291, Abcam) is included as a loading control.



Lanes	Normalised	Ratio
A549	69.7	14.52
A549 **DuP 697**	4.8	
MSTO-211H	86.4	10.16
MSTO-211H **DuP 697**	8.5	

Figure 13.4 Western blotting image demonstrating expression of BID. Western blotting demonstrated a significant decrease in expression of full length BID in the MSTO-211H and A549 cells following treatment with DuP-697. The primary antibody against full length BID used here (#ab32060, Abcam) is expected to detect a band size of approximately 22 kD. Alpha tubulin (#ab7291, Abcam) is included as a loading control.

13.4 **Discussion**

We have previously confirmed that COX-2 is over-expressed in MPM samples which suggests that novel anticancer therapies targeted at this pathway may be useful in mesothelioma patients (O'Kane, Cawkwell et al. 2005). In addition, we have demonstrated that the COX-2 inhibitor DuP-697 enhanced the cytotoxic effect of Pemetrexed in mesothelioma cell lines, including MSTO-211H (O'Kane, Eagle et al. 2010). It is important to understand the molecular mechanism of action of novel agents before possible clinical testing and DuP-697 has not been widely researched. Two previous studies have suggested a role in inducing apoptosis via the caspase pathway (Churchman, Baydoun et al. 2007;

Peng, Zhang et al. 2008). In the present study we have explored the molecular mechanism of action of DuP-697 using an antibody microarray proteomic platform. We have identified 32 unique differentially expressed proteins which were associated with DuP-697 treatment for 72 hours. Of these, 20 proteins demonstrated significant (≥ 1.8-fold) differential expression in both the MSTO-211H mesothelioma and A549 lung cancer cell lines. Using some of the data from these, and other, experiments we have recently described Zyxin as the commonest repeatedly identified DEP (RIDEP) when using this proteomic platform (Hodgkinson, Elfadl et al. 2011) and therefore the selection of proteins for further analysis must be carefully considered. The analysis of the 32 DEPs using IPA indicated that 5 proteins, BCL2L1 (Bcl-xL), BID, CHUK (IKK), FASLG and RAF1, were associated with the Apoptosis Signalling canonical pathway. Following a positive signal for apoptosis, activated caspase 8 cleaves inactive, cytosolic, full length BID into active truncated BID (tBID), which localises to the mitochondrial membrane (Danial 2007; Song, Chen et al. 2010; Strasser, Cory et al. 2011). The anti-apoptotic proteins BCL-2 and BCL2L1 (Bcl-xL) block the escape of cytochrome C from the mitochondria, by preventing Bax from forming channels in the mitochondrial membrane, until activated tBID is localised to the membrane (Danial 2007; Song, Chen et al. 2010; Strasser, Cory et al. 2011). The caspase pathway of apoptosis has previously been implicated as the *in vitro* mechanism of action for DuP-697 (Churchman, Baydoun et al. 2007; Peng, Zhang et al. 2008). The onset of apoptosis may be associated with decreased levels of full length BID, due to its cleavage into tBID, and decreased levels of the anti-apoptotic protein BCL2L1 (Bcl-xL). Our western blot data would support these suggested protein changes following administration of DuP-697 for 72 hours.

The caspase pathway of apoptosis has previously been implicated as the *in vitro* mechanism of action for DuP-697, with upregulation of caspase 3, 8 and 9 being observed in hypothesis-driven experiments in normal proliferating endothelial cells or leukaemia cells (Churchman, Baydoun et al. 2007; Peng, Zhang et al. 2008). At the 72 hour time-point, which we examined here, we did not identify differential expression of caspase 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 or pro-caspase 8 in either MSTO-211H or A549 cells. However this may be due to the return of these proteins to basal levels within 72 hours since the upregulation of caspase 3, 8 and 9 was noted within 8 hours in HUVECs (Churchman, Baydoun et al. 2007).

COX-2 is a key enzyme involved in the metabolism of Arachidonic acid resulting in the production of PG, particularly PGE2, which plays an important role in tumour progression. COX-2 inhibitors may act by inhibition of COX-2, but the exact mechanism of how COX-2 inhibitors exert an anti-neoplastic effect is currently unknown. Indeed, several studies have suggested that COX-2 inhibitors may act independently of COX-2 (Kern, Haugg et al. 2006; Lou, Fatima et al. 2006; Pang, Zhou et al. 2007; Schonthal 2007). In our antibody microarray experiments, differential expression of COX-2 was not observed in either cell line after treatment with DuP-697 for the duration selected (72 hours). In future work, the expression of COX-2 and the individual proteins within the apoptosis signalling pathway, which we have implicated here, could now be examined over a time-course of treatment with DuP-697.

We have demonstrated that the antibody microarray proteomic platform can be used to explore the molecular mechanism of a COX-2 inhibitor. This will prove useful in gaining a more thorough understanding of novel agents which may have clinical applications. Specific COX-2 inhibitors, such as DuP-697, may have a future therapeutic role in MPM.

Our proteomic analysis suggests that the anti-proliferative effect of DuP-697, which was previously seen in mesothelioma cell lines, may be exerted via the induction of apoptosis. DuP-697, or other COX-2 inhibitors such as Celecoxib or Rofecoxib, may act as an effective apoptosis sensitiser when combined with chemotherapy drugs such as Pemetrexed and further studies are required to test this hypothesis.

Chapter 14. Conclusions

In summary, this study has demonstrated that

- PTEN protein expression is undetectable in 27% of archival MPM tissue samples and is reduced in another 27% of MPM tissue samples as analysed by immunohistochemistry.
- Both the 5LOX and 12LOX proteins were up regulated in 73% and 83% of archival MPM tissue samples respectively.
- EGFR protein was expressed in MSTO-211H, NCI-H2052, NCI-H2452 and A549 cells as analysed by flow cytometry.
- PTEN protein expression was absent in MSTO-211H cells, weakly expressed in A549 cells and strongly expressed in NCI-H2052 and NCI-H2452 cells as analysed by western blot.
- Our study is the first to evaluate the EGFR, KRAS and BRAF mutation in NCI-H2052, NCI-H2452 and MSTO-211H cells. NCI-H2052, NCI-H2452, MSTO-211H and A549 cells harboured wild type EGFR TK domain and there was no BRAF mutations seen. A549 cells demonstrated a single KRAS mutation. No KRAS mutations were seen in H2052, NCI-H2452 and MSTO-211H cells.
- We demonstrated that EGFR inhibitors (Cetuximab and Gefitinib) were not very effective as single agents in MPM cells. Our study is the first to evaluate the cytotoxic effect of Cetuximab in MPM.
- The COX-2 inhibitor (Celecoxib) did demonstrate cytotoxicity, but at doses significantly higher than those that can be achieved therapeutically.

- MTOR inhibitors (Rapamycin and Ku0063794) demonstrated significant cytotoxicity in NCI-H2052, NCI-H2452 and A549 cells, but not in MSTO-211H cells. Our study is the first to evaluate the cytotoxic effect of MTOR kinase inhibitor (Ku0063794) in MPM.
- Combined inhibition of Cetuximab and Gefitinib with Celecoxib, Rapamycin and Ku0063794 demonstrated enhanced inhibition in NCI-H2052, NCI-H2452 and A549 cells when Cetuximab was combined with Celecoxib and in MSTO-211H cells when Cetuximab was combined with Ku0063794 cells.
- We successfully carried out a pilot study using antibody microarray as a novel platform to identify mechanism of action of COX-2 inhibitor (DuP-697) and demonstrated that DuP-697 may cause cytotoxicity by activating apoptosis.

14.1 **Implications of using *in vitro* cell line models**

Our cytotoxicity studies are based on cell lines grown in monolayer which is a simple and convenient way of demonstrating the effectiveness of drugs in cancer cells. There are several advantages to using cell lines as they are easy to grow, store and handle. They are homogenous and available in unlimited amount. However, culturing the cells over prolonged duration may alter their morphology and genotype (Bahia, Ashman et al. 2002; Burdall, Hanby et al. 2003; Watson, Bahia et al. 2004). Under normal circumstances a tumour microenvironment is made up of cancer cells, inflammatory cells, cytokines, extracellular matrix (containing stromal fibroblasts and epithelial cells), blood vessels and endothelial cells (Bissell and Radisky 2001). Significant interactions take place within different cells via cell-cell cross talk and cytokines. This complex tumour microenvironment plays an important role in the morphology and phenotype of cancer

initiation and progression. Cell lines grown as monolayers are immortalised cells derived from cancer tissues and lack the extracellular matrix, inflammatory cells, blood vessels and endothelial cells significantly altering the tumour microenvironment. *In vivo* experiments or cells grown in a three dimensional model may represent the phenotype and the morphology of cancer cells more accurately as there is less alteration of the tumour microenvironment (Smalley, Lioni et al. 2006). Barbone *et al* (2008) grew MPM cells in a three dimensional multicellular spheroid model and demonstrated that the spheroids acquired resistance to a variety of apoptotic stimuli such as TRAIL, ribotoxic stressors, HDAC1 and proteosome inhibitors, which were highly effective in cells grown as monolayers (Barbone, Yang et al. 2008). As a result, validation of the MTS assays in our study may need to be done using three dimensional spheroid models or by using *in vivo* models such as mouse xenografts.

14.2 **Is this the end of EGFR inhibition in MPM?**

Preclinical studies, including our study, have demonstrated that EGFR TKI such as Gefitinib have no significant cytotoxic effect in MPM cell lines at clinically relevant doses (Janne, Taffaro et al. 2002; Nutt, O'Toole et al. 2009). Clinical studies evaluating the effect of Gefitinib and Erlotinib (first generation EGFR TKIs) have demonstrated lack of clinical benefit in early phase trials in MPM (Govindan, Kratzke et al. 2005; Garland, Rankin et al. 2007). The likely reason for the lack of benefit from EGFR TKI may be as a result of lack of activating EGFR mutations in MPM. Various authors have failed to demonstrate the presence of EGFR TK activating mutations in MPM (Section 2.2.7) (Cortese, Gowda et al. 2006; Destro, Ceresoli et al. 2006; Okuda, Sasaki et al. 2008). Our study has demonstrated that EGFR mutations are not seen in MPM cell lines. The second generation EGFR TKIs irreversibly inhibit the TK activity of the EGFR resulting in longer and more potent inhibition of the receptor. The second generation EGFR TKIs such as Afatinib (BIBW

2992, Boehringer Ingelheim) and Dacomitinib (PF-00299804, Pfizer) have shown to be anti-neoplastic in EGFR mutation positive NSCLC (Ou 2012) (See appendix B for list of all drugs and targets for the targeted biological agents used). In a Phase II trial in NSCLC, Dacomitinib when compared to Erlotinib has shown to improve PFS in patients with wild type EGFR and KRAS (11.1 weeks *versus* 8 weeks; p= 0.047) (Ou 2012). The Phase III trial (ARCHER 1009; ClinicalTrials.gov Identifier: NCT01360554) comparing Dacomitinib to Erlotinib in advanced NSCLC patients with wild type EGFR is currently ongoing. If second generation irreversible EGFR TKIs demonstrate better efficacy compared to first generation reversible EGFR TKIs in tumours with wild type EGFR, then their evaluation in patients with MPM (with wild type EGFR) may demonstrate a therapeutic potential.

To date there have not been any studies evaluating the effect of EGFR monoclonal antibodies such as Cetuximab in MPM. Biomarkers of Cetuximab resistance such as KRAS and BRAF mutations are not seen in MPM (Sections 2.2.8 and 2.2.9). Our study has demonstrated that KRAS and BRAF mutations are not seen in MPM cell lines. Given that KRAS and BRAF mutations are not seen in MPM and that EGFR is expressed in MPM, we expected to find significant anti-proliferative activity with Cetuximab in MPM cell lines. This was however not seen in our study. The current role of other anti-EGFR monoclonal antibodies such as Panitumumab and Matuzumab® (EMD 72000, Merk Serono) in MPM are currently not known. Given that Cetuximab has so far been not very effective in MPM cells in our study, the benefit of other similar anti-EGFR monoclonal antibody may be doubtful in cell line models, but will need further evaluation in *in vivo* models. One of the mechanisms of resistance to anti-EGFR therapy may be that the downstream signalling pathways are activated by other pathways such as COX-2/PGE2 pathway or co-activation

of other growth factor receptors may mediate the activation of PI3K/AKT/MTOR pathway (**Figure 14.1**). HGF/MET, IGF-1R and VEGFR are frequently up regulated and activated along with EGFR in MPM and the HGF/MET, IGF-1R and VEGFR inhibitors have demonstrated significant activity against MPM cells (Mukohara, Civiello et al. 2005; Jagadeeswaran, Ma et al. 2006; Whitson and Kratzke 2006; Kai, D'Costa et al. 2009; Nutt, O'Toole et al. 2009; Brevet, Shimizu et al. 2011). Inhibition of multiple targets in combination with EGFR may overcome anti-EGFR therapy resistance induced as a result of the activation of other growth factor receptors.

The studies done to date evaluating the incidence of KRAS, BRAF and PIK3CA in MPM are very few and with small number of tissue samples and cell lines and this needs further evaluation with a larger number of MPM tissue samples.

EGFR is the most common growth factor expressed in MPM and its role as a target for anti-cancer therapy is not the end, but a modest, albeit a frustrating beginning. The complex molecular interaction of EGFR with other growth factors, its downstream signalling pathways, complicated feedback inhibitions and the role played by activating and resistant mutations of the EGFR and its other downstream signalling proteins make this an extremely difficult target to understand and manipulate for therapeutic advantage. Further work is required to understand the role played by EGFR in the pathogenesis of MPM and its mechanism of resistance. Inhibition of multiple targets, along with EGFR, may be of benefit and should be the next phase of the study. The suggested targets to co inhibit would be pan EGFR inhibition along with EP receptor, MET, IGFR and VEGFR in various combinations and also in combination with PI3K/AKT/MTOR pathway inhibition which plays an important and a central role in the oncogenic phenotype of MPM. In clinical practice inhibition of various targets is associated with toxicities which may be generic or

specific to the target inhibited (Widakowich, de Castro et al. 2007). Our ability to appropriately combine multiple target inhibitors is likely to be limited due to the varied and increased toxicity profile of the drug and target combinations.

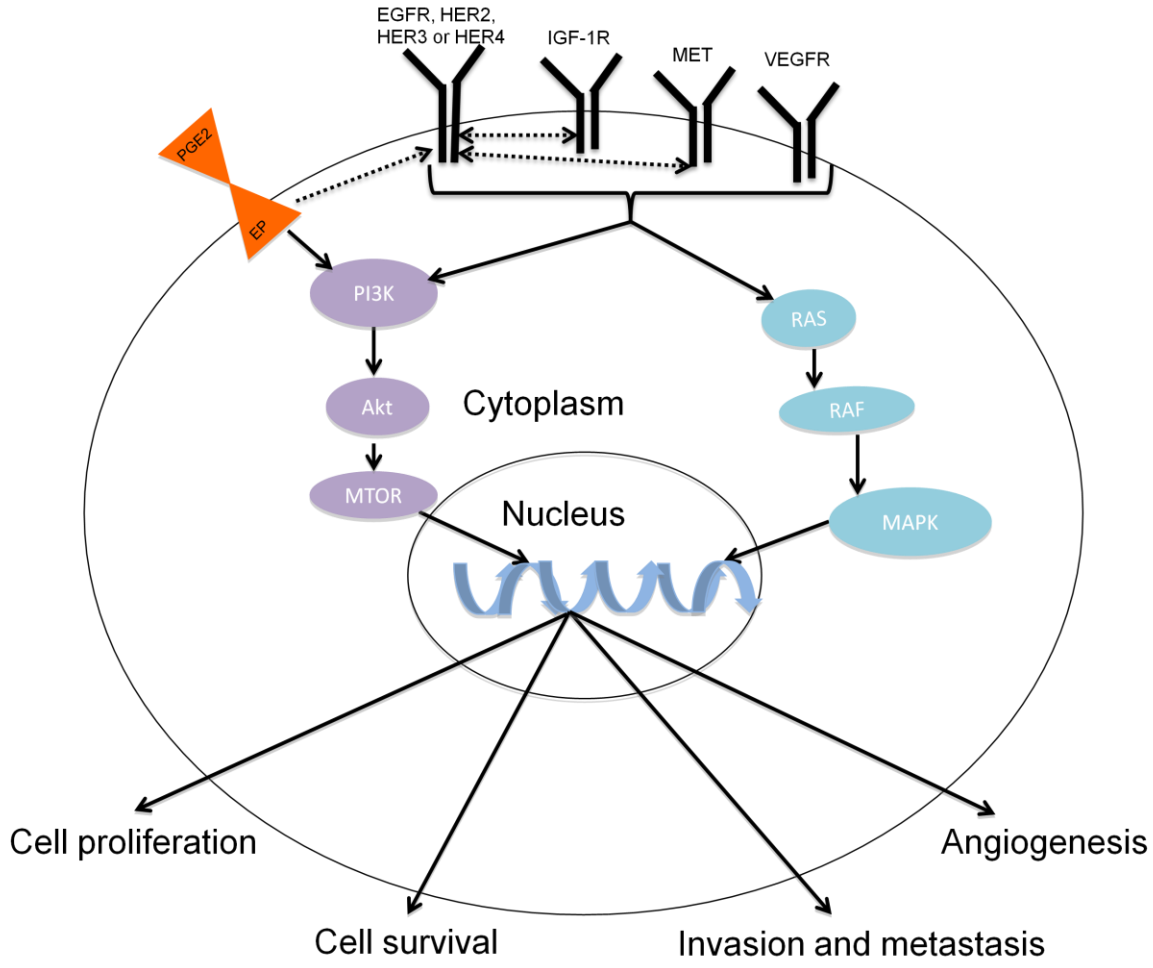


Figure 14.1 Interaction between cell surface receptors. This figure demonstrates the interaction between EGFR family and other cell surface receptors PGE2/EP, HGF/MET, IGF-1R and VEGFR and the likely mechanism of resistance to EGFR. Figure adapted and modified from (Doebele, Oton et al. 2010).

14.3 **MTOR pathway and MPM – There is still work to do.**

We have demonstrated that NCI-H2052, NCI-H2452 and A549 cells were sensitive to the cytotoxic effects of the MTORC1 inhibitor Rapamycin at clinically relevant doses and the MTOR kinase inhibitor Ku0063794. Various other studies have demonstrated significant inhibitory effects of Rapamycin in MPM cell lines as well as MPM spheroids (Altomare, You et al. 2005; Kim, Wilson et al. 2005; Barbone, Yang et al. 2008; Wilson, Barbone et al. 2008). However, inhibition of MTORC1 may result in inhibition of the inhibitory feedback loop via the p70S6K/IRS1/ PI3K pathway resulting in activation of AKT and increased cell survival. Inhibition of MTOR kinase may overcome this effect by inhibiting MTORC2 which is an activator of AKT. In our study Ku0063794 has demonstrated significant cytotoxic effects in NCI-H2052, NCI-H2452 and A549 cells for the first time. There are various MTOR kinase inhibitors undergoing early phase clinical trials such as OSI-027 (OSI), Phase I; AZD-8055 (AstraZeneca), Phase I/II; AZD-2014 (AstraZeneca), Phase I and INK128 (Intellikine), Phase I (Zaytseva, Valentino et al. 2012). The inhibitory effect of these MTOR kinase inhibitors will need further evaluation in MPM at clinically relevant doses. MTOR inhibition can also be combined with other targeted drugs such as PI3K inhibitors (XL-147 (Exelixis) and BKM120 (Novartis)) to overcome the inhibitory effects of the p70S6K/IRS1/ PI3K negative feedback loop and enhance its therapeutic effect. The newer MTOR kinase inhibitors also inhibit the p110α, β and γ isoforms of PI3K thereby completely blocking the effect of PI3K / AKT signalling. Some of the dual MTOR kinase / PI3K inhibitors currently undergoing clinical trials are NVP-BEZ235 (Novartis), Phase I/II; XL765 (Exelixis/Sanofi-Aventis), Phase I/II; SF1126 (Semafore), Phase I; GSK2126458 (GlaxoSmithKline), Phase I; BGT226 (Novartis), Phase I/II; GDC0980 (Genentech), Phase I; PF-04691502 (Pfizer), Phase I/II and PF-05212384 (PKI-587,

Pfizer), Phase I (Zaytseva, Valentino et al. 2012). The effect of dual (MTOR kinase / PI3K) kinase inhibitors will need further evaluation in MPM.

In our study the combined inhibition of EGFR and MTOR failed to demonstrate any significant therapeutic advantage at clinically relevant doses except in MSTO-211H cells where the combination of Ku0063794 and Cetuximab enhanced the cytotoxic effect of Cetuximab. One of the reasons for resistance to EGFR inhibition could be that the growth factor mediating the MTOR signalling pathway in NCI-H2052, NCI-H2452 and A549 may be independent of EGFR. Significant co activation of IGF-1R, VEGF and MET/HGF along with EGFR has been seen in MPM and inhibition of these growth factors in combination with MTOR inhibition using multiple kinase inhibitors may enhance the therapeutic effect when compared with inhibition of any single target alone. The next phase of this translational research should involve the evaluation of MTOR dual kinase inhibitors in MPM cells, initially as single agents and subsequently in combination with multiple growth factor receptor inhibitors (**Figure 14.2**).

Since there was a significant inhibition in MPM cell lines by Rapamycin at clinically relevant doses, the next phase would be to trial this in a clinical setting, either a single agents after failure of chemotherapy or in combination with chemotherapy. Currently there are two Phase II clinical trials ongoing which are evaluating the benefits of the Rapamycin rapalog Everolimus in patients with MPM (Section 3.1.3). The outcome of these trials will provide significant information on the importance of inhibiting MTOR in patients with MPM.

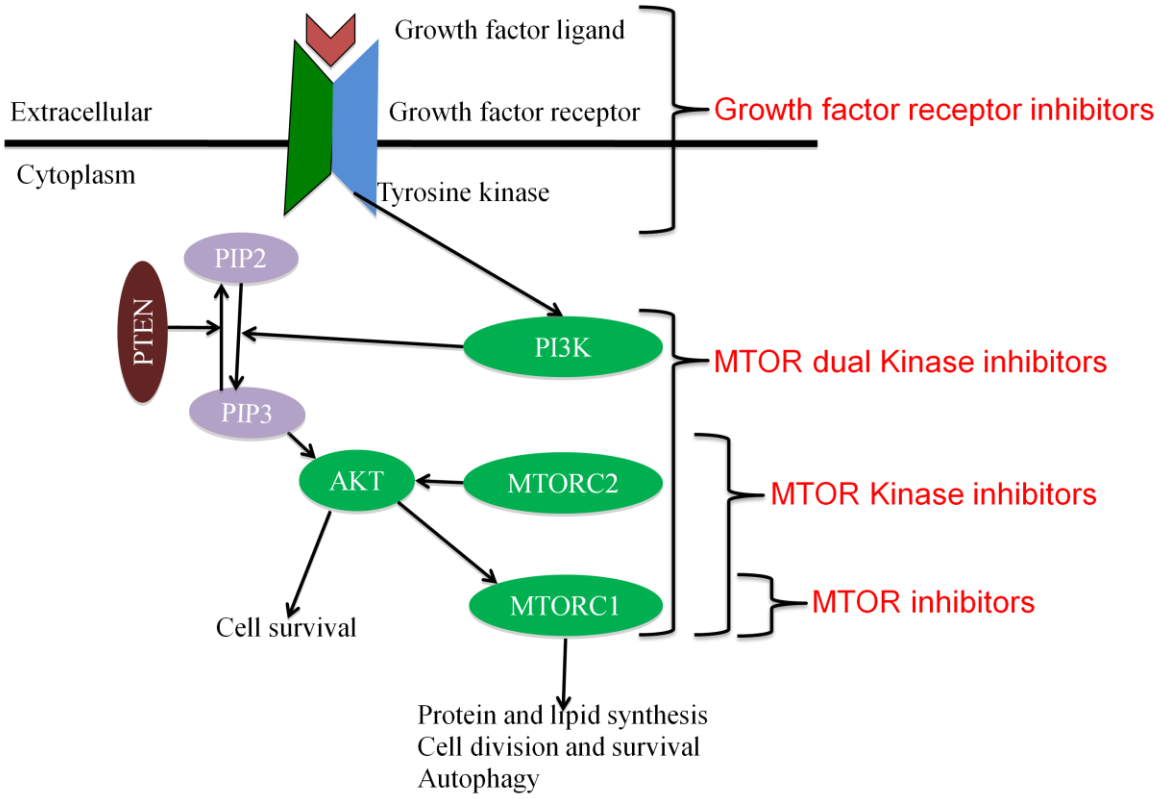


Figure 14.2 MTOR and Growth factor receptor inhibitors. This figure demonstrates the extent of inhibition of growth factor receptor and the PI3K/AKT/MTOR pathway using various MTOR and growth factor receptor inhibitors.

14.4 **Arachidonic acid metabolism in MPM**

Arachidonic acid metabolizing enzymes COX-2 and 5LOX are upregulated in MPM suggesting that both the metabolic pathways may be active (Section 8.3.3, **Figure 4.1**, and **Figure 4.2**). Since the metabolism of Arachidonic acid by COX-2 and 5LOX are interrelated, there is a likely possibility that inhibiting either enzyme may shift and increase the metabolic activity to the other enzyme resulting in neoplastic activity. In a study by Duffield-Lillico *et al* (2009), healthy volunteers when treated with Celecoxib demonstrated a reduction in the level of urinary PGE-M (metabolite of PGE2 secreted in urine) levels, but an increase in the urinary LTE4 levels, demonstrating a shift in the metabolism of

Arachidonic acid from the COX-2 pathway to the 5LOX pathway (Duffield-Lillico, Boyle et al. 2009). In colon cancer cell lines (Caco-2 and HT29) treatment with Celecoxib resulted in significant reduction of PGE2 production, but an increase in the production of cysteinyl Leukotriene (5LOX metabolite) demonstrating a shift in the metabolic activity (Cianchi, Cortesini et al. 2006). Similarly treatment of the colon cancer cells with a 5LOX inhibitor (MK886) resulted in a reduction in the production of cysteinyl Leukotriene and an increase in the production of PGE2. Combined inhibition with Celecoxib and MK886 resulted in the reduction of both PGE2 and cysteinyl Leukotriene in both the cell lines. Similar additive effect of COX-2 and 5LOX inhibition has been demonstrated by other studies in colon cancer, oral cancer and oesophageal cancers (Chen, Wang et al. 2004; Li, Sood et al. 2005; Ye, Wu et al. 2005; Shi, Lv et al. 2011) In MPM, COX-2 inhibitors and 5LOX inhibitors have independently demonstrated cytotoxic effect in MPM cell lines (Marrogi, Pass et al. 2000; Romano , Catalano et al. 2001; DeLong, Tanaka et al. 2003; Catalano, Graciotti et al. 2004; O'Kane, Eagle et al. 2010). Inhibiting both the 5LOX and COX-2 pathways by using both COX-2 and 5LOX inhibitors simultaneously or dual COX-2/5LOX inhibitor (Licofelone®, Merckle GmbH, Germany), may target the shift of metabolic pathways resulting in an enhanced therapeutic benefit and needs further evaluation.

In MPM, COX-2 inhibition with Celecoxib has demonstrated significant cytotoxic effect, but at doses significantly higher than those achieved clinically. The exact mechanism of action of Celecoxib is currently a focus of investigation and various studies have suggested that the mechanism may be independent of COX-2/PGE2 inhibition (Tegeder, Pfeilschifter et al. 2001; Maier, Schilling et al. 2004; Pyrko, Soriano et al. 2006; Tong, Wu et al. 2006). In our study we used antibody microarray as a pilot to evaluate the mechanism of action of

the COX-2 inhibitor DuP-697 and a similar study may be required to identify the mechanism of action of Celecoxib in MPM and identify further targets that may be therapeutically manipulated.

14.5 **Research in MPM**

There is a significant paucity of research in MPM. In the UK in 2008, there were 1,967 patients diagnosed with mesothelioma, 47,693 patients diagnosed with breast cancer, 17,960 patients diagnosed with lung cancer and 17,894 patients diagnosed with colorectal cancer (http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/ accessed 20/03/2012). A brief survey conducted in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) on 20/02/2012 and searching the tumour type under the heading of Title/abstract and deducting any review articles, showed that the total number of manuscripts in MPM was 1450, in comparison to breast cancer (124,478), lung cancer (64,419) and colorectal cancer (37,940). Using the same search criteria in clinicaltrials.gov (http://clinicaltrials.gov/), the total number of clinical trials in MPM were 97, in comparison to breast cancer (4,606), lung cancer (3,877) and colorectal cancer (2,421). This suggests that MPM is proportionately (in relation to the incidence) significantly under researched and needs more research input to better understand the pathophysiology of the disease and identify important targets that can be therapeutically manipulated to benefit patients.

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Appendix A (MTS Optimization)

Estimation of duration of exposure to MTS reagent

MSTO-211H cell lines were seeded in a 96 well plate at cell density of 5000cells per well, 10000 cells per well and 20000 cells per well in triplicates. After 4 days of incubation, MTS reagent was added to each well and colorimetric reading taken after 1hr, 2 hrs and 3 hrs interval. Manufacturer's recommendation was to read the plate between 1 to 4 hours (**Table A.1**).

Table A.1 Table showing true absorbance values after adding MTS reagent at 1hr, 2 hrs and 3 hrs interval.

MSTO-211H cells	1 hr reading	2 hrs reading	3 hrs reading
5000 cells	0.33	0.56	0.7
10000 cells	0.58	0.88	1.07
20000 cells	0.73	1.08	1.34

The best readings were observed after 3 hrs and hence that was taken forward.

Optimal cell seeding density for cell growth

NCI-H2052, NCI-H2452, MSTO-211H and A549 cell lines were seeded in a 96 well plate at cell density of 5000cells per well, 10000 cells per well and 20000 cells per well in triplicates with and without 0.01% DMSO (drug carrier) and incubated for 4 days (**Table A.2)**. Growth of the cells was calculated at the end of 4 days by MTS assay read at 3 hrs. The aim was to achieve maximal growth without exceeding 80% confluence at the end of 4 days 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 t othe total area of the well by light microscopy (magnification 20X). Maximal growth was seen in MSTO-211H cell line at 5000 cells per well with 70-80% confluence. Growth was also seen in NCI-H2052 and A549 cell lines, at seeding density of 5000cells per well, but the confluence of these cells were nearing 100%. NCI-H2452 cell lines did not show significant growth at seeding density of 5000 cells per well. The experiment was repeated for NCI-H2052, NCI-H2452 and A549 cell lines with a seeding density of 1000, 2500 and 5000 cells per well. Maximal growth was seen in 1000 cell per well for NCI-H2052, NCI-H2452 and A549 cell lines. Confluence was seen to be 70% to 80% in the cell lines at this seeding density. There was no cytotoxic effect of 0.01% DMSO noted.

Calculation of percentage of growth inhibition

An example of a single calculation of A549 cell line treated with 50μM Celecoxib is shown in **Table A.3**. All subsequent calculations done to estimate the growth response curve were based on this template.

Once the growth of treated cell as a percentage of control cells was calculated the average of the three independent experiments were taken and Average of the three experiments was calculated along with the standard error of mean. The average reading of all the doses was taken an plotted onto a XY scatter plot in Microsoft excel work sheet along with the error bars to denote the standard error of mean. Growth inhibition (50%) was calculated after adding the trend line equation on the graph.

Table A.2 This table demonstrates the % of cell growth and the effect of 0.01% DMSO in cell lines depending upon their seeding density.

Cell lines	Cell seeding density	% Growth	% Growth with 0.01% DMSO
NCI-H2052	1000	218.40	263.47
	2500	141.75	161.28
	5000	27.22	22.42
	5000	186.9	167.68
	10000	92.37	94.89
	20000	76.65	81.65
NCI-H2452	1000	165.54	166.65
	2500	35.53	41.30
	5000	10.95	9.84
	5000	52.18	58.33
	10000	17.78	17.22
	20000	8.65	10.16
MSTO-211H	5000	127.41	171.08
	10000	96.95	115.95
	20000	68.05	79.93
A549	1000	405.67	414.45
	2500	128.96	120.92
	5000	44.34	32.29
	5000	106.28	96.38
	10000	15.06	12.45
	20000	5.15	4.32

Table A.3 Example calculation of the growth of treated cell as a % of control cells.

A549 cells with 50µM Celecoxib					
D2 reading					
Blank	0.13	0.13	0.11		Average= 0.12
Baseline reading	0.49	0.52	0.53		
	0.54	0.40	0.39		Average= 0.48
True Baseline reading (a) =(Baseline reading [0.48]– blank [0.12])					0.35
D5 reading					
Blank	0.13	0.11	0.14		Average=0.13
Control absorbance	2.15	2.46	2.45		
	2.12	2.21	1.96		Average=2.23
True Control absorbance (b)=(control absorbance [2.23]-blank [0.13])					2.1
Treated absorbance	0.82	0.88	0.78		
	0.76	0.89	0.90		Average=0.84
True Treated absorbance (c)=(Treated absorbance [0.84]-blank [0.13])					0.71
Growth of control cells (d) = [(b-a)/a]*100 = [(2.1-0.35)/0.35]*100					494.15%
Growth of treated cells (e) = [(c-a)/a]*100 = [(0.71-0.35)/0.35]*100					100.71%
Growth of treated cells as a % of control cells (f) = (((e-d)/d)*100)+100 = (((100.71-494.15)/494.15)*100)+100					20.38%

Appendix B (List of Drugs and Targets)

Table B.1 List of targeted drugs used in text.

Drug	Alternative names	Manufacturer	Drug Targets
AA-861	SC-200570		5LOX
AG1024			IGF-1R Tyrosine Kinase
AZD-8055		Astra Zeneca	MTORC1, MTORC2
AZD-2014		Astra Zeneca	MTORC1, MTORC2
Bevacizumab	Avastin®	Genentech Inc	VEGF
BGT226		Novartis	MTORC1, MTORC2, PI3K
BIBW 2992	Afatinib®	Boehringer Ingelheim	Irreversible EGFR Tyrosine Kinase (2^{nd} Generation)
BKM120		Novartis	PI3K
Cediranib	AZD2171	Astra Zeneca	VEGF Tyrosine Kinase
Celecoxib	Celebrex®, Onsenal®	Pfizer	COX-2
Cetuximab	IMC-C225, Erbitux®	ImClone Systems Incorporated	EGFR
DuP-697			COX-2
Everolimus®	RAD001	Novartis	MTORC1
GDC0980		Genentech	MTORC1, MTORC2, PI3K
Erlotinib	OSI-774, Tarceva®	OSI Pharmaceuticals	Reversible EGFR Tyrosine Kinase (1^{st} Generation)
Flurbiprofen			Non selective COX
Gefitinib	ZD1839, Iressa®	AstraZeneca	Reversible EGFR Tyrosine Kinase (1^{st} Generation)

GSK2126458		GlaxoSmithKline	MTORC1, MTORC2, PI3K
Indomethacin			Non selective COX
INK128		Intellikine	MTORC1, MTORC2
Ku0063794		AstraZeneca	MTORC1, MTORC2
Lapatinib	GW572016, Tyverb®	GlaxoSmithKline	EGFR, HER2
ML3000	Licofelone®	Merckle GmbH	COX-2, 5LOX
LY294002			PI3K
Matuzumab	EMD 72000	Merck Serono	EGFR
MK886			FLAP (5LOX activating protein)
NDGA			Non selective LOX
NS398			COX-2
NVP-AEW541		Novartis	IGF-1R Tyrosine Kinase
NVP-BEZ235		Novartis	MTORC1, MTORC2, PI3K
OSI-027		Osi Pharmaceuticals	MTORC1, MTORC2
Panitumumab	ABX-EGF, Vectibix®	Amgen	EGFR
PF-00299804	Dacomitinib®	Pfizer	Irreversible EGFR Tyrosine Kinase (2nd Generation)
PF-04691502		Pfizer	MTORC1, MTORC2, PI3K
PF-05212384	PKI-587	Pfizer	MTORC1, MTORC2, PI3K
Ranpirnase	Onconase®	Tamir Biotechnology	Ribonuclease enzyme
Rapamycin	Rapamune®	Wyeth	MTORC1

		Pharmaceuticals	
Rofecoxib	Vioxx®,Ceoxx®	Merck	COX-2
Semaxanib	SU5416	SUGEN	VEGF Tyrosine Kinase
SF1126		Semafore	MTORC1, MTORC2, PI3K
Sorafinib	Nexavar®	Bayer	VEGF Tyrosine Kinase, PDGFR Tyrosine Kinase, Raf kinases
SU11274			c-MET Tyrosine Kinase
Temsirolimus	CCI-779	Wyeth Pharmaceuticals	MTORC1
Thalidomide			VEGF
Valproic acid			HDAC
Vatalanib	PTK787, PTK/ZK	Bayer & Novartis	VEGFR, PDGFR, c-kit
Vorinostat	Zolinza®	Merck	HDAC
XL-147	SAR245408	Exelixis	PI3K
XL-765	SAR245409	Exelixis	MTORC1, MTORC2, PI3K

Appendix C (Ethics approval letter)

HULL AND EAST RIDING LOCAL RESEARCH ETHICS COMMITTEE

c/o Faculty of Health
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22 November 2000

Dr. L Cawkwell
Lecturer in Cancer Genetics
Acad. Department of Oncology
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R & D Building, Castle Hill Hospital
HU16 5JQ

Dear Dr Cawkwell

AN IMMUNO STAINING STUDY OF PROGNOSIS AND RESPONSE TO CHEMOTHERAPY IN PATIENTS WITH MALIGNANT MESOTHELIOMA

LREC NUMBER: 11/00/212

Your application for this study was considered at a meeting of the Local Research Ethics Committee on 20 November 2000.

I am pleased to say that ethical approval was granted.

If you have not already done so, you should ensure that you also have the approval of the management in the Trust(s) in which the research is to take place.

Yours sincerely

Professor S R Killick
Chairman

The following members were involved in the ethical review of the study:

Professor SR Killick	Dr E Baguley	Dr C Brophy	Dr R Calvert	Mrs E Dakkak	Mr M Davidson
Mrs C Metcalfe	Dr D Ryan	Mrs H Thornton-Jones		Mrs P Webb	Coun. K West

Hull and East Riding Local Research Ethics Committee Members

Professor SR Killick (Chairman)	Dr E Baguley	Dr CJ Brophy	Mrs E Dakkak	Mr M Davidson	Mr GS Duthie
Mrs C Metcalfe	Dr PF Newman	Dr D Ryan	Mrs P Webb	Councillor K West	

Appendix –D (MTA - Celecoxib and Cetuximab)

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Global Research & Development

March 25, 2010

Donnie W. Owens
Compound Transfer Manager
Strategic Alliances

Dr. Vijay Agarwal
University of Hull, Hull, UK
Research Lab 2nd Floor, Daisy Bldg. Entrance 2, Castle Hill Hospital, Castle Road Hull
Hull
UK - HU16 5JQ

Dear Dr. Agarwal:

We have received your (the "Investigator") request for a sample of a following Pfizer Compound(s) SC-58635, (the "Material"), to be used in your laboratory under your supervision. Please complete the attached Statement of Investigation Form, which will inform Pfizer of your intended use of our compound. Before the Material can be sent to you, we will require that you confirm your agreement to the conditions of delivery by signing the Compound Transfer Agreement ("Agreement") below and, have the Agreement signed by an authorized individual at University of Hull, Hull, UK. The execution of this Agreement places no obligation on Pfizer to supply the Material to you. Any decision to supply the Material will be made at Pfizer's sole discretion upon review of the completed Statement of Investigation Form.

1. PARTIES OF THE AGREEMENT:

The parties to this Agreement are Pfizer and its Affiliates ("Pfizer") and University of Hull, Hull, UK, (the "Institution"), effective as of March 25, 2010.

2. SCOPE OF WORK:

The research ("Research") is described in the Statement of Investigation Form, which is attached to and made part of this Agreement as Exhibit A. The use of the Material, is limited to the Research under the Investigator's direction.

3. MATERIAL:

(a) The Investigator and the Institution acknowledge that the Material is for laboratory use only and is not for consumption by, or treatment of, humans or non-laboratory animals.

(b) The Investigator and the Institution will use the Material in compliance with all applicable

federal, state and local laws, regulations and ordinances. The Investigator and the Institution will not make the Material available to any third party or to any person who is not subject to the Investigator's direct supervision.

(c) At the conclusion of the Research, the Investigator will return any unused portion of the Material to Pfizer at the following address: Manager, Sample Bank, Pfizer Global Research & Development, Eastern Point Road, Groton, Connecticut 06340-5146.

4. REPORT:

The Investigator will inform Pfizer of the results of the Investigator's work with the Material in the Research ("Results"). The Investigator will inform Pfizer in writing of the Results at the conclusion of the Research or within one (1) year of receiving the Material, whichever is sooner. The Results will be provided as a written report to Compound Transfer Manager, Medical Business Operations, MS 8200-30 Eastern Point Road, Groton, CT.

5. PUBLIC DISCLOSURE:

(a) Pfizer shall not publish any of the Investigator's data without the Investigator's written permission.

(b) Pfizer encourages the exercise of academic freedom and shall have no right to prevent the Investigator and the Institution from publishing or presenting any findings from the Research. Notwithstanding the foregoing, the Investigator and the Institution acknowledge Pfizer's role as discoverer of the Material and, before they publish or present any findings from the Research relating to the Material, they will provide Pfizer with an opportunity to react to such findings. In the case of any publications planned by the Investigator or the Institution, they will provide Pfizer with a copy of the proposed submission for publication at least forty-five (45) days before its submission. In the case of any oral presentation to a third party, they will provide Pfizer with copies of any audiovisual materials, including slides, at least twenty-one (21) days before the presentation.

6. INTELLECTUAL PROPERTY:

Neither the Investigator nor the Institution shall have any property rights in the Material or in any information that Pfizer discloses to either of them relating to the Material. Except and only to the extent necessary for the Investigator to use the Material in the Research, neither the Investigator nor the Institute is granted any license, express or implied, under any Pfizer patent or patent application, or under any patent or patent application under which Pfizer is licensed.

7. INVENTION:

(a) "Invention" means any invention or discovery, patentable or not, relating to the manufacture, use or sale of the Material which is first conceived or first reduced to practice by the Investigator, by any person under the Investigator's supervision, or by the Institution in performance of the

Research.

(b) The Institution will inform Pfizer in writing of any Invention promptly upon its occurrence.

(c) The Institution hereby grants Pfizer a worldwide, perpetual license in and to all Inventions. Such license shall be royalty-free, irrevocable and nonexclusive, to make, use, import, offer for sale and sell the subject matter of such Inventions.

Sincerely,

Donnie W. Owens 3/25/10

Donnie W. Owens

Date

INSTITUTION I represent to have authority to execute this Agreement on behalf of Institution:	**INVESTIGATOR** while not a party to this agreement, acknowledges that they have read this agreement and understands their obligations as an Institution employee
By : UNIVERSITY OF HULL	
Institution Signature	Investigators Signature
Printed Name	Printed Name
Title **Mr Jonathan Cant** **Research Grants & Contracts Manager**	Date
Date 20 APRIL 2010	

do/58457

Please complete and submit to Donnie.w.owens@pfizer.com

THIS FORM MUST BE TYPED

STATEMENT OF INVESTIGATION FORM

Date: March 22, 2010	Request Number: XXXXXX	Sponsor Approval: Write in
Compound Requested: Celebrex (Celecoxib)		Amount: 50 mg
Investigator Name: Dr. Vijay Agarwal		Institution: University of Hull, Hull, UK
Investigator Title: Doctor		Department: Post Graduate Medical Institute
Phone: 00447894967114 Fax: 00441482 461874 e-mail: Vijay.Agarwal@hey.nhs.uk		Mailing Address: Research Laboratories (2nd Floor) Daisy Building Entrance 2 Castle Hill Hospital Castle Road Hull HU16 5JQ UK
Animal Study ☐ No		

Please type a detailed explanation of the proposed studies using Pfizer compound(s) in the space provided below

Study Title: Overcoming resistance to tyrosine kinase inhibitors in malignant pleural mesothelioma

Key Words: mesothelioma, EGFR, tyrosine kinase inhibitor, COX2

Objective:

Mesothelioma is a rare tumour that is increasing in incidence in the UK with a likely peak from 2011 to 2015[1]. Therapeutic options remain limited. Destro et al[2] documented EGFR immunoreactivity in 34 out of 61 (55.7%) malignant pleural mesothelioma cases. Cancer and Leukemia Group B (CALGB 30101)[3] looked at EGFR expression score by immunohistochemistry in 28 patients. It was categorized as low (EGFR 1+ or 2+) or high (EGFR 3+) expression and 97% had EGFR over-expression (2+ or 3+). Two phase II studies of the receptor tyrosine kinase inhibitors, gefitinib[3] and erlotinib[4], have shown no activity in this tumour type. Inspite of there being significant EGFR over-expression in Mesothelioma it is surprising that receptor tyrosine kinase inhibitors do not work. Prostaglandin E2 (PGE2) derived from intratumoural COX-2 has been shown to transactivate the EGFR inhibitors in many tumours[5]. It is hypothesized that a similar mechanism of transactivation may exist in mesothelioma cells conferring resistance of EGFR activation.

Background:

COX-2 is highly over expressed in malignant pleural mesothelioma. Our group obtained archival samples from 86 malignant pleural mesothelioma patients (histological subtype: 42 epithelial, 28 biphasic and 16 sarcomatoid) [6]. Over-expression of COX-2 was detected by immunohistochemical analysis. Overall 51/86 (59%) tumour sections demonstrated COX-2 over-expression. The frequency varied with histological subtype with 31/42 (73%) of epithelial sections, 14/28 (50%) of biphasic sections and 6/16 (37%) of sarcomatoid sections recorded as positive. Kaplan Meier survival analysis indicated that over-expression of COX-2 was significantly related to improved prognosis ($P < 0.001$) and was an independent prognostic factor in multivariate analysis. We also looked to see whether selective COX-2 inhibitors were capable of inhibiting the growth of mesothelioma cell lines. Three mesothelioma cell lines (MSTO-211H, NCI-H2052 and NCI-H2452) and three selective COX-2 inhibitors (nDuP-697, nNS-398 and nPTBS) were used. The IC50 values obtained were as follows: MSTO-211H cells with DuP-697 (231μM) and with PTPBS (180μM);

NCI-H2052 cells with DuP-697 (86μM) and with PTPBS (236μM). IC50 values were not achieved using the any inhibitors on the cell line NCI-H2452 or using the inhibitor NS-398 in any cell line. This study concluded that COX-2 specific inhibitors do have a cytotoxic effect on some Mesothelioma cell lines. We also demonstrated that the combination of either of the specific COX-2 inhibitors DuP-697 or NS-398 with pemetrexed enhanced the cytotoxicity of this drug by at least 4-fold in mesothelioma cell lines [7]. COX2 inhibitor Celecoxib has previously been shown to have a cytotoxic effect in malignant mesothelioma cell lines[8].

Chen et al carried out the in vitro study using Celecoxib combined with EGFR-tyrosine kinase inhibitor ZD1839 on Non small cell lung cancer cell lines. The study concluded that Celecoxib combined with EGFR inhibitors like Gefitinib led to stronger inhibition of related cell signal transduction pathways[9].

Celecoxib is the most commonly used COX2 inhibitor in clinical practice with a good safety profile. This project will explore the cytotoxic effects of Celecoxib on its own and in combination with Gefitinib in mesothelioma cell lines. Depending on the results, its mechanism of action will be studied using proteomics. If any promising combinations are identified then the combination may form a basis of clinical trials. So far there are no published reports of any studies with Celecoxib and Gefitinib in mesothelioma call lines.

Research Protocol:

Materials:

Mesothelioma cell lines MSTO-211H, NCI-H2052 and NCI-H2452 are obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium. The lung cancer cell line (A549), which is selected as a positive control is obtained from the European Collection of Cell Cultures (ECACC) and maintained in RPMI 1640 medium. The medium will be supplemented with 10% (v/v) fetal bovine serum, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

The antibodies for western blot will include

Antibody	Company	Catalogue number
EGFR	Abcam	Ab2430
Phospho-EGFR	Cell signalling	#2236
AKT	Abcam	Ab8805
Phospho-AKT	Cell signalling	#4058
ERK	Santa cruz	Sc-154
Phospho- ERK	Santa cruz	Sc-7383
COX-2	Abcam	ab52237
EGF	Invitrogen	PMG8044

Gefitinib is purchased commercially from Tocris Bioscience (Cat No 3000).

Methodology:
Mesothelioma cell lines (MSTO-211H, NCI-H2052 and NCI-H2452) will be grown in cell culture. NSCLC cell line A549 will act as a control. Cytotoxic effects of Celecoxib on its own and in combination with Gefitinib will be studied by MTT/MTS assays on the mesothelioma cell lines. For inhibitors that demonstrate an antiproliferative effect we will investigate the mode of action further. Analysis of the cellular pathways and specific molecular targets involved in the mechanisms of action of COX2/EGFR pathway inhibitors in mesothelioma cell lines will be undertaken using global proteome profiling techniques. Protein samples will be extracted in triplicate at time zero (untreated cells) and up to 48 hours post-treatment with the pathway inhibitor. Each post-treatment protein sample will be compared directly to the untreated sample using our established complementary proteomic approaches based on 2-dimensional polyacrylamide gel electrophoresis combined with mass spectrometry (2D-MS) and antibody microarrays. This will allow a high-resolution analysis of the proteomic changes, which occur in each cell line during a time-course exposure to each agent under test. The identification of proteins and cellular pathways which are affected by the drugs under test could lead to further studies in which the expression of these proteins is manipulated to increase the efficacy of therapy in mesothelioma and will also highlight novel drug targets. Any novel treatment

strategies will be progressed towards clinical trials in mesothelioma patients.

Research Team: Our group at the University of Hull is dedicated to research in cancer proteomics. We have a team of scientists and oncologists based at Castle Hill Hospital in Hull. The Hospital has recently benefited from the addition of the new £6 million Daisy medical research building and the new £70 million Queens Oncology and Hematology Centre, providing state-of-the-art cancer care. Our Cancer Biology Proteomics Group has developed proteomic methodologies over the past 6 years for application to cancer biology with recent publications in Cancer and Molecular Cancer Therapeutics. We are undertaking a focused research programme targeting novel therapeutic strategies for mesothelioma.

References:

1. *Mesothelioma mortality in Great Britain, Health and Safety executive, December 2003.* See http://www.hse.gov.uk/statistics/causdis/proj6801.pdf.
2. Destro, A., et al., *EGFR overexpression in malignant pleural mesothelioma. An immunohistochemical and molecular study with clinico-pathological correlations.* Lung Cancer, 2006. **51**(2): p. 207-15.
3. Govindan, R., et al., *Gefitinib in patients with malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B.* Clin Cancer Res, 2005. **11**(6): p. 2300-4.
4. Garland, L.L., et al., *Phase II study of erlotinib in patients with malignant pleural mesothelioma: a Southwest Oncology Group Study.* J Clin Oncol, 2007. **25**(17): p. 2406-13.
5. Pai, R., et al., *Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy.* Nat Med, 2002. **8**(3): p. 289-93.
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7. O'Kane, S.L., et al., *COX-2 specific inhibitors enhance the cytotoxic effects of pemetrexed in mesothelioma cell lines.* Lung Cancer, 2009.
8. Catalano, A., et al., *Preclinical evaluation of the nonsteroidal anti-inflammatory agent celecoxib on malignant mesothelioma chemoprevention.* Int J Cancer, 2004. **109**(3): p. 322-8.
9. Chen, L., et al., *Selective COX-2 inhibitor celecoxib combined with EGFR-TKI ZD1839 on non-small cell lung cancer cell lines: in vitro toxicity and mechanism study.* Med Oncol, 2008. **25**(2): p. 161-71.

Please complete and submit to Donnie.w.owens@pfizer.com

Please type a brief justification for the amount of compound(s) requested.

We will be using a dose range of 1 to 100 micro molar concentrations per well of Celecoxib in a 96 well plate. This will require approximately 10microgram of celecoxib per well. To cover 80 wells, we will need 800 microgram per well. We will be doing on an average about 50 experiments requiring 40 milligram of Celecoxib. To account for wastage, please provide 50 milligram of Celecoxib.

Material Transfer Agreement
("the Agreement")

Between

Merck KGaA
Frankfurter Str. 250
64293 Darmstadt
Germany

(hereinafter referred to as "MERCK")

and

University of Hull
~~Dr. Vijay Agarwal~~
~~Research Laboratories (2nd Floor), Daisy Building, Entrance 2~~
Cottingham Road
Hull HU6 7RX
England

(hereinafter referred to as "RECIPIENT")

(each party hereinafter referred to as PARTY or commonly as "PARTIES".)

WHEREAS, MERCK has developed or has control over a pharmaceutical substance identified as Cetuximab ("COMPOUND").

WHEREAS, RECIPIENT has the necessary equipment and is authorized to conduct experiments, such as, but not limited to in vitro laboratory experiments and experiments using animal models.

WHEREAS, RECIPIENT is desirous of obtaining from MERCK the right to evaluate the COMPOUND for its use in Oncology as further outlined in the attached Appendix (hereinafter referred to as "EVALUATION") and MERCK is willing to make the COMPOUND available to RECIPIENT for such EVALUATION upon the terms and conditions presented herein.

Now, therefore, the PARTIES agree as follows:

I. RECIPIENT as used in this Agreement shall be understood as RECIPIENT as well as the affiliated companies of RECIPIENT as mentioned in the Appendix. Affiliated companies are defined as any entity, which owns or controls RECIPIENT, or which is owned or controlled by RECIPIENT, each by ownership of at least 50 % of the outstanding voting stock of such entity.

II. MERCK shall provide to RECIPIENT twenty (20 mg) milligrams of the COMPOUND along with such data and information relating to the COMPOUND as necessary to enable RECIPIENT to conduct the EVALUATION. Such data and information shall hereinafter be collectively referred to as "INFORMATION".

III. RECIPIENT, for a period of 12 months, shall have a non-exclusive, non-transferable license (or sublicense), without the right to sub-license, to use the COMPOUND and the INFORMATION supplied by MERCK, solely to conduct the EVALUATION, and confirms that COMPOUND will only be used in accordance with applicable laws and will not be used

in humans. Any other use, including, without limitation, use for commercial purposes shall be prohibited. Use for commercial purposes as used herein shall mean inter alia, use for screening, production, sale or license, and also the use or transfer of products, processors, or other materials or INFORMATION derived from or identified through the use of the COMPOUND.

RECIPIENT shall not file and shall not allow to file any patent application or other intellectual property right based on INFORMATION or COMPOUND, or the results of the EVALUATION, derived or achieved from the work under this Agreement (such results, whether patentable or not, hereinafter referred to as "RESULTS").

During the term of this Agreement and for **five (5) years** thereafter, RECIPIENT agrees to keep confidential any and all INFORMATION as well as the COMPOUND obtained from MERCK and not to disclose or provide such INFORMATION to any third party without prior written consent of MERCK. The confidentiality obligation shall not apply to INFORMATION which

a) was known to RECIPIENT prior to the disclosure by MERCK as evidenced by RECIPIENT's written records; or

b) was or becomes public knowledge through no fault of RECIPIENT; or

c) was obtained by RECIPIENT from a third party entitled to use and disclose the same as a matter of right; or

d) that RECIPIENT is required to disclose by law, or by any regulatory or governmental authority to which RECIPIENT may be subject, on prior written notice to MERCK so that MERCK may determine whether to seek a protective order or other appropriate remedy. In the event that such a protective order or other remedy is not obtained before the time such disclosure is so required, RECIPIENT will disclose only that portion of the INFORMATION to the requesting body which is so required to be disclosed.

IV. Title to the COMPOUND and the INFORMATION shall always be and remain with MERCK. The COMPOUND and the INFORMATION represent significant investment on the part of MERCK, and is considered proprietary to MERCK. RECIPIENT therefore agrees to retain control over the COMPOUND and the INFORMATION and further agrees that the COMPOUND and the INFORMATION will only be used by or through the direct supervision of RECIPIENT in its laboratories under suitable conditions and not to transfer or make available the COMPOUND and the INFORMATION to others not under the direct supervision of RECIPIENT without prior written approval of MERCK.

V. Upon receipt of COMPOUND and/or INFORMATION, RECIPIENT shall complete the EVALUATION as outlined in the attached Appendix, such EVALUATION to be completed within the period mentioned in clause III above, but in no event shall such EVALUATION last longer than the duration of this Agreement as per clause VIII.

VI. At the end of the EVALUATION, RECIPIENT shall provide to MERCK the detailed RESULTS, which may be used by MERCK for any purposes without any restrictions, except as otherwise provided herein.

Any invention being part of the RESULTS, which does not incorporate or otherwise contain COMPOUND or INFORMATION or are related to them shall be owned by RECIPIENT and RECIPIENT may file for patent protection of such invention. RECIPIENT shall grant MERCK a license, at no cost, to use such RECIPIENT discovery for research purposes, and MERCK shall further have the right of first refusal to obtain a commercial license, with the right to grant sublicenses, to make, have made, use and sell products incorporating the RECIPIENT

discovery. Such right shall be exercised during a period of ninety (90) days after the receipt of written notification of such offer by MERCK.

Any RESULTS, which incorporate, or otherwise contain the INFORMATION or COMPOUND or are related to them, shall be jointly owned by RECIPIENT and MERCK. If such RESULTS contain patentable inventions, MERCK shall be entitled to file for patent protection in the name of RECIPIENT and MERCK, provided that RECIPIENT shall have a ninety (90) days period to review any patent application before its submission. RECIPIENT agrees not to license such jointly owned discovery without the prior written consent of MERCK. The costs of prosecution and maintenance of such patent application shall be shared equally between the PARTIES.

VII. In order to protect MERCK's proprietary rights, RECIPIENT agrees not to make publications that are based upon, make reference to or directly or indirectly relate to INFORMATION, COMPOUND or RESULTS, without the prior written approval of MERCK.

VIII. This Agreement shall come into force upon signature of the last to sign PARTY and shall terminate one year thereafter, unless earlier terminated by the PARTIES in accordance with this Agreement.

IX. This Agreement may be terminated in writing by either PARTY at any time during the term of this Agreement:

a) if the other PARTY has materially breached one of its essential obligations hereunder by causes and reasons within its control and has not cured such default within thirty days after the receipt of a certified letter from the other PARTY requesting correction of the default; or

b) upon filing institution of bankruptcy, reorganization, liquidation, or receivership proceedings by or against the other PARTY.

X. In case of early termination of this Agreement, RECIPIENT will provide to MERCK the detailing RESULTS of the EVALUATION available at the time of termination. All such RESULTS may then be used by MERCK without any limitation.

XI. Any notice or request required or permitted under this Agreement shall be made by either PARTY to the other by personal delivery or by first class mail, postage prepaid, to the addresses mentioned above, or to such other address or person as either PARTY shall designate by written notice to the other. Notice shall be deemed effective upon receipt.

XII. This Agreement shall be binding upon and inure to the benefit of the successors of the PARTIES hereto, but shall not be assignable by either PARTY hereto without the prior written consent of the other PARTY, which consent shall not be unreasonably withheld.

XIII. The termination of this Agreement for any reason whatsoever shall not be deemed a release, nor shall it relieve either PARTY from any obligation under this Agreement, which may have accrued prior thereto.

XIV. Nothing in this Agreement shall be deemed to create a relationship of agency or to constitute the PARTIES as partners or joint ventures and shall not create any obligation to the PARTIES to enter into further agreements.

XV. RECIPIENT agrees to indemnify, defend and hold harmless MERCK from and against any and all loss, claim, damage, liability or expense (including reasonable attorney's fees) arising out of or in connection with the possession, use or other activity relating to the COMPOUND and/or INFORMATION by RECIPIENT, any employee or any third party involved by RECIPIENT to fulfill its obligations under this Agreement.

XVI. The invalidity or unenforceability of any provision of this Agreement shall not affect or limit the validity or enforceability of any other provisions hereof.

XVII. The COMPOUND and INFORMATION is provided to enable the RECIPIENT to conduct the EVALUATION. MERCK GIVES NO WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, SAFETY, EFFICACY, POTENCY, PURITY OR ACTIVITY, OR THAT ITS USE WOULD NOT INFRINGE ANY PATENT OR INTELLECTUAL PROPERTY RIGHT OF ANY THIRD PARTY. THE RECIPIENT HEREBY IRREVOCABLY WAIVES ANY STATUTORY WARRANTIES OF ANY KIND RELATING TO THE COMPOUND OR INFORMATION.

XVIII. This Agreement constitutes the entire and only Agreement between the PARTIES relating to this subject matter; all prior negotiations, representations, agreements, and understandings are superseded by this Agreement. Save as otherwise herein expressly provided no variations of or to this Agreement or any of its provisions shall be effective unless made in writing and signed by both PARTIES.

IN WITNESS WHEREOF, the PARTIES have caused this Agreement to be executed by their authorized representatives and effective as of the date first above written.

Merck KGaA

i.V. i.V.

Dr. A. Blaukat Dr. S. Heitz

Date: November 19, 2009

RECIPIENT

Mr Jonathan Cant
Research Grants & Contracts Manager

Date: 8 DECEMBER 2009

Appendix: Research proposal

Title

Overcoming resistance to EGFR - tyrosine kinase inhibitors (TKI) in mesothelioma

Investigator

Name:	Dr. Vijay Agarwal
Institution:	The Postgraduate Medical Institute, University of Hull and Hull York Medical School.
Address:	Research Laboratories (2nd Floor) Daisy Building Entrance 2 Castle Hill Hospital Castle Road Hull HU16 5JQ England
Phone/Fax:	Tel: 01482 461871, 07894967114(M) Fax: 01482 461874
E-mail:	Vijay.Agarwal@hey.nhs.uk
Homepage:	http://www2.hull.ac.uk/pgmi/cancer/cancer%20biology%20proteomics.aspx http://www2.hull.ac.uk/pgmi/cancer/oncology.aspx

Introduction

(Provide a short introduction describing the scientific background of your proposal and the current knowledge it is based on; not more than ½ page)

Mesothelioma is a rare, aggressive tumour of the serosal surfaces, such as the pleura and peritoneum. The incidence of malignant pleural mesothelioma (MPM) is rising in most European countries[1] and current therapeutic options remain limited. The median overall survival with current standard chemptherapy (Alimta - Cisplatin) regime is 12 months[2]. Currently, there are no clinically proven targeted treatment options in MPM.

Epidermal Growth Factor Receptor (EGFR) has shown to be over-expressed in MPM by various investigators[3-6]. EGFR Tyrosine Kinase Inhibitor's (TKI's) like Gefitinib[5] and Erlotinib[7] have not shown any efficacy in Phase II clinical trials. In non-small cell lung cancer (NSCLC), the presence of activating mutations in the TKI domain is associated with increased response to EGFR TKI inhibitors[8, 9]. As these activating mutations are not seen in MPM tissue samples[10, 11], it may be one of the reasons why TKI's don't work in MPM.

EGFR can also be inhibited by using monoclonal antibodies like Cetuximab, which act on the extracellular domain of the EGFR receptor. Presence of KRAS mutations in colorectal cancer and NSCLC are associated with resistance to monoclonal antibodies[12]. These KRAS mutations are not present in MPM[13-16].

Our group has shown that COX-2 in overexpressed in 51 of the 86 MPM tissue samples studied[17] and selective COX-2 inhibitors inhibit 3 different Mesothelioma cell lines (MSTO-211H, NCI-H2052 and NCI-H2452)[18]. COX-2 is known to transactivate EGFR pathway[19] and hence may induce resistance to EGFR inhibitors. Blocking of both EGFR inhibitors and COX-2 pathway may be necessary to induce a therapeutic effect in MPM.

Hypothesis (one sentence)

In the presence of EGFR over-expression, absence of activating mutations at the EGFR - TKI domain and absence of KRAS mutations in MPM, it is hypothesized that monoclonal antibodies like Cetuximab may be effective as opposed to TKI inhibitors which have failed, as a targeted treatment modality in MPM.

Experimental Strategy

(Briefly explain your experimental strategies; not more than 1 page)

This project will explore the EGFR pathway inhibition by Cetuximab and study the cytotoxic effects of single agent Cetuximab in mesothelioma cell lines. Depending on the results, its synergism with COX-2 inhibitors and chemotherapy will be studied. The COX2 inhibitors flurbiprofen (#1769), DuP-697 (#1430) and NS-398 (#0942) will be purchased off the shelf from Tocris Bioscience. Cisplatin will be purchased from Sigma–Aldrich (#P4394).

Mesothelioma cell lines MSTO-211H, NCI-H2052 and NCI-H2452 are obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium. The lung cancer cell line (A549), which is selected as a positive control is obtained from the European Collection of Cell Cultures (ECACC) and maintained in RPMI 1640 medium. The medium will be supplemented with 10% (v/v) foetal bovine serum, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

The antibodies for western blot will include:
EGFR - Abcam (Ab2430)
Phospho-EGFR -Cell signalling (#2236)
AKT - Abcam (Ab8805)
Phospho-AKT- Cell signalling(#4058)
ERK- Santa cruz (Sc-154)
Phospho- ERK - Santa cruz(Sc-7383)
COX-2 - Abcam(ab52237)
EGF - Invitrogen(PMG8044)

Methodology:
Mesothelioma cell lines (MSTO-211H, NCI-H2052 and NCI-H2452) will be grown in cell culture. NSCLC cell line A549 will act as a control. All cell lines will be stimulated with EGF to look for phosphorylation of EGFR, AKT and ERK by western blot. All cell lines will then be incubated with Cetuximab at various time intervals and then reattempt stimulation with EGF to look for blocking of EGFR/AKT/ERK phosphorylation by western blot.

If Cetuximab successfully blocks EGFR pathway, then cytotoxic effects of Cetuximab on the cell lines will be studied by MTT assays. Furthers synergistic cytotoxic effects of Cetuximab with COX-2 inhibitors flurbiprofen, DuP-697 and NS-398 and chemotherapy agent Cisplatin will be studied.

For inhibitors that demonstrate an antiproliferative effect we will investigate the mode of action further. Analysis of the cellular pathways and specific molecular targets involved in the mechanisms of action of EGFR pathway inhibitors in mesothelioma cell lines will be undertaken using global proteome profiling techniques. Protein samples will be extracted in triplicate at time zero (untreated cells) and up to 48 hours post-treatment with the Cetuximab. Each post-treatment protein sample will be compared directly to the untreated sample using our established complementary proteomic approaches based on 2-dimensional polyacrylamide gel electrophoresis combined with mass spectrometry (2D-MS) and antibody microarrays. This will allow a high-resolution analysis of the proteomic changes, which occur in each cell line during a time-course exposure to each agent under test.

Research Team:

My work will be supervised by Professor Michael Lind(Professor and Lead Oncologist in Department of Academic Oncology) and Dr. Lynn Cawkwell (Lecturer in Cancer Genetics). Our group at the University of Hull is dedicated to research in cancer proteomics. We have a team of scientists and oncologists based at Castle Hill Hospital in Hull. The Hospital has recently benefited from the addition of the new £6 million Daisy medical research building and the new £70 million Queens Oncology and Haematology Centre, providing state-of-the-art cancer care. Our Cancer Biology Proteomics Group has developed proteomic methodologies over the past 6 years for application to cancer biology with recent publications in Cancer and Molecular Cancer Therapeutics. We are undertaking a focused research programme targeting novel therapeutic strategies for mesothelioma.

References:
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5. Govindan, R., et al., Gefitinib in patients with malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B. Clin Cancer Res, 2005. 11(6): p. 2300-4.
6. Edwards, J.G., et al., EGFR expression: associations with outcome and clinicopathological variables in malignant pleural mesothelioma. Lung Cancer, 2006. 54(3): p. 399-407.
7. Garland, L.L., et al., Phase II study of erlotinib in patients with malignant pleural mesothelioma: a Southwest Oncology Group Study. J Clin Oncol, 2007. 25(17): p. 2406-13.
8. Lynch, T.J., et al., Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med, 2004. 350(21): p. 2129-39.
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Amount of Antibody required for your study

2 milligrams

Appendix E (List of antibodies in antibody microarray kit)

Table E.1 List of antibodies in Panorama XPRESS Profiler725 antibody microarray Kit (Sigma Aldrich #XP725)

ANTIBODY	SIGMA No.
14-3-3 □□	T5942
Acetylated Protein	A5463
Actin	A5060
Actin	A3853
Actin, α-Smooth Muscle	A5228
β-Actin	A1978
β-Actin	A2228
□-Actinin	A5044
Actopaxin	A1226
AP2	A7107
β1 and β2-Adaptins	A4450
I-Afadin	A0349
AFX	A8975
AFX (FOXO4)	A5854
AKR1C3	A6229
Aly	A9979
β-Amyloid	A8354
Amyloid Precursor Protein, C-Terminal	A8717
Amyloid Precursor Protein, N-Terminal	A8967
Amyloid Precursor Protein, KPI Domain	A8842
Androgen Receptor	A9853
Annexin V	A8604
Annexin VII	A4475
Anti Cy3+Cy5	C0992
AOP1	A7674
AP-1	A5968
AP-2□	A0844
AP Endonuclease	A2105
Apaf1, N-Terminal	A8469
Apoptosis Inducing Factor (AIF)	A7549
APRIL, Extracellular Domain	A1726
APRIL, Extracellular Domain 2	A1851
ARC, C-Terminal	A8344
ARNO (Cytohesin-2)	A4721
Arp1□/Centractin	A5601
ARP2	A6104
ARP3	A5979
ARTS	A3720
ARTS	A4471

ASAP1/Centaurin β4	A4227
ASC-2	A5355
ASPP1	A4355
ASPP2	A4480
ATF-1	A7833
ATF2	A4086
phospho-ATF-2 ($pThr^{69,71}$)	A4095
ATM	A6093
Anti Cy3+Cy5	C0992
ATM	A6218
Aurora-B	A5102
BACE-1	B0806
BACH1	B1310
BAD	B0559
BAF57	B0436
BAK	B5897
BAP1	B9303
Bax	B3428
Bax	B8429
Bax	B8554
Bax	B9054
Bcl-10	B7806
Prion protein	P0110
Bcl-10	B0431
Seladin	S4697
Bcl-2	B9804
Bcl-2	B3170
Bcl-x	B9304
Bcl-x_L	B9429
BID	B4305
BID	B3183
Bim	B7929
Anti Cy3+Cy5	C0992
CDK5	C6118
Bmf, N-Terminal	B1684
Bmf, C-Terminal	B1559
BNIP3	B7931
BOB.1/OBF.1	B7810
Brg1/hSNF2β	B8184
BTK, C-Terminal	B0811
BTK, N-Terminal	B0686
BUB1	B0561
BUBR1	B9310
c-Abl	A5844
c-Cbl	C9603
c-erbB-2	E2777

c-erbB-3	E8767
c-erbB-4	E5900
phospho-c-Jun (pSer^{63})	J2128
phospho-c-Jun (pSer^{73})	J2253
c-Myc	M4439
c-Myc	C3956
Uvomorulin/E-Cadherin	U3254
N-Cadherin	C2542
N-Cadherin	C2667
Pan Cadherin	C1821
Anti Cy3+Cy5	C0992
Calbindin-D-28K	C7354
Calcineurin (□-Subunit)	C1956
Caldesmon	C6542
Calmodulin	C7055
Calnexin	C4731
Calponin	C2687
Calreticulin	C4606
Calretinin	C7479
Claspin	C7867
CaM Kinase IV (CaMKIV)	C2851
CaM Kinase Kinase □(CaMKK□)	C7099
CaM Kinase IIα (CaMKIIα)	C6974
CaM Kinase IV (CaMKIV)	C9973
CASK/LIN2	C4856
Casein Kinase 2β	C3617
Caspase 2	C7349
Caspase 3	C9598
Caspase 3, Active	C8487
Caspase 4	C4481
Caspase 4	C3392
Caspase 5	C6979
Caspase 6	C7599
Caspase 7	C7724
Anti Cy3+Cy5	C0992
Caspase 7	C1104
Caspase 8	C3101
Caspase 8	C2976
Caspase 8	C4106
Pro-Caspase 8	C7849
Caspase 9	C7729
Caspase 9	C4356
Caspase 10	C8351
Caspase 10	C1229
Caspase 11	C1354
Caspase 12	C7611

Caspase 13 (ERICE)	C8854
Catalase	C0979
α-E-Catenin	C8114
□-N-Catenin	C8239
□Catenin	C2081
β-Catenin	C7207
β-Catenin	C7082
phospho-β-Catenin (pThr^{41})	C8616
phospho-β-Catenin (pSer^{33}/pSer^{37})	C4231
phospho-β-Catenin (pSer^{45})	C5615
phospho-β-Catenin (pSer^{33})	C2363
δ-Catenin/NPRAP	C4864
Anti Cy3+Cy5	C0992
Cathepsin D	C0715
Cathepsin L	C2970
Caveolin-1	C3237
CD40	C5987
Cdc14A	C2238
Cdc25c	C0349
Cdc25A	C9479
Cdc27	C7104
Cdc6	C0224
Cdc7 Kinase	C6613
Cdh1	C7855
Cdk1^{p34cdc2}	C4973
Negative Control	NA
Cdk4	C8218
Cdk6	C8343
Cdk-7/cak	C7089
TBP	T1827
CENP-E	C7488
Centrin	C7736
Chk1	C9358
Chk2	C9108
Chk2	C9233
Chondroitin Sulfate	C8035
Anti Cy3+Cy5	C0992
Ciliated Cell Marker	C5867
CIN85	C8116
Casein Kinase 2α	C5367
Clathrin Light Chain	C1985
Clathrin Heavy Chain	C1860
CNPase	C5922
Cofilin	C8736
Coilin	C1862
Collagen, Type IV	C1926

Connexin 32	C3470
Negative Control	NA
Connexin- 32	C6344
Connexin- 43	C8093
Connexin- 43	C6219
β-COP	G6160
Cortactin	C6987
Corticotropin Releasing Factor	C5348
COX II	C9354
Crk-L	C0978
Crk II	C0853
Csk	C7863
CtBP1, N-Terminal	C9491
CtBP1, C-Terminal	C8741
Anti Cy3+Cy5	C0992
CUG-BP1	C5112
Cyclin A	C4710
Cyclin B_1	C8831
Cyclin D_1	C5588
Cyclin D_1	C7464
Cyclin D_2	C7339
Cyclin D_3	C7214
Cyclin H	C5351
Cystatin A	C3095
Cytohesin-1	C8979
Cytokeratin peptide 4	C5176
Cytokeratin CK5	C7785
Cytokeratin peptide 7	C6417
Cytokeratin 8.12	C7034
Cytokeratin 8.13	C6909
Cytokeratin peptide 13	C0791
Cytokeratin Peptide 17	C9179
Cytokeratin peptide 18	C1399
Cytokeratin peptide 19	C6930
Pan Cytokeratin	C2931
DAPK	D2178
phospho-DAPK ($pSer^{308}$)	D4941
DAP Kinase 2	D3191
Anti Cy3+Cy5	C0992
Daxx	D7810
DcR1	D3566
DcR2	D3188
DcR3	D1814
DEDAF	D3316
Desmin	D1033
Desmosomal Protein	D1286

Destrin/ADF	D8940
Dnase I	D0188
Dnase II	D1689
DNMT1	D4567
DNMT1	D4692
DOPA Decarboxylase	D0180
DP2	D7438
DR3	D3563
Negative Control	NA
DR4	D3813
DR5	D3938
DR6	D1564
DRAK1	D1314
Dystrophin	D8168
Dystrophin	D8043
E2F1	E9026
Anti Cy3+Cy5	C0992
E2F1	E8901
E2F2	E8776
E2F3	E8651
E2F4	E8526
E6AP	E8655
EGF receptor	E3138
ERK5 (Big MAPK-BMK1)	E1523
Elastin	E4013
ELKS	E4531
Endothelial Cell Protein C Receptor	E6280
Endothelial Cells	E9653
Endothelin	E0771
Epidermal Growth Factor	E2520
Episialin (EMA)	E0143
ERP57	E5031
Estrogen Receptor	E0521
Estrogen Receptor	E1396
Exportin T	E1531
Ezrin	E8897
F1A	F3428
FADD	F8053
Focal Adhesion Kinase ($pp125^{FAK}$)	F2918
FAK Phospho ($pSer^{772}$)	F9051
Anti Cy3+Cy5	C0992
phospho-FAK Phospho ($pSer^{910}$)	F9301
phospho-FAK ($pTyr^{397}$)	F7926
phospho-FAK ($pTyr^{577}$)	F8926
Falkor/PHD1	F5303
Fas (CD95/Apo-1)	F4424

Fas Ligand	F2051
Fas Ligand	F1926
FBI-1/PAKEMON	F9429
Fibroblast Growth Factor-9	F1672
Fibronectin	F0791
Fibronectin	F3648
Fibronectin	F7387
Filamin	F1888
Filensin	F1043
FKHR (FOXO1a)	F6928
FKHRL1 (FOXO3a)	F2178
FKHRL1 (FOXO3a)	F1304
FLIPγ/δ, C-Terminal	F9925
FOXC2	F1054
FOXP2	F6304
FANCD2	F0305
FXR2	F1554
FRS2 (SNT-1)	F9052
Anti Cy3+Cy5	C0992
G9a Methyltransferase	G6919
Glutamic Acid Decarboxylase 65 (GAD 65)	G4913
Glutamic Acid Decarboxylase 65 (GAD 65)	G5038
Glutamic Acid Decarboxylase (GAD65/67)	G5163
GADD 153 (CHOP-10)	G6916
$GAP1^{IP4BP}$	G6666
GAPDH	G8795
GATA-1	G0290
Gelsolin	G4896
Gemin 2	G6669
Gemin 3	G6544
GFAP (Glial Fibrillary Acidic Protein)	G9269
GFAP (Glial Fibrillary Acidic Protein)	G3893
Growth Factor Independence-1 (GFI)	G6670
Glutamate receptor NMDAR 2a	G9038
Glutamine Syntethase	G2781
Glycogen Synthase Kinase-3β (GSK-3β)	G7914
Glycogen Synthase Kinase-3 (GSK-3)	G4414
Glycogen Synthase Kinase-3 (GSK-3)	G6414
Granzyme B	G1044
Grb-2	G2791
GRK 2	G7670
GRP1	G6541
Anti Cy3+Cy5	C0992
GRP 75	G4170
GRP78/BiP	G8918

GRP94	G4420
hABH1	A8103
hABH2	A8228
hABH3	A8353
hBRM/hSNF2α	H9787
HAT1 (Histone acetyltransferase 1)	H7161
HDAC-1	H3284
HDAC-1	H6287
HDAC-2	H3159
HDAC-2	H2663
HDAC-3	H6537
HDAC-3	H3034
HDAC-4	H9411
HDAC-4	H9536
Negative Control	NA
HDAC-5	H4538
HDAC-5	H8163
HDAC-6	H2287
HDAC-7	H2537
HDAC-7	H6663
HDAC-8	H6412
Anti Cy3+Cy5	C0992
HDAC-10	H3413
HDAC-11	H2913
HDRP/MITR	H9163
Heat Shock Factor 1	H4163
Heat Shock Factor 2	H6788
Heat Shock Protein 25	H0148
Heat Shock Protein 27	P1498
Heat Shock Protein 27/25	H2289
Heat Shock Protein 70	H5147
Heat Shock Protein 90	H1775
Heat Shock Protein 110	H7412
Heat Shock Protein 110	H7287
Acetyl Histone H3 (Ac-Lys^{9})	H9286
Acetyl Histone H3 (Ac-Lys^{9})	H0913
Acetyl- & phospho-Histone H3 (Ac-Lys^{9}, Ser^{10})	H9161
Acetyl- & phospho-Histone H3 (Ac-Lys^{9}, Ser^{10})	H0788
Dimethyl Histone H3 (diMe-Lys^{4})	D5692
Dimethyl Histone H3 (diMe-Lys^{9})	D5567
methyl-Histone H3 (Me-Lys^{9})	H7162
phospho-Histone H2AX (pSer^{139})	H5912
phospho-Histone H3 (pSer^{10})	H6409
phospho-Histone H3 (pSer^{28})	H9908

phospho-Histone H3 (pSer^{10})	H0412
Anti Cy3+Cy5	C0992
SUV39H1 Histone Methyl Transferase	S8316
HMG-1	H9537
hMps1	M5818
hnRNP-A1	R4528
hnRNP-A1	R9778
hnRNP-A2/B1	R4653
hnRNP-C1/C2	R5028
hnRNP-K/J	R8903
hnRNP-L	R4903
hnRNP-Q	R5653
hnRNP-U	R6278
hnRNP M3-M4	R3777
hPlk1	P5998
hPlk1	P6123
hSNF5/INI1	H9912
iASPP	A4605
IFI-16	I1659
I□B□	I0505
IKK□	I6139
ILK	I0783
ILK	I1907
ILP2	I4782
Negative Control	NA
Anti Cy3+Cy5	C0992
Importin-□1	I9658
Importin-□3	I9783
Importin-□5/7	I9908
INCENP	I5283
ING1	I3659
□-Internexin	I0282
JAB 1	J3395
JAB 1	J3020
JAK 1	J3774
c-Jun N-Terminal Kinase	J4500
JNK, Activated (Diphosphorylated JNK)	J4750
KCNK9 (TASK-3)	K0514
Kaiso	K4263
KIF17	K3638
KIF3A	K3513
KSR	K4261
Ku Antigen	K2882
L1CAM	L4543
l/s-Afadin	A0224
Laminin	L9393

Laminin-2 (□-2 Chain)	L0663
LAP2 (TMPO)	L3414
Leptin	L3410
Anti Cy3+Cy5	C0992
LIM Kinase 1	L2290
LIN-7	L1538
LIS1	L7391
LKB1	L7917
LDS1	L4793
Mad1	M8069
Mad2	M8694
MADD	M5683
MAFF	M8194
MAGI-1	M5691
MAGI-2	M2441
MAP Kinase, Activated/Monophosphorylated (Phosphothreonine ERK-1&2)	M7802
MAP Kinase, Monophosphorylated Tyrosine	M3682
MAP Kinase, Activated (Diphosphorylated ERK-1&2)	M9692
MAP Kinase, Monophosphorylated Threonine	M3557
MAP Kinase (ERK-1)	M7927
MAP Kinase (ERK1+ERK2)	M5670
MAP Kinase Activated Protein Kinase-2 (MAPKAPK-2)	M3550
MAP Kinase Phosphatase-1 (MKP-1)	M3787
MAPK non phosphorylated ERK	M3807
MAP Kinase 2 (ERK-2)	M7431
MAP Kinase Kinase (MEK, MAPKK)	M5795
MAP2 (2a+2b)	M2320
Anti Cy3+Cy5	C0992
MAP1	M4278
MAP1 (Light Chain)	M6783
MAP1b	M4528
MAP2	M9942
MBD1	M6569
MBD2a	M7568
MBD2a,b	M7318
MBD4	M9817
MBDin/XAB1	M1944
MBNL 1	M3320
MCH	M8440
Mcl-1	M8434
MDC1	M2444

MDM2	M8558
MDM2	M4308
MDM2	M7815
MDMX	M0445
MeCP2	M9317
MeCP2	M7443
MeCP2	M6818
MEKK4	M7194
Melanocortin-3 Receptor	M4937
MGMT	M3068
Anti Cy3+Cy5	C0992
Mint2	M3319
LRRK2 (PARK8)	L3044
MRP1	M9192
MRP2	M3692
□-MSH	M0939
MSH6	M2445
MSH6	M2820
MSK-1	M5437
MTA 2	M7569
MTA1	M1320
MTA1	M7693
MTA2/MTA1L	M7818
MTA3L	M0819
MTBP	M3566
mTOR	T2949
Munc-18-1	M2694
Munc-13/1	M6194
MyD88	M9934
Myosin	M1570
Myosin Iβ (Nuclear)	M3567
Myosin IIA	M8064
Myosin IX/Myr5	M5566
Negative Control	NA
Anti Cy3+Cy5	C0992
Myosin Light Chain Kinase	M7905
Myosin Va	M4812
Myosin Va	M5062
Myosin VI	M0691
Myosin VI	M5187
NBS1 (Nibrin)	N9287
NBS1 (Nibrin)	N3037
NBS1 (Nibrin)	N3162
Nck-2	N2911
Nedd 8	N2786
Nerve Growth Factor-β	N3279

Nerve Growth Factor Receptor	N5408
Nerve growth factor receptor (NGFR p75)	N3908
Neurabin I	N4412
Neurabin II (C-terminal)	N5037
Neurabin-II	N5162
Neurofibromin	N3662
Neurofilament 160	N2787
Neurofilament 200	N4142
Neurofilament 200	N0142
Neurofilament 200	N5389
Neurofilament 68	N5139
Neurofilament 160/200	N2912
Anti Cy3+Cy5	C0992
NF-□B	N8523
NAK (NF□B-Activating Kinase)	N2661
NG2	N8912
Nicastrin	N1660
Nitric Oxide Synthase, Brain (b-NOS)	N2280
Nitric Oxide Synthase, Brain (b-NOS)	N7155
Nitric Oxide Synthase, Endothelial (e-NOS)	N9532
Nitric Oxide Synthase, Endothelial (e-NOS)	N3893
Nitric Oxide Synthase, Endothelial (e-NOS)	N2643
Nitric Oxide Synthase, Inducible (i-NOS)	N7782
Nitric Oxide Synthase, Inducible (i-NOS)	N9657
Notch1	N6786
Nitrotyrosin	N0409
NTF2	N9527
Nuf2	N5287
O-GlcNAc Transferase	O6264
OP-18/Stathmin	O0138
Ornithine Decarboxylase (ODC)	O1136
p115/TAP	P3118
$p120^{ctn}$	P1870
$p130^{CAS}$	C0354
$p14^{arf}$	P2610
$p16^{INK4a/CDKN2}$	P0968
Anti Cy3+Cy5	C0992
$p19^{INK4d}$	P4354
p21WAF1/Cip1	P1484
p300/CBP	P2859
$p34^{cdc2}$	C3085
p35 (Cdk5 Regulator)	P9489
p38 MAP Kinase, Non-Activated	M8432

p38 MAPK	M0800
p38 MAPK activated (diphosphorylated p38)	M8177
Negative Control	NA
p53	P5813
p53	P6874
phospho-p53 (pSer^{392})	P8982
p53DINP1/SIP	P4868
p53R2l	P4993
p53 BP1	B4561
p53 BP1	B4436
p57^{kip2}	P2735
p63	P3362
p63	P3737
PABP	P6246
PAD14	P4749
phospho-PAK1 (pThr^{212})	P3237
Par-4 (Prostate Apoptosis Response-4)	P5367
Anti Cy3+Cy5	C0992
□Parvin	P5746
Parkin	P6248
PARP	P7605
Paxillin	P1093
PCAF	P7493
Proliferating Cell Nuclear Antigen (PCNA)	P8825
PDK 1	P3110
Pen-2	P5622
Peripherin	P5117
Peroxiredoxin 3	P1247
PERP	P5243
Phospholipase A2 group V	P5242
Phosphoserine	P5747
Phosphothreonine	P6623
Phosphotyrosine	P1869
Phospholipase C □1 (PLC □1)	P8104
PhosphatidylSerine Receptor (PSR)	P1495
Negative Control	NA
PIAS-x	P9498
Negative Control	NA
PINCH-1	P9371
Protein Kinase B□Akt1	P2482
Protein Kinase B□Akt1	P1601
Anti Cy3+Cy5	C0992
phospho-PKB (pSer^{473})	P4112
phospho-PKB (pThr^{308})	P3862
Protein Kinase C (PKC)	P5704

Protein Kinase C□	P4334
Protein Kinase Cβ$_1$	P3078
Protein Kinase Cβ$_1$	P6959
Protein Kinase Cβ$_2$	P3203
Protein Kinase Cβ$_2$	P2584
Protein Kinase C□	P8083
Protein Kinase Cδ	P8333
Protein Kinase Cε	P8458
Protein Kinase Cζ	P0713
Protein Kinase Cη	P8090
Protein Kinase D	P3987
PKR	P0244
Plakoglobin (Catenin □)	P8087
Platelet-Derived Growth Factor Receptor β	P7679
Plectin	P9318
PML	P6746
Presenilin-1 (S182)	P7854
Prion Protein	P5999
PRMT1	P6871
PRMT1	P6996
Anti Cy3+Cy5	C0992
PRMT2	P0748
PRMT3	P9370
PRMT4	P4995
PRMT5	P0493
PRMT6	P6495
PRMT6	P2996
Proliferating Cell Protein Ki-67	P6834
Protein Phosphatase 1□	P7979
Protein Phosphatase 1□	P7607
Protein Phosphatase 2A□ (PP2A□)	P8998
Protein S	P4555
Protein Tyrosine Phosphatase PEST	P9109
PSF	P2860
PTEN	P7482
PTEN	P3487
PUMA/bbc3, C-Terminal	P4618
PUMA/bbc3, N-Terminal	P4743
Pyk2	P3902
AP2 beta	
phospho-Pyk2 (pTyr$^{579/580}$)	P6989
Negative Control	NA
Rab5	R7904
Anti Cy3+Cy5	C0992
Rab 7	R8779

Rab9	R5404
RAD1	R5029
Rad17 (C-terminal)	R8029
Raf-1/c-Raf	R2404
Raf-1	R5773
phospho-c-Raf (pSer^{621})	R1151
RAIDD, Internal Domain	R9775
RAIDD	R5275
RALAR	R8529
Ran	R4777
PIASy	P0104
RAP1	R8154
RbAp48/RbAp46	R3779
Reelin	R4904
Retinoblastoma	R6775
phospho-Retinoblastoma (pSer^{795})	R6878
RhoE	R6153
RICK, C-Terminal	R9650
RIP (Receptor Interacting Protein)	R8274
RNase L	R3529
ROCK-1	R6028
ROCK-2	R8653
Anti Cy3+Cy5	C0992
Rsk1	R5145
S-100	S2644
S-100 (□-Subunit)	S2407
S-100 (β-Subunit)	S2532
S-Nitrosocysteine	N5411
S6 Kinase	S4047
SAPK3	S0315
Spectrin (□ and β)	S3396
Serine/Threonine Protein Phosphatase 2 A/A	P8109
Serine/Threonine Protein Phosphatase 1β	P7484
Serine/Threonine Protein Phosphatase 1□1	P7609
Serine/Threonine Protein Phosphatase 2 A/B□	P5359
Serine/Threonine Protein Phosphatase 2 A/B′ pan2	P8359
Serine/Threonine Protein Phosphatase 2C □□	P8609
AP2 gamma	A3108
SGK	S5188
SH-PTP2 (SHP-2)	S3056
Siah2	S7945
Sin3A, N-terminal	S4445
Sin3A, C-Terminal	S6695

Sir2	S5313
SIRP□1 (SHPS-1)	S1311
Sirt1	S5196
Anti Cy3+Cy5	C0992
SKM1 (Skeletal Muscle Type 1)	S9568
Beta tubulin III (neuronal)	
SLIPR/MAGI-3	S1190
SLIPR/MAGI-3	S4191
Smad4 (DPC4)	S3934
SMC1L1	S6446
SMN	S2944
□□-SNAP, C-terminus	S9444
SNAP-23	S2194
SNAP-25	S9684
SNAP- 29	S2069
Sos1	S2937
Sp1	S9809
Spred-2	S7320
Striatin	S0696
Substance P Receptor	S8305
SMAC/Diablo	S0941
SUMO-1	S8070
SUMO-1 (C-terminal)	S5446
Survivin	S8191
Synaptotagmin	S2177
Synaptopodin	S9442
Synaptopodin	S9567
Anti Cy3+Cy5	C0992
SynCAM	S4945
□1 Syntrophin	S4688
□1 Syntrophin	S4813
Syntaxin	S0664
Syntaxin 6	S9067
Syntaxin 8	S8945
□-Synuclein	S3062
Negative Control	NA
Tal	T1075
Tal	T1200
TAP	T1076
Tau	T9450
phospho-Tau (pSer$^{199/202}$)	T6819
Tau	T5530
Tenascin	T2551
Thimet Oligopeptidase 1	T7076
TIS7	T2576
Tumor Necrosis Factor Soluble	T1815

Receptor II	
Tob	T2948
TOM22	T6319
Topoisomerase-I	T8573
TRAIL	T3067
TRAIL	T9191
Anti Cy3+Cy5	C0992
Transforming Growth Factor-β, pan	T9429
Transportin 1	T0825
TRF1	T1948
Tropomyosin	T2780
Tropomyosin (Sarcomeric)	T9283
Tryptophane Hydroxylase	T0678
TSG101	T5826
□Tubulin	T6074
□Tubulin	T6199
□-Tubulin	T5201
β-Tubulin I	T7816
□-Tubulin I+II	T8535
□ -Tubulin III	T5076
β-Tubulin IV	T7941
□Tubulin	T5326
□Tubulin	T3559
□ -Tubulin	T3320
ε-Tubulin	T1323
Tubulin, Polyglutamylated	T9822
Tubulin, Tyrosine	T9028
Tumor Necrosis Factor-□	T8300
Tumor Necrosis Factor-□	T2824
Nanog	N3038
Anti Cy3+Cy5	C0992
TWEAK Receptor/Fn-14	T9700
Tyrosin hydroxylase	T2928
$U2AF^{65}$	U4758
Ubiquitin	U0508
Ubiquitin C-terminal Hydrolase L1	U5133
Ubiquitin C-terminal Hydrolase L1	U5258
Pinin	P0084
Vanilloid Receptor-1	V2764
VDAC/Porin	V2139
Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1)	V4762
Vesicular GABA Transporter	V5764
VGLUT 1	V0389
VGLUT 2	V2639
Vimentin	V6389

Vinculin	V4505
Vitronectin	V7881
WAVE	W0392
WSTF	W3516
Y14	Y1253
ZAP-70	Z0627
Zip Kinase	Z0134
Zyxin	Z0377
GAPDH	G8795
Anti Cy3+Cy5	C0992