CHARACTERIZATION OF MTOR INHIBITION AND AUTOPHAGY INHIBITION IN NON-SMALL CELL LUNG CANCER

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Dedicated to my family.

My mom, Karen, my dad, Bob, my brother, Matt,

and my husband, Byron.

CHARACTERIZATION OF MTOR INHIBITION AND AUTOPHAGY INHIBITION IN NON-SMALL CELL LUNG CANCER

by

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Abstract

Lung cancer continues to be the leading cause of cancer related death in both men and women. Pre-clinical studies of targeted therapies are needed in order to improve upon the chemotherapeutics that are currently in use. The ability to identify subsets of patient tumors which will respond to a particular targeted agent using biomarkers to indicate an acquired vulnerability will improve selection of effective therapeutics and minimize time and money wasted on ineffective drugs. The goal of this dissertation has been to characterize NSCLC response to mTOR inhibition and determine whether there are any molecular biomarkers that can predict response. mTOR is a central regulator of several pro-oncogenic signaling pathways and plays a role in cell growth, proliferation, metabolism, and inhibition of autophagy. Early studies examining mTOR inhibitors were limited by a lack of proper patient selection and the inability of first generation drugs to completely inhibit mTOR signaling. In the present study, we screened a panel of well-characterized NSCLC cell lines with three mTOR inhibitors, classical mTORC1

inhibitor rapamycin, and two novel dual mTORC1/2 inhibitors, Torin1 and AZD8055 in order to identify potential biomarkers that may be used to predict response to these agents. Additionally, in order to further characterize vulnerabilities to mTOR related genes within lung cancer subsets, we performed a genetic knockdown screen individually targeting 55 genes in this important pathway. Because inhibition of mTOR frequently leads to a cytostatic rather than cytotoxic effect, mTOR targeting agents may have greater utility when used in combination with other chemo- and targeted-agents. Therefore, we screened the three mTOR inhibitors in combination with the chemotherapy doublet paclitaxel/carboplatin or the targeted agent erlotinib. Finally, mTOR inhibition and other drug treatments have been shown to lead to autophagy activation. This process of cellular "self-eating" is thought to protect cancer cells from low nutrient availability and therapy induced stress. We screened NSCLC cells for their response to autophagy inhibitors alone and in combination with chemo- and targeted-therapy agents. The studies described in this thesis led us to the following conclusions. A subset of NSCLCs are more responsive to rapamycin than to mTORC1/2 inhibition by Torin1, or AZD8055, and sensitivity to mTOR inhibition is associated with RTK activation such as ERBB2 amplification or EGFR mutation or amplification, while KRAS and/or LKB1 mutations were associated with resistance. RNAi knockdown of various components related to mTOR signaling and autophagy produce a heterogeneous growth effect response in NSCLCs cells, and potentially define subset-specific vulnerabilities. mTOR inhibitors sensitize NSCLC cells to standard targeted- and chemotherapy agents erlotinib and paclitaxel/carboplatin doublet in an additive or synergistic manner, with the greatest level of synergy occurring in cell lines which are resistant to single agent therapies, including those with KRAS mutations. Finally, inhibition of autophagy using chloroquine is not likely to be a successful therapeutic approach in lung cancer as no significant growth effect was seen at physiologically relevant concentrations and no sensitization to standard chemo- or targeted-therapies were observed.

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ADC- adenocarcinoma

ALK- anaplastic lymphoma kinase

AMPK- AMP-activated protein kinase

BCR- breakpoint cluster region

BRAF- v-raf murine sarcoma viral oncogene homolog B1

CaMK- Ca2+/calmodulin-dependent protein kinase

CI- combination index

DAG- diacyglycerol

DMSO- dimethylsulfoxide

dsRNA- double stranded RNA

EGF/EGFR- epidermal growth factor/ receptor

FACS- fluorescence activated cell sorting

FBS- fetal bovine serum

G6P/G6PDH- glucose-6-phosphate/dehydrogenase

GAPDH- glyceraldehyde-3-phosphate dehydrogenase

GC-MS- gas chromatography-mass spectrometry

GEF- guanine nucleotide exchange factor

GFP- green fluorescent protein

GLS- glutaminase

HBEC- human bronchial epithelial cells

HBSS- Hank's balanced salt solution

HCC- Hamon Cancer Center

HIF- hypoxia inducible factor

HK2- hexokinase 2

iBMK- immortalized baby mouse kidney

IP- intraperitoneal

KRAS- Kirsten rat sarcoma viral oncogene homolog

KSFM- keratinocyte free medium

LOH- loss of heterozygosity

MAPK- mitogen-activated protein kinase

MATRIX- MicroArray Transformation in Microsoft Excel

miRNA- micro-RNA

MITF- microphthalmia- associated transcription factor

mTOR- mechanistic target of rapamycin

NAD- nicotinaminde adenine dinucleotide

NCI- National Cancer Institute

NDRG1- N-myc downstream regulated gene 1

NGS- next generation sequencing

NOD/SCID- non-obese diabetic/severe combined immunodeficiency

NSCLC- non-small cell lung cancer

PBS- phosphate buffered saline

PDAC- pancreatic ductal carcinoma

PE- phosphatidylethanolamine

PH- pleckstrin homology

PI- propidium iodide

PI3K- phosphoinositol-3 kinase

PIP- phosphatidylinositol phosphate

PKC- protein kinase C

PLC- phospholipase

PtdIns- phosphatidylinositol

PTEN- phosphatase and tensin homolog

qPCR- quantitative polymerase chain reaction

RCC- renal cell carcinoma

RISC- RNA-induced silencing complex

RMPI- Roswell Park Memorial Institute Medium

RNAi- RNA interference

RTK- receptor tyrosine kinase

SCC- squamous cell carcinoma

SCLC- small cell lung cancer

SCR- scrambled siRNA

SGK- serum- and glucocorticoid-induced protein kinase

SH2- Src homology 2

shRNA- short hairpin RNA

siRNA- short interfering RNA

SKT11- serine/threonine kinase 11

TGF- transforming growth factor

TKI- tyrosine kinase inhibitor

TMS- trimethylsilyl

TOX- toxic siRNA

TSC2- tuberous sclerosis complex 2

UTR- untranslated region

UTSW- University of Texas Southwestern

Chapter 1: The Role of mTOR Signaling, Autophagy, and Targeted Therapy in Lung Cancer

I. Introduction to Lung Cancer

Lung Cancer Epidemiology and Aetiology

Lung cancers are the leading cause of cancer related deaths in both men and women worldwide. In the United States, cancers of the lung and bronchus account for 28% of cancer deaths in males and 26% of cancer deaths in females (Figure 1.1) (Siegel, Naishadham et al. 2013). For the past 50 years it has been understood that tobacco smoking is the single primary cause of lung carcinoma (Talley, Kushner et al. 2004, Pillai and Ramalingam 2014). However about 25% of new lung cancer cases occur in non-smokers (Sun, Schiller et al. 2007). Genetic factors, exposure to environmental and occupational toxins including radon, inorganic fibers such as asbestos, toxic chemicals, and pollution may also contribute to the cellular insults that sometimes result in the development of lung cancer (Sun, Schiller et al. 2007, Favoni and Alama 2013).

Tumorigenesis refers to the process by which cancer develops. The development of cancer is thought to be a multi-step process in which cells become invasive through a series of molecular changes that lead to deregulated growth. The activation of oncogenes and the loss of tumor suppressors drive tumorigenesis. Though specific molecular changes may vary depending on the type of cellular insults and the affected tissue type, several acquired capabilities, or hallmarks are integral to cancer formation. In 2000, Hanahan and Weinberg proposed six hallmarks that cancer cells acquire in succession that ultimately enable them to become malignant. These include replicative immortality, sustaining proliferative signaling, resisting cell death, evading growth suppressors, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). Since then, several additional

hallmarks have been proposed and include avoiding immune destruction, deregulation of cellular energetics, genome instability, and tumor-promoting inflammation (Hanahan and Weinberg 2011). Greater understanding of the complex biology of cancer has the potential to advance the development of successful therapeutics, since often mutations and abnormal gene expressions that drive cancer growth can serve as druggable targets (Li, Kung et al. 2013). Phenotypic and genetic classifications of lung tumors, which may help identify the driving oncogenes, are becoming increasingly important for the development and selection of the most appropriate approach to therapy for individual patients whose tumor histology and molecular drivers are known.

Lung Cancer Classification

Histological

Lung cancers can be divided into two major groups, small cell lung cancer (SCLC), which account for ~30% of cases, and non-small cell lung cancer (NSCLC) which comprise the remaining 70-85%. The former classification represents a small proportion of lung cancer cases and arises from neuroendocrine cells of the bronchus. The present review will focus mainly on NSCLC. NSCLCs are further divided into subclasses based on histological features. These include adenocarcinoma (35-40%), squamous cell carcinoma (SCC) (25-30%), large cell carcinoma (10-15%), or possibly a mixture of these histotypes (Figure 1.2) (Favoni and Alama 2013, Li, Kung et al. 2013). Smoking history is associated with any of the major histological subtypes, but has a stronger association with SCC, while adenocarcinoma is the commonest subtype found in never-smokers (Sun, Schiller et al. 2007).

Accurate histological classification of lung tumors is essential for selection of the most successful anti-cancer agents, as the efficacy of a particular targeted therapeutic agent is often predictable based on the tumor's histology. One example of this phenomenon includes the exclusion of treatment of squamous cell cancers with the anti-folate drug pemetrexed due to known resistance (Stewart 2010).

Histology classification may be beneficial for selection or exclusion for a therapy, but additional molecular classification of tumors is usually required in order to fine-tune the selection of a therapeutic (or combination of therapeutics) for the successful future of personalized medicine in this disease.

Molecular

New genomic data have increased the current knowledge of various molecular subsets based on driver mutations that exist in NSCLC (Kris MG, Johnsen BE et al. 2011). A significant number of mutations occur in signaling proteins that are crucial to cell survival and proliferation, and result in deregulated activation of pro-growth signals. Two abundant driver mutations found in NSCLC include those in KRAS and EGFR. Mutations in KRAS are present in more than 20% of adenocarcinomas, and mutations in EGFR are found 5-15% of the time. A number of less abundant but potentially targetable driver mutations that have recently been identified include ALK fusions (5-15%), ERRB2 (aka Her2) mutations (<5%), PIK3CA mutations (<5%), BRAF mutations (<5%), MEK (aka MAP2K1) mutations (<5%), MET mutations and amplifications (<5%). Mutations in AKT1 are found very rarely (Figure 1.2) (Kris MG, Johnsen BE et al. 2011, Pao and Girard 2011, Li, Kung et al. 2013, Pillai and Ramalingam 2014).

Alternately or in addition to oncogene activation, lung cancer cells may also have molecular alterations in tumor suppressors. These normal functions of tumor suppressors tend to limit cell growth by inactivating pro-growth signaling pathways or inducing cell death in response to genomic instability and metabolic stress (Hanahan and Weinberg 2011). In cancer cells these “brake” signals can be lost through inactivating mutations, gene loss, or promoter methylation to prevent expression. Genes commonly mutated in NSCLC include p53 (60%), P16 (INK4a) (60%), STK11 (10%), and PTEN (<10%) (Toyooka, Mitsudomi et al. 2011). While typically tumor suppressors are not directly targetable using drugs, alterations that affect the function of a tumor suppressor may affect how the cell responds to certain therapeutic agents.

The past two decades have brought an abundance of knowledge about molecular characteristics of lung cancer. However, nearly 50% of cases still have no known driver mutation that can be identified (Li, Kung et al. 2013). Studies that continue to classify molecular driver mutations are currently underway. These are made possible only with new technologies that allow high throughput genome-wide classifications of large sample sizes.

Standard of Care

The choice for NSCLC therapy options is complicated by the wide range of tumor characterizations including the type, stage and spread of the disease and patient performance status. Surgery, though often not feasible, is the first choice for localized NSCLC, followed by chemo- and radio-therapy for advanced stage tumors. Chemotherapy combined with targeted agents is also common in NSCLC (Favoni and Alama 2013).

First -line chemotherapy for NSCLC usually consists of a standard chemotherapy doublet including a taxane such as paclitaxel, and a platinum agent like carboplatin in an adjuvant or neo-adjuvant setting (Brahmer and Ettinger 2007). These agents are used to inhibit spindle formation and thus inhibit the cell cycle and to cause extensive DNA damage which will ultimately kill the cell (Yvon, Wadsworth et al. 1999). Other common chemotherapy agents include etoposide, gemcitabine, and vinorelbine (Favoni and Alama 2013). Radiation therapy is also frequently used for the treatment of lung tumors with and without concurrent chemotherapy (O'Rourke and Macbeth 2010). In the past decade, chemotherapy regimens have not improved survival for lung cancer patients. Despite the availability of new agents for the treatment of NSCLC, no new chemotherapy regimens have been found to offer a significant advantage over any other (Schiller, Harrington et al. 2002). The most promising novel therapies will likely move away from chemotherapeutics and employ new cancer genomic data into the development of molecularly targeted agents.

Oncogene Addiction and Targeted Therapy

The concept of "oncogene addiction" has propelled the use of targeted agents into the spotlight. In this phenomenon, the growth signaling pathways of cancer cells become deregulated and overly activated to the point that the cells can become dependent on the continued stimulation of these growth signals that drive tumor growth (Figure 1.3) (Sharma and Settleman 2010). Cancer researchers are attempting to exploit these cancer driver dependencies by developing agents that target a particular molecule of such deregulated pathways, often a kinase that may also be mutated or amplified to contribute to its over-activation. Use of targeted agents generally requires the clinician to be able to identify which pathways are most deregulated (and presumably those on which the tumor cells are dependent) in a patient's tumor in order to "personalize" the chosen therapy with the most effective targeted agent for that person's tumor.

Already, the successful use of targeted therapy is being employed to personalize therapy for lung cancer patients with particular vulnerabilities. The first successful targeted therapy for lung cancer came with the development of agents targeting the epidermal growth factor receptor (EGFR) (Pao and Girard 2011). EGFR was known to be amplified in lung tumors and its deregulated signaling leads to the activation of several downstream pathways and increased proliferation. However, in an unselected population, EGFR tyrosine kinase inhibitor (TKI), gefitinib, plus best supportive care failed to improve survival (Thatcher, Chang et al. 2005). Researchers noticed that a small population of patients that consisted largely of never-smokers of Asian origin had a significantly better response to gefitinib. Further studies indicated that that the tumors in these patients had something in common; a somatic mutation in the EGFR gene, which led to its constitutive activation and sensitivity to EGFR inhibition (Kosaka, Yatabe et al. 2004, Lynch, Bell et al. 2004, Paez, Jänne et al. 2004, Pao, Miller et al. 2004). EGFR TKIs showed increased binding affinities to the mutant forms of the receptor, and the cancer cells were

addicted to EGFR signaling (Carey, Garton et al. 2006, Yun, Boggon et al. 2007). Successful results in a trial that selected patients with EGFR activating mutations propelled these agents into the spotlight as the first targeted therapies to exploit a lung tumor's acquired vulnerability and enable a personalized therapeutic approach for lung cancer treatment (Mok, Wu et al. 2009).

Later, a chromosomal rearrangement that leads to the formation of a gene fusion of the echinoderm microtubule-associated protein-like 4 (EML4) gene with the anaplastic lymphoma kinase (ALK) (EML4-ALK fusion protein) was identified. This gene fusion leads to activation of ALK signaling and a malignant phenotype was noted in young male non-smoker lung cancer patients. A new ALK targeted therapy, crizotinib, is being studied and has been shown to shrink or stabilize the tumors of 90% of the patients whose tumors harbor this gene fusion (Pillai and Ramalingam 2012).

Although countless attempts have been made, pharmacologically targeting KRAS has been notoriously difficult, and limited by a lack of specificity. Farnesyltransferase inhibitors that inhibit post-translational modifications of the Ras protein were tested, but also inhibited WT KRAS activity essential for normal cell growth (Caponigro, Casale et al. 2003, Sunaga, Shames et al. 2011). An RNA interference approach that specifically targeted mutant *KRAS* mRNA found that NSCLCs that harbored mutations in *KRAS* were growth suppressed *in vitro* and *in vivo* following shRNA-mediated knockdown, but did not lead to apoptosis or complete loss of tumorigenicity. KRAS knockdown, however, increased sensitivity to p38 and EGFR inhibitors, suggesting that drug combinations may be an appropriate approach to targeting KRAS mutant tumors (Sunaga, Shames et al. 2011).

A primary focus of cancer research presently aims to classify lung tumors according to the molecular drivers that they contain, and to develop targeted therapies to exploit these vulnerabilities. Oncogene identification is the first step in the process of development of targeted therapies, which then require prospective clinical evaluation. Next generation sequencing (NGS) technologies allow for rapid

genome-wide characterization of DNA, mRNA, chromatin structure, and DNA methylation patterns. These technologies are being rapidly applied to a clinical setting as tools for understanding molecular mechanisms in tumors cells, discovery of novel drug targets, and screening candidate patients for clinical trials (Li, Kung et al. 2013). Genomic technologies and pre-clinical and clinical evaluation of novel targets have the potential to transform lung cancer therapy strategies into molecularly- based personalized care to the benefit of each patient.

II. Detailed Overview of Oncogenic Signaling in Lung Cancer

EGFR Signaling

The ErbB family of tyrosine kinases is an important group of signaling proteins that are often deregulated in cancer. The epidermal growth factor receptor (EGFR) was the first described member of this family of proteins, which also consists of ErbB2, ErbB3, and ErbB4 (Herbst 2004). These receptor tyrosine kinases (RTKs) share certain structural similarities including an extracellular ligand binding region, a trans-membrane region, and an intracellular tyrosine kinase domain (Herbst 2004).

The preliminary step in activation of signaling occurs when a ligand binds to the receptor. Epidermal growth factor (EGF) and transforming growth factor (TGF)-α are the most important EGFR specific ligands (Herbst 2004). Ligand binding induces the receptors to form homo- or hetero-dimers with other members of the ErbB family at the cell surface, and promotes a conformational change that stimulates cross-phosphorylation of the two receptors in a dimer at distinct tyrosine residues in the intracellular tyrosine kinase domain leading to their activation (van der Geer, Hunter et al. 1994). These phosphorylated tyrosine residues can then serve as binding sites for substrates, adaptor proteins or structural proteins that contain SH2 (Src homology 2)- or PTB (phosphotyrosine binding)- domains (Seet, Dikic et al. 2006).

One well known pathway initiated by EGFR, and important for signaling in cancer is the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway. Grb2 is an adaptor protein that binds phosphotyrosine residues on EGFR, and recruits the guanine nucleotide exchange factor (GEF), SOS, which activates the G-protein Ras. This event leads to the activation of downstream serine/threonine kinases Raf, MEK, and MAPK/ERK. Activation of this pathway leads to the modulation of transcription factors including ETS-1, c-Myc, and c-Jun, which alter the expression of genes that regulate the functions such as the cell cycle (Haagenson and Wu 2010). Another well characterized pathway initiated by EGFR is the Phosphatidylinositol-3-Kinase (PI3K)/Akt pathway. Signaling from this pathway promotes proliferation and survival. PI3K/Akt signaling will be discussed in detail below since its importance for mechanisms of acquired resistance to tyrosine kinase inhibitors has brought this pathway into the spotlight.

Phospholipases also play a role in signaling downstream of EGFR. Phospholipase C (PLC)-γ binds to EGFR via an SH2 domain where it is phosphorylated to promote its function to hydrolyze PIP_2 to form diacylglycerol (DAG) and inositol 1,4,5, triphosphate (IP_3) (Anderson, Koch et al. 1990). IP_3 releases Ca^{2+} from intracellular stores, which activates Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and DAG activates protein kinase C (PKC) (van der Geer, Hunter et al. 1994). Activation of this pathway also results in pro-survival and anti-apoptotic gene expression and has been implicated in tumor progression (Lo, Hsu et al. 2006). In another pathway, STAT family proteins are also able to bind to EGFR and become activated following a tyrosine phosphorylation. STATs are transcription factors that participate in the regulation of differentiation, proliferation, survival, apoptosis and angiogenesis and have also been implicated in tumorigenesis of several cell types (Calò, Migliavacca et al. 2003).

Role of EGFR in Lung Cancer

EGFR is a central signaling protein that regulates a diverse range of biological processes including gene expression, cellular proliferation, angiogenesis, and inhibition of apoptosis. As such, it is also commonly deregulated in cancer and serves as a mechanism for cancer cells to gain self-sufficiency in growth signals, increase replicative potential, evade apoptosis, stimulate angiogenesis, and promote metastasis. In NSCLC, EGFR is over-expressed in 40-80% of cases (Herbst 2004). This over-expression is usually the result of gene amplification (Prenzel, Fischer et al. 2001), and leads to the strong signal transduction that contributes to growth and invasiveness of the cancer, and poor prognosis of the patient (Herbst 2004).

Activating mutations of EGFR are also commonly found in NSCLC and contribute to the deregulated signaling necessary for tumorigenesis. EGFR activating mutations occur in the first four exons of the tyrosine kinase domain and can consist of point mutations, deletions, or insertions. The 'classic' activating mutations include in frame deletions in exon 19 encompassing residues 747-749 (ΔLRE), or a single point mutation in exon 21, in which leucine 858 is exchanged for an arginine (L858R) (Gazdar 2009). These mutations destabilize the inactive state of the receptor and secure it in its active conformation. For instance, leucine 858 is part of a group of hydrophobic residues that, in the inactive state, form a short helix that displaces the regulatory C-helix from the active site. In the active state, this helix is not formed and these residues make up the N-terminal portion of the activation (A) loop. When the L858 residue is replaced with arginine, which is much larger and positively charged, the short helix cannot stably form, thus securing the active conformation over the inactive conformation and results in an enzyme with fifty-fold greater activity (Yun, Boggon et al. 2007).

As a result of the activating mutations, the EGFR becomes largely independent of ligand binding. Also, EGFR activating mutations contribute to the phenomenon of "oncogene addiction". EGFR mutant tumors come to depend on the pro-survival signaling induced by EGFR over-activation. This effect is further enhanced in cases when the mutant allele is specifically amplified (Gazdar and Minna 2005).

These activating mutations lead to increased proliferation and provide a growth advantage. They also, however, have provided a useful target for therapy. The great majority of EGFR mutant tumors are sensitive to inhibition of signaling by small molecule tyrosine kinase inhibitors (TKIs) (Gazdar 2009).

Other mutations, however, confer resistance to the EGFR to TKIs. The most prevalent of these is the so-called "gatekeeper mutation", a point mutation in which threonine 790 is replaced with methionine (T790M). This mutation lies at the back of the ATP binding cleft and restores the ATP binding affinity to wild-type levels, which significantly reduces the occupancy of EGFR TKIs and limits their efficacy (Yun, Mengwasser et al. 2008). Therefore, new strategies that are effective at inhibiting TKI resistant forms of EGFR will be required to treat tumors expressing the T790M resistance mutation.

PI3K-AKT-mTOR Signaling

The PI3K/Akt pathway is an important signaling cascade initiated by growth factor activation that is important in promoting cell growth, proliferation, survival, motility, and has been implicated in driving tumor progression (Figure 1.4) (Vivanco and Sawyers 2002). Also, this pathway consistently becomes reactivated in TKI resistant tumors, and the ability of TKIs to inhibit signaling from this pathway is lost when resistance is acquired (Rexer, Engelman et al. 2009). Three classes of PI3K exist; however, the most relevant for oncogenesis is Class IA which will be discussed.

Phosphoinositol (PI)-3K is a lipid kinase and a heterodimer made up of the regulatory subunit p85 and the catalytic subunit p110. Three genes encode regulatory subunits of PI3K (p85α, β, and γ). p85α is the most well-known and contains two SH2 domains surrounding a domain that constitutively binds the p110 catalytic subunit. p85 also contains SH3 and BCR (breakpoint cluster region) homology domain which are thought to negatively regulate catalytic activity of p110. There are also three genes encoding p110 (α, β, γ) which contain p85 and Ras interacting domains, a C2 domain for membrane anchoring, and a kinase domain (Vivanco and Sawyers 2002).

Following ligand mediated activation of RTKs, p85-p110 is recruited to the phospho-tyrosine residues on the RTK via the SH2 domain of p85. The interaction between the RTK and p85 may remove p85's inhibition of p110 activity through conformational changes (Yu, Zhang et al. 1998). Ras can also bind and activate PI3K. The recruitment of the PI3K complex also brings it into close contact with its substrates, primarily PtdIns(4,5)P_2 (PIP_2). PI3K phosphorylates PIP_2 to form PtdIns(3,4,5)P_3 (PIP_3), which acts as a second messenger. The phosphatase, PTEN, regulates signaling by PI3K by removing the phosphate group from PIP_3, thus, converting it back to PIP_2. In doing so, PTEN acts as a tumor suppressor that can reduce oncogenic signaling of PI3K. Many cancers lose PTEN expression, leading to further deregulated signaling (Toyooka, Mitsudomi et al. 2011).

PIP_3 recruits proteins that contain pleckstrin-homology (PH)-domains to the membrane. As a central component of many signaling pathways, the serine/threonine kinase Akt (also known as Protein Kinase B (PKB)) is the most important of such proteins. In order for Akt to become activated, it requires recruitment to the plasma membrane, and phosphorylation on Thr308 (Bellacosa, Chan et al. 1998). An additional phosphorylation on Ser473 allows Akt to become maximally activated (Alessi, Andjelkovic et al. 1996). The binding of the PH-domain leads to a conformational change that exposes these two phosphorylation sites (Alessi, Deak et al. 1997). Another PH domain containing serine/threonine kinase, PDK1, is also recruited to the membrane and phosphorylates Akt on Thr308 (Alessi, James et al. 1997). mTORC2 (mTOR-rictor) is the primary kinase that phosphorylates Akt on Ser473 (Sarbassov, Guertin et al. 2005).

Hundreds of putative Akt effectors have been identified, but only a few will be described here. Downstream targets of Akt have functions in cell survival, growth and proliferation, autophagy, angiogenesis, and metabolism. Pro-apoptotic BH3-only protein, BAD, is a direct target of Akt, which adds an inhibitory phospho-group. Akt can also inhibit FOXO transcription factors, which have pro-apoptotic targets including BIM and Fas ligand, by phosphorylating them, leading to their export out of

the nucleus. E3 ubiquitin ligase, HDM2, responsible for initiating p53 degradation, is also a direct target for Akt. Akt promotes cell growth by inhibiting tuberous sclerosis complex 2 (TSC2), allowing Akt to indirectly activate mTORC1. Akt can also phosphorylate the p27 cyclin-dependent kinase inhibitor which prevents p27 localization to the nucleus rendering it unable to inhibit the cell cycle, thus promoting proliferation. Recently, Akt was found to directly inhibit autophagy by phosphorylating Beclin1 (Wang, Wei et al. 2012). Targets such as GSK3, TSC2, and PRAS40 can also drive proliferation and regulate metabolism. Akt plays a role in angiogenesis by activating endothelial nitric oxide synthase (eNOS), which then releases NO leading to stimulation of angiogenesis (Manning and Cantley 2007). As an important signaling pathway required for promoting tumor progression, the PI3K/Akt pathway is a valuable target for cancer therapies.

MTOR

The mechanistic (or mammalian) target or rapamycin (mTOR) is a serine-threonine kinase that has a central role in incorporating signals from growth factor, amino acid, glucose, and oxygen sensing pathways, in order to regulated growth, proliferation, autophagy, and protein synthesis in response to availability of nutrients (Laplante and Sabatini 2012).

mTOR was first identified through experimentation with the small molecule, rapamycin, which is an anti-fungal agent discovered in an Easter Island soil sample containing the bacterium *Streptomyces hygroscopicus*. The island's name, Rapa Nui, lent itself to the moniker of the newly discovered agent, which found its first clinical use as an immunosuppressant for organ transplantations. The target of rapamycin (TOR) was discovered in 1991 through studies to look for knockdowns that led to the resistance of *Saccharomyces cerevisiae* to rapamycin. The mammalian TOR (mTOR) was identified in 1994 (Chiu, Katz et al. 1994, Sabatini, Erdjument-Bromage et al. 1994).

mTOR exists in two distinct complexes with non-overlapping functions (Figure 1.5) (Loewith, Jacinto et al. 2002). mTOR complex 1 (mTORC1) is defined by the inclusion of regulatory associated protein of TOR (Raptor), and also includes mammalian lethal with SEC13 protein 8 (MLST8, aka GβL). AKT1S1 (aka PRAS40) and DEPTOR are both negative regulators of mTORC1 (Laplante and Sabatini 2012). This complex is responsible for the majority of the classical nutrient sensing and growth control functions of mTOR signaling. mTORC1 is stimulated by growth factor signaling through the PI3K-Akt pathway. When Akt becomes activated, it can directly phosphorylate mTOR, or can activate it indirectly by phosphorylating and inactivating the tuberous sclerosis complex 2 (TSC2). This inactivation enables the GTP-binding protein Rheb to be maintained in its GTP-bound state, thus allowing the activation of mTOR (Laplante and Sabatini 2012). mTOR complex 2 (mTORC2) is defined by the inclusion of the rapamycin insensitive component of TOR (Rictor), and also includes MLST8, proline rich 5 (PRR5, aka PROTOR), and mammalian stress-activated protein kinase associated protein 1 (MAPKAP1, aka mSIN1). DEPTOR is also a negative regulator of mTORC2 (Laplante and Sabatini 2012). This complex also appears to be regulated by growth factor signaling, but in an as-of-yet undefined manner. mTORC2 has been shown to regulate the cytoskeleton by stimulated F-actin stress fibers, and, importantly, phosphorylates AKT on Ser473 leading to its complete activation (Sarbassov, Guertin et al. 2005). This activity places mTORC2 in an important pro-oncogenic role due to the array of cellular processes regulated by AKT.

The amino acid sensing components that regulate mTOR are primarily associated with the lysosome. Through a vacuolar ATPase (V-ATPase), amino acids enter the lysosomal lumen and activate a complex with guanine exchange factor (GEF) activity called Ragulator (Bar-Peled, Schweitzer et al. 2012). Ragulator ultimately activates mTOR by changing the Rag GTPase heterodimers (Rag A or B with Rag C or D) to their active GTP-bound state. When the Rag proteins are active, they recruit mTORC1 to the lysosomal surface where it can interact with its activator, Rheb. When the Rags are GDP bound, mTORC1 is inactivated and leaves the lysosomal membrane. Recently, an octameric complex called the

Gator complex has been identified, which interacts with the Rag GTPases (Bar-Peled, Chantranupong et al. 2013). Gator consists of two sub-complexes, Gator1, which contains subunits DEPDC5, Nprl2, and Nprl3, and Gator2, which contains subunits Mios, WDR24, WDR59, Seh1L, and Sec13. Gator1 has GTPase-activating protein (GAP) activity for RagA and B, and thus inhibits mTORC1 activity. This activity is tumor suppressive and several components of Gator1 are lost through mutation in human cancer, leading to the hyper-activation of mTOR, insensitivity to amino acid starvation, and hypersensitivity to rapamycin. In contrast, inhibition of Gator2 suppresses mTORC1 signaling (Bar-Peled, Chantranupong et al. 2013).

Pathways that sense the energy status of the cell also regulate mTOR. As ATP levels drop and ADP levels rise the AMP-activated protein kinase (AMPK) becomes activated. Full AMPK activation also requires STK11/LKB1, which forms a complex with sterile-20 related adaptor (STRAD), and mouse protein 25 (MO25), which maintain STK11 in an active state. AMPK phosphorylates TSC2, which inactivates Rheb and leads to inhibition of mTORC1 signaling (Shaw 2009). Hypoxia leads to the inactivation of mTOR through the protein regulated in development and DNA damage response 1 (REDD1). The expression of REDD1 is up-regulated by the hypoxia inducible factor (HIF-1) (Jin, An et al. 2007). This over expression is sufficient to inactivate mTOR, and requires TSC1/2, but the details of how Redd1 acts in inhibition of mTOR remain to be elucidated.

Downstream of mTORC1

The best characterized downstream function of mTOR signaling is the initiation of protein translation. mTORC1 phosphorylates the eukaryotic initiation factor 4E (eIF4E) binding proteins (4E-BPs). Nuclear-encoded mRNAs have a 5',7-methyl guanosine cap, to which the small protein eIF-4E binds. Under pro-growth conditions, eIF-4E forms a complex, termed the eIF-4F complex, together with eIF-4G scaffold protein, eIF-4A helicase, and eIF-4B regulatory protein. 4E-BP1 prevents the formation

of this complex and inhibits the activity of eIF-4E (Thoreen, Kang et al. 2009). Phosphorylation of 4E-BP1 by mTOR releases eIF4E from inhibition by the 4E-BPs and allows for the translation pre-initiation complex assembly at the 5' end of mRNAs. mTORC1 also activates S6K which recruits eIF4B to the initiation complex, and the small ribosomal subunit protein S6 (Gingras, Raught et al. 2001) . Notable proteins whose synthesis are controlled by mTOR regulated translation include HIF1α, cell cycle regulating proteins, c-MYC, cyclin D1, OC1, and cell survival and proliferation proteins, SURVIVIN, XIAP, and BCL2 (Pause, Belsham et al. 1994, Gingras, Kennedy et al. 1998, De, Miskimins et al. 2013).

A mechanism of how TOR regulates progression through the cell cycle has recently been described in yeast. TOR regulates a pathway that post-transcriptionally controls M phase cyclin stability. In early M phase the levels of B-cyclin, CLB2, mRNA increase, and then are rapidly degraded at the end of M phase. A mitotic exit-regulating kinase, Dbf2, phosphorylates and activates arginine methyl-transferase, Hmt1, which promotes the nuclear localization of hnRNPs through methylation, and their association with CLB2 mRNA, as well as the stability of CLB2 mRNA. When TOR is inhibited by starvation or treatment with rapamycin, the phosphatase Pph22 is recruited to Hmt1, which causes its deactivation, preventing the methylation of hnRNPs and the accumulation of CLB2 mRNA and delay of M phase (Messier, Zenklusen et al. 2013).

Autophagy

mTORC1 activity also inhibits autophagy (Laplante and Sabatini 2012). This cellular process is responsible for the degradation of proteins, damaged organelles and other cellular components so their constituents can be re-used (Rubinsztein, Codogno et al. 2012). Autophagy is activated during periods when supplies of nutrients are scarce, when it makes more sense to recycle cellular components in order to maintain only the essential activities for viability. The primary autophagy initiation kinase, ULK1, is inhibited through phosphorylation by mTOR on Ser757. This phosphorylation prevents the

interaction of ULK1 with AMPK, which activates it through phosphorylation on Ser317 and Ser777 under glucose starvation (Egan, Kim et al. 2011).

The role of autophagy in cancer is multifaceted, as it has the potential to be both tumor suppressive and tumor promoting. Autophagy is activated during environmental stress in order to minimize damage and promote cellular senescence, but may also allow tumor cells to survive nutrient deficiency, hypoxia, and chemotherapy induced stresses (Mathew, Karantza-Wadsworth et al. 2007). This discrepancy in the role may depend on the stage of tumor progression and the presence of certain oncogenic changes. For example, in non-transformed cells, autophagy prevents transformation by removing ROS and damaged organelles, while in transformed cells, autophagy can promote survival of cancer cells in low nutrient conditions, and protect against chemotherapy induced damage (Mah and Ryan 2012). The presence of oncogenic Ras has been associated with a dependence on autophagy for survival. Ras-driven tumors have significant levels of energy depletion, which increases the dependency on autophagy to buffer the demand for energy through the preservation of mitochondrial function (Guo, Chen et al. 2011). Similarly, in an oncogenic KRAS driven mouse model of pancreatic ductal carcinoma (PDAC), genetic ablation of autophagy permitted the accumulation of low-grade, pre-malignant intraepithelial neoplasia lesions, but blocked progression to high grade lesions and PDAC. However, when p53 was lost concurrently with oncogenic KRAS, loss of autophagy didn't block tumor progression, but accelerated tumor onset (Rosenfeldt, O'Prey et al. 2013).

Downstream of mTORC2

Much less is known about the downstream effectors of mTORC2. As already mentioned, mTORC2 plays a role in the maintenance of the actin cytoskeleton, and phosphorylates AKT (Sarbassov, Guertin et al. 2005). mTORC2 may also play a role in the regulation of solute carriers, importantly, those which facilitate the transport of glucose into the cell (Garcia-Martinez and Alessi 2008). It has

been shown to phosphorylate the hydrophobic motif of serum- and glucocorticoid- induced protein kinase 1 (SGK1), similarly to its activation of the structurally related Akt (García-Martínez and Alessi 2008, Hong, Larrea et al. 2008). SGK1 is a member of the AGC family of proteins and regulates cellular processes such as ion transport and growth, and has two related family members, SGK2 and SGK3, which may also be regulated by mTORC2. SGK1 phosphorylates N-myc downstream regulated gene 1 (NDRG1), which is involved in stress responses, hormone responses, cell growth, and differentiation. SGK1 and 3 also phosphorylate and inactivate the ubiquitin ligase NEDD4L. NEDD4L regulates the cell surface expression of the epithelial sodium channel ENaC and glucose transporter SGLT1 (Dieter, Palmada et al. 2004).

Alterations in the mTOR Signaling Pathway in NSCLC

A variety of mechanisms exist in which components of the mTOR signaling pathway may be altered in order to give cancer cells a growth advantage. These potentially include mutation (constitutive activation of an oncogene or loss of a tumor suppressor), gene amplification, and alteration of expression through epigenetic or other mechanisms. KRAS mutations are among the most common somatic mutations seen in NSCLC patients, observed in 30% of cases. Ras mutations may lead to constitutive activation of PI3K signaling by directly binding and activating it. Mutations in EGFR are also well documented in NSCLC occurring in approximately 10% of cases, but most often in patients with no history of tobacco use. Both of these well recognized oncogenes have been reported to coincide with an increase in mTOR activity (Conde, Angulo et al. 2006). Mutations in p110 catalytic or the p85 regulatory subunits of PIK3CA have also been observed, but occur relatively infrequently in NSCLC; and mutations in AKT1 are even rarer, occurring in less than 2% of cases (Pao and Girard 2011). Alterations in PIK3CA and AKT are seen slightly more frequently in squamous cell carcinomas (SCC) than in

adenocarcinomas (ADC) of the lung (Pao and Girard 2011, Li, Kung et al. 2013). Despite the central role of mTOR in controlling cell growth and proliferation, mutations in this gene are never seen in NSCLC.

PI3K pathway activation may also occur through loss of tumor suppressor activity. Phosphatase PTEN acts inversely to PI3K by removing the phosphate group added by the kinase, thus deactivating downstream PI3K signaling. However, mutations in this gene obliterate this function and occur in approximately 5% of NSCLCs (Toyooka, Mitsudomi et al. 2011). Loss of another tumor suppressor STK11/LKB1 activates this pathway through the failure of the nutrient sensing regulation of mTOR. Nonsense mutations in STK11 often lead to degradation of any remaining transcripts and complete loss of the protein. Mutations in STK11 occur in about 20% of patients, often co-occurring with Ras mutations, but generally mutually exclusive with loss of PTEN (Gill, Yang et al. 2011, Dearden, Stevens et al. 2013). In some human cancers, such as breast cancer and glioblastoma, loss of the tumor suppressor activity of the Gator1 complex, which inhibits mTOR signaling has been identified (Bar-Peled, Chantranupong et al. 2013). However, such mutations have not been found in lung cancer.

Gene amplification is another mechanism often found in cancer cells that can lead to hyper-activation of oncogenic signaling. Cancer cells with mutations in Ras or EGFR often also have multiple gene copies. Amplifications of another ERBB family member, ErbB2, are also common, and often result in sensitivity to ERBB targeted therapies (Sos, Michel et al. 2009). PI3K amplifications are also found in NSCLC (Angulo, Suarez-Gauthier et al. 2008). Such alterations often mean that expression of these genes is very high and activation of pro-growth signaling is maximally activated.

III. Targeting Oncogenic Signaling in Lung Cancer

EGFR Based Therapies

Gefitinib (Iressa, ZD1839; AstraZeneca Pharmaceuticals) and Erlotinib (OSI-774, Tarceva; OSI Pharmaceuticals, Genentech) are two orally active reversible tyrosine kinase inhibitors (TKIs) that have

been shown to have anti-cancer activity in EGFR mutant tumors. These small molecules compete with ATP for its binding site on the intracellular domain (Herbst 2004). EGFR molecules with activating mutations have reduced binding affinity for ATP compared to the wild-type enzyme. Furthermore, mutant EGFR binds gefitinib twenty times more tightly than the wild-type ($K_{d\ (L858R)}$ = 2.6 ± 0.2 nM vs. $K_{d\ (WT)}$ = 53.5 ± 1.8 nM)(Yun, Boggon et al. 2007). As a result, mutant EGFRs are strongly inhibited by TKIs, and patient tumors expressing these proteins respond to TKI treatment.

Other small molecules that can be used to target EGFR to treat cancer include irreversible inhibitors. These have a similar structure to the reversible inhibitors, but have a 6-4-(dimethylamino)crotonamide group, which act as a Michael-acceptor that reacts with the nucleophilic thiol side chain found on cysteine residues (Wissner and Mansour 2008, Yun, Mengwasser et al. 2008). Cysteine-797 at the edge of the ATP binding cleft is the target for irreversible inhibitors that inhibit EGFR. The drugs form a covalent bond with this residue and prevent the binding of ATP (Yun, Mengwasser et al. 2008). These agents are beneficial because they can be effective in treating tumors expressing TKI resistant EGFR (Doebele, Oton et al. 2010). Some examples of irreversible inhibitors currently under investigation include BIBW 2992 (Afatinib,Boehringer Ingelheim), HKI-272 (Neratinib; Wyeth Pharmaceuticals), and PF00299804 (Pfizer) (Doebele, Oton et al. 2010). The former, afatinib, was recently approved for use as a first-line treatment for lung cancer patients with EGFR mutations (Malin 2013). In addition to possibly targeting EGFR TKI resistant mutants, other benefits of these agents include the fact that they don't required prolonged blood circulating levels. The formation of a covalent bond with the target allows for a more persistent biological effect, which depends on the rate of receptor turnover rather than time in the circulation (Wissner and Mansour 2008).

Another approach for targeting EGFR in cancer is the use of monoclonal antibodies to inhibit EGFR activity. Antibody therapies have greater specificity for EGFR compared to small molecules, and inhibition can be achieved at lower concentrations. However, this type of therapy must be administered

intravenously and can induce an immune response that can limit the efficacy of the therapy (Herbst 2004). Cetuximab (IMC0C225, Erbitux; ImClone Systems Incorporated and Bristol-Myers Squibb Company) is an example of an EGFR targeting monoclonal antibody, that is approved for use in colorectal and head and neck cancer (Ganjoo and Wakelee 2007). It is a chimeric human-murine antibody in which portions of a mouse EGFR antibody were attached to the complementarity determining region of human immunoglobulin. It functions by blocking the binding of ligands to the receptor in order to prevent its activation. However, this therapy is not as effective when altered forms of EGFR are present, due to the ligand independence seen in EGFR mutants (Dassonville, Bozec et al. 2007).

Toxicity of EGFR Targeting Therapies

Toxicities from EGFR targeting therapies are generally milder than those from cytotoxic chemotherapy. A common side effect from EGFR inhibitors is a skin rash that is thought to be the result of inhibition of EGFR in the skin. Interestingly, the development of a skin reaction positively correlates with response to therapy (Cunningham, Humblet et al. 2004). EGFR plays an important role in the developing epidermis (Nanney, Stoscheck et al. 1990) and is also expressed in adult skin, particularly in highly proliferative cell types including basal keratinocytes and eccrine sweat ducts (Nanney, Magid et al. 1984). In the skin, EGFR participates in processes such as stimulating growth of the epidermis, inhibiting inflammation, protecting against damage from UV light, and accelerating wound healing. The use of EGFR inhibitors to treat tumors will also reduce the levels of EGFR activity in basal keratinocytes, which leads to growth arrest, premature differentiation, and accumulation of non-viable cells that contribute to the skin rashes associated with EGFR tyrosine kinase inhibitors (Giovannini, Gregorc et al. 2009).

Long term treatment with EGFR targeted therapies has also resulted in cardiovascular toxicities. Members of the ERBB receptor family are essential for cardiovascular development, and also play a role in adult cardiac homeostasis (Barrick, Yu et al. 2008). Cetuximab treatment has been associated with cardiac toxicity, which can be life threatening or fatal, but only on rare occasions (Chaudhary and Gajra 2010). One study demonstrated that female Black-6 mice treated with TKIs EKB-569 or AG-1478 experienced alterations in the thickness of the left ventricular wall and cardiac function due to increased apoptosis (Barrick, Yu et al. 2008). Such cardiovascular toxicities have become apparent since EGFR targeting treatments are being used as chronic rather than acute therapy, and emphasize a need for continued examination of toxicities relevant for EGFR targeting agents.

Targeted mTORC1/2 for Cancer Therapy

Due to the central role of mTORC1/2 for the survival of cancer cells, the idea of targeting mTOR signaling for the treatment of cancer has been well established. Initially, rapamycin, and a number of analogous small molecules were tested treatment of a variety of cancer types. In a phase III trial in patients with advanced renal cell carcinoma (RCC) that had progressed with sunitinib or sorafenib, a rapamycin analogue, everolimus (RAD001) was found to prolong progression free survival to 4.0 months, compared to 1.9 months in the placebo treated arm (Motzer, Escudier et al. 2008). The success of this study led to FDA approval in 2009 for this indication (Coppin 2010). In other cancer types, trials examining mTOR targeted therapies have had only modest success. A phase II “window of opportunity” trial found that second-line temsirolimus (CCI-779) in NSCLC patients had acceptable toxicity and a median progression free survival of 2.3 months and overall survival of 6.6 months. These results were not robust enough to meet the defined criteria for success. Therefore, researchers concluded that single agent targeted agent trials in an unselected patient population is not the appropriate strategy,

and suggested a need for the development of predictive biomarkers and rational drug combinations (Reungwetwattana, Molina et al. 2012).

The new generation of mTOR inhibitors aims to improve the level of pathway inhibition through dual targeting. A number of agents that compete with ATP binding and inhibit mTOR kinase activity are in development. mTOR kinase inhibitors improve upon the allosteric inhibition by rapamycin analogues with their ability to inhibit both mTORC1 and mTORC2. Two such agents, Torin1 and AZD8055 will be discussed in greater detail in Chapter 3. Pre-clinical studies comparing mTOR kinase inhibitors with rapamycin indicate an improved anti-cancer effect, presumably due to superior inhibition of mTOR activity (Benjamin, Colombi et al. 2011).

Another approach to pharmacologically inhibit this important signaling pathway is the use of dual PI3K/mTOR inhibitors. One such agent, NVPBEZ235 (Novartis), has been shown to be effective in preclinical studies, and is currently being tested in Phase I/II trials in solid tumors, singly and in combinations (Benjamin, Colombi et al. 2011). Although this drug has already been found to be ineffective in KRAS mutant tumors, combinations with another targeted agent, AZD6244, which inhibits MAPK/ERK signaling, led to significant tumor regression in a KRAS transgenic mouse model (Engelman, Chen et al. 2008).

The relatively modest success of mTOR inhibitors in clinical trials suggest that these agents may provide greater clinical benefit when used in combination therapy. Since continued activation of PI3K signaling is an important mechanism of resistance to EGFR-TKIs, it has been suggested that multi-targeted strategies that combine EGFR inhibitors with downstream mTOR inhibition may improve the efficacy of these single agents (Gridelli, Maione et al. 2008). A preclinical evaluation of such a combination found that in resistant NSCLC cell lines, everolimus restored gefitinib sensitivity by improving inhibition of signaling and having a growth inhibitory effect (La Monica, Galetti et al. 2009).

A small Phase I study tested oral daily 5 mg everolimus with oral daily 250 mg gefitinib and found that 2 of 8 evaluable patients exhibited radiographic responses with this combination (Milton, Riely et al. 2007). A separate phase I trial using 5 mg/day everolimus with 150 mg erlotinib had similar results, and demonstrated that this combination had acceptable tolerability and improved disease control rates compared to erlotinib alone in previously treated metastatic or unresectable NSCLC (Papadimitrakopoulou, Soria et al. 2012).

Targeting Autophagy

The inhibition of mTOR has been shown to result in increased levels of autophagy due to mTOR's role in suppressing autophagy initiation. This increase in autophagy has the potential to limit the effectiveness of therapy, as this process can increase cancer cell survival during times of stress. A number of therapies have been shown to induce autophagy, which has been suggested as a protective mechanism (Mizushima, Levine et al. 2008). Because of this potential protective role, autophagy inhibition, alone or in combination with other therapeutics, has been suggested as a strategy for cancer therapy. Chloroquine and the related hydroxychloroquine are autophagy inhibiting drugs that are frequently used in the treatment of malaria, and are currently under investigation as anti-cancer agents. A phase II trial in NSCLC to examine the combination of standard chemotherapy for lung cancer, plus hydroxychloroquine is currently recruiting patients (NCT01649947).

Objectives of this study

In this study, the utility of mTOR or autophagy inhibition individually or in combination with standard chemotherapy or EGFR targeted therapy was examined by screening a large panel of NSCLC cell lines for their response to a variety of pharmacologic and genetic inhibitors. Chapter 3 will discuss the characterization of NSCLC response to allosteric and active site mTOR inhibitors in order to identify potential biomarkers, which may enable improved patient selection for this class of targeted therapy.

Through these studies we determined that alteration along the EGFR/ERBB2 axis predicts sensitivity to mTOR inhibition, and provides rationale for the use of these drugs for treatment of tumors that are resistant to EGFR targeted therapies. In Chapter 4, an RNAi screen to characterize potential mTOR related vulnerabilities in NSCLC is described. The response of NSCLC cell lines to a panel of siRNAs targeting mTOR and autophagy related genes produces a heterogeneous response, which can be used to define subset-specific vulnerabilities. Chapter 5 describes the results of our studies using drug combinations incorporating mTOR inhibitors with standard chemo- and targeted therapy regimens. We suggest such combination strategies are likely to be beneficial in improving current treatment approaches, even in tumors that are resistant to the individual agents. Finally in Chapter 6, we investigate the potential of autophagy inhibition, alone and in combination with standard chemo- and targeted therapies in NSCLC. Here we find that autophagy inhibition is not likely to be a successful therapeutic approach in lung cancer as no significant growth effect was seen at physiologically relevant drug concentrations, and no sensitizations to standard chemo- and targeted therapies were observed. Pre-clinical evaluations of novel targeted therapies, such as those performed in this study, are required in order to design clinical trials that select patient subsets that are most likely to benefit from the proposed therapy or combination of therapies.

IV. Figures

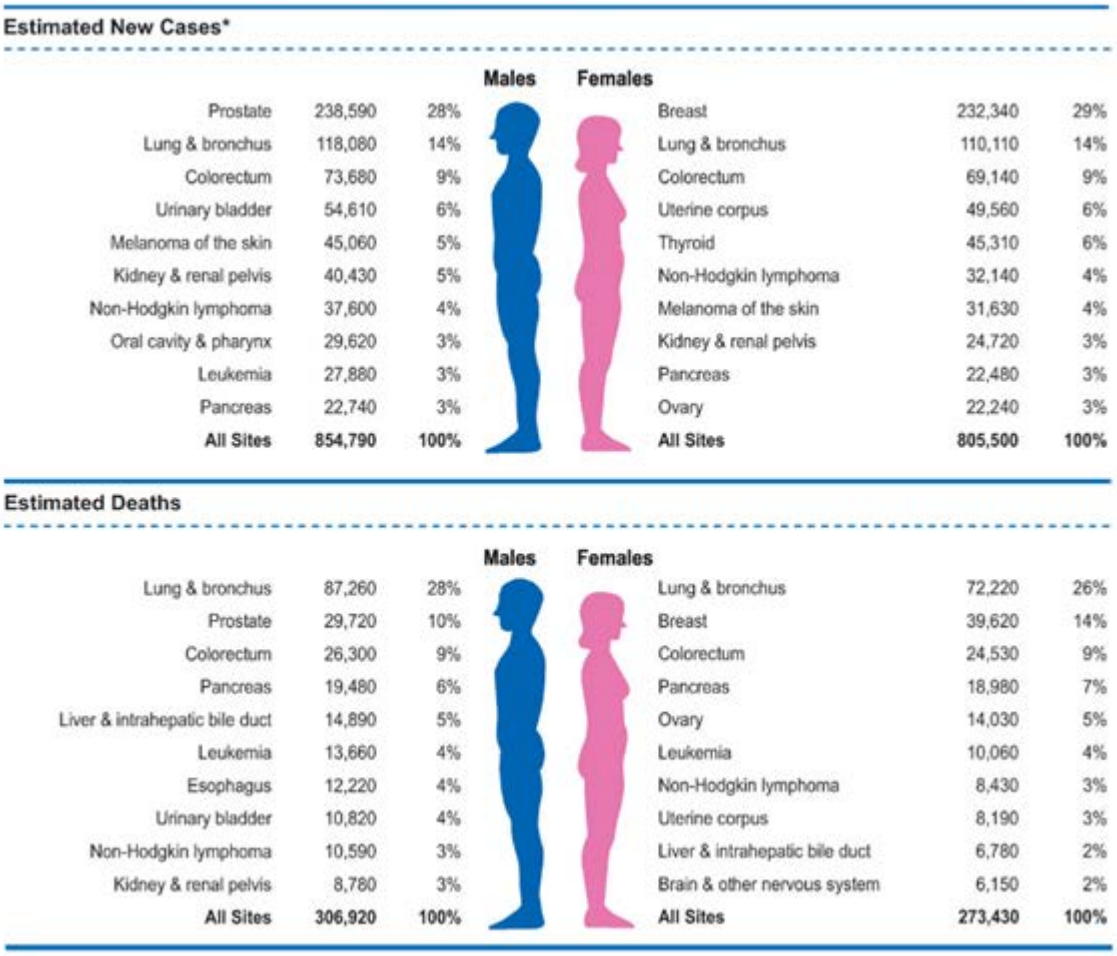


Figure 1.1: Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2013

*Estimates are rounded to the nearest 10 and exclude basal cell and squamous cell skin cancer and in situ carcinoma except urinary bladder (Siegel et al., 2013)

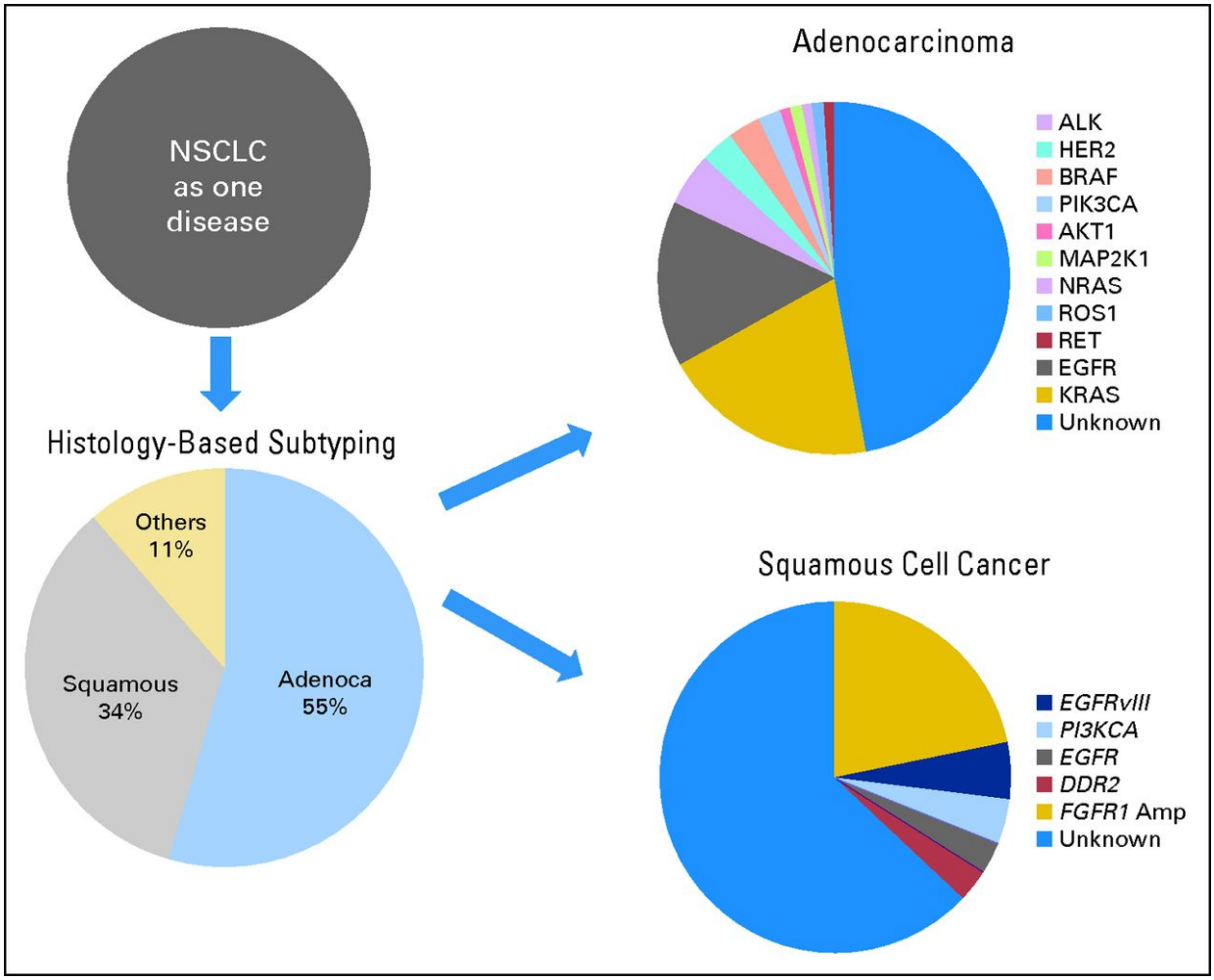


Figure 1.2: Histologic and Molecularly Based Classification of NSCLC
EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; MAP2K1, mitogen-activated protein kinase kinase 1(Li, Kung et al. 2013).

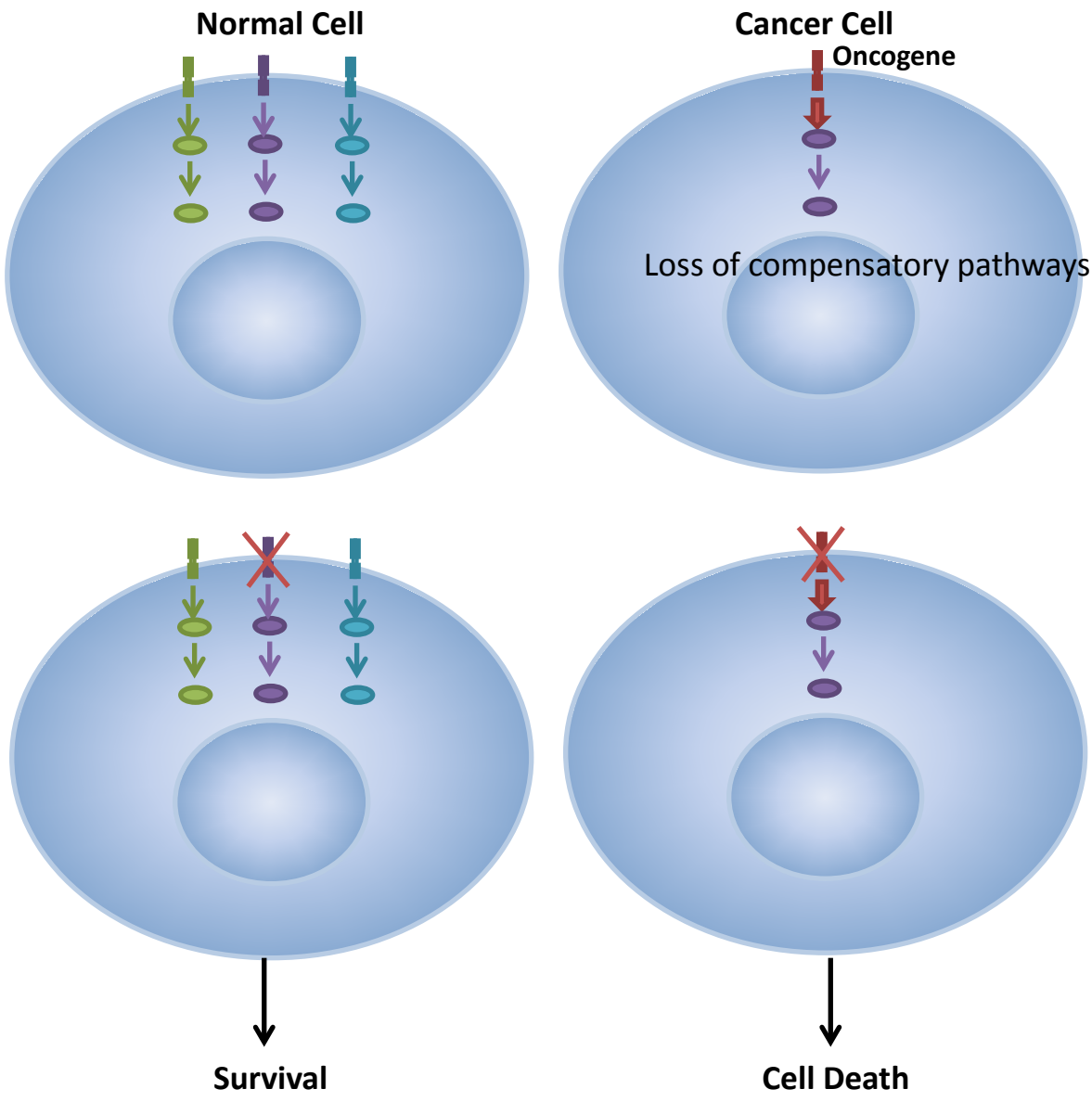


Figure 1.3: Oncogene Addiction Leads to Targetable Vulnerabilities in Cancer Cells
Cancer cells acquire abnormalities in oncogenes and tumor suppressor genes resulting in aberrant activation of signaling pathways often concurrent with loss of compensatory pathways resulting in addiction. Targeting these acquired vulnerabilities with small molecules or neutralizing antibodies exploits these cancer cell dependencies with minimal effect on normal cells.

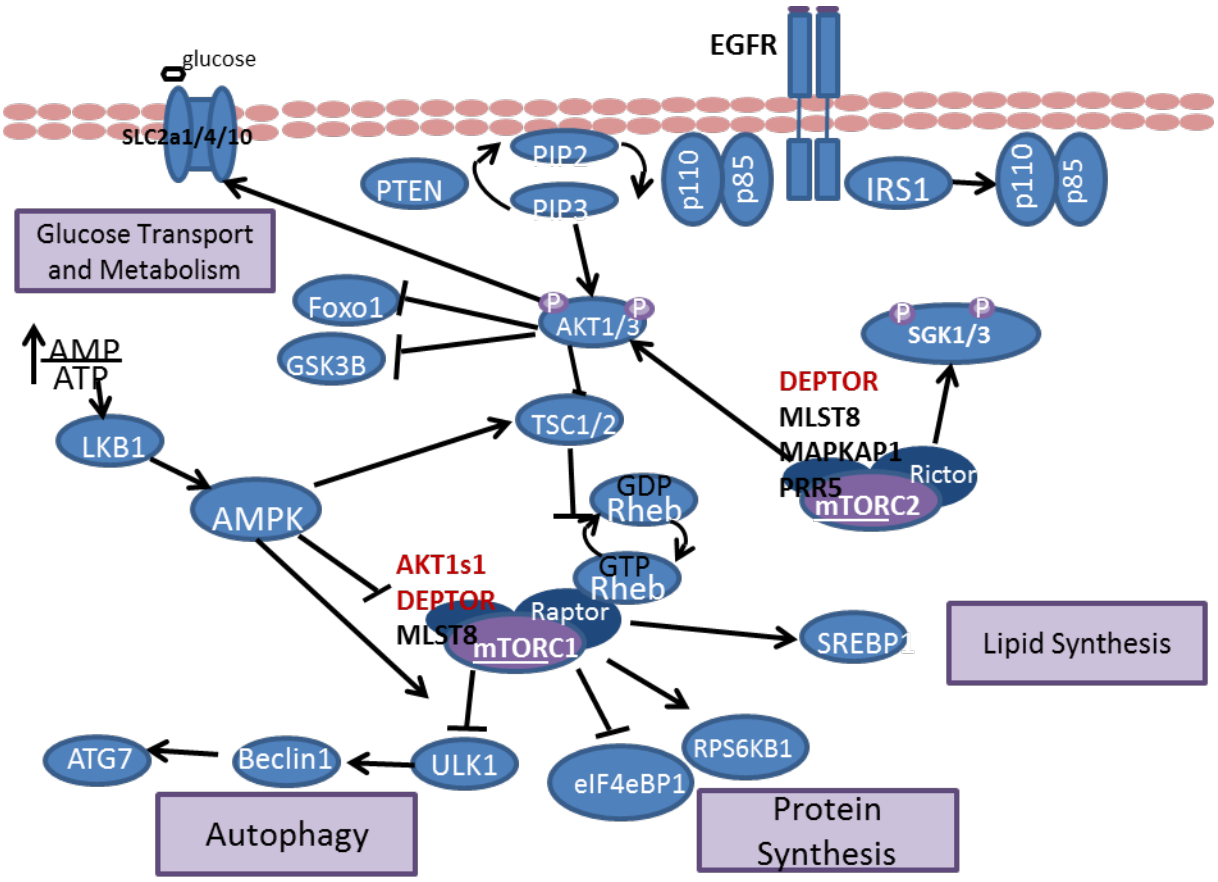


Figure 1.4: Schematic Representation of mTOR Signaling Pathway
mTOR is regulated in response to growth factors and the presence of nutrients and controls outputs including regulation of glucose transport and metabolism, autophagy, protein and lipid synthesis.

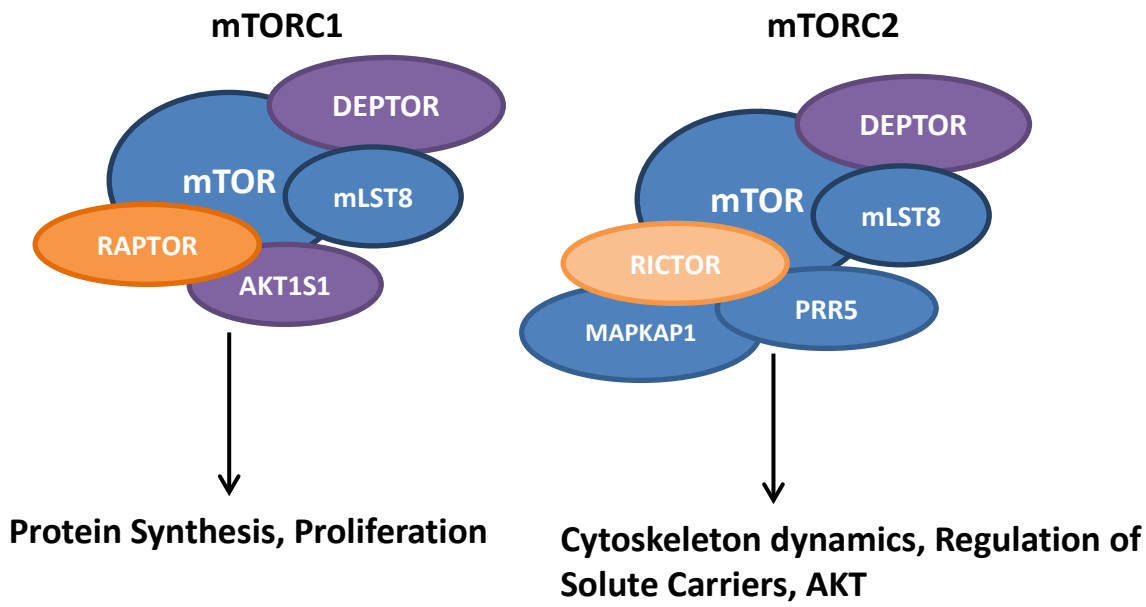


Figure 1.5: mTOR Exists in 2 Complexes with Unique Functions
Both mTOR complexes consist of the mTOR kinase and mammalian lethal with SEC13 protein (mLST8) in addition to mTOR itself. mTORC1 is defined by the inclusion of the regulatory associated protein or TOR (Raptor) and AKT1 substrate 1 (AKT1S1) which acts as a negative regulator. mTORC2 is defined by the inclusion of rapamycin insensitive component of TOR (RICTOR), and also includes mammalian stress-activated protein kinase associated protein 1 (MAPKAP1), proline rich 5 (PRR5). DEPTOR is a negative regulator of both complexes.

Chapter 2: Materials and Methods

Cell Culture

The majority of the human NSCLC cell lines used in this study was derived by the laboratories of Dr. Adi Gazdar and Dr. John Minna. Cell lines named with the prefix H were established at the National Cancer Institute and those with the prefix HCC were established in the Hamon Center for Therapeutic Oncology at UTSW (Gazdar, Girard et al. 2010). A few cell lines were purchased from the American Type Culture Collection (ATCC) and can be identified by their alternate naming schemes. Cancer cells were maintained in RPMI-1640 (Sigma) supplemented with 5% fetal bovine serum (FBS) (referred to as R5) and incubated at 37 degrees C in a humidified atmosphere with 5% CO2 (Phelps, Johnson et al. 1996). Long term stocks of NSCLC cells are frozen in FBS containing 0.1% DMSO in liquid nitrogen freezers.

Also used in this study is a panel of human bronchial epithelial cells (HBECs) that serves as cell culture models of "normal" lung epithelium. These cells were previously established in the Minna Lab and are immortalized for cell culture through ectopic expression of *CDK4* and *hTERT*. HBECs are maintained in keratinocyte serum free media (KSFM) (Life Technologies, Inc.) supplemented with bovine pituitary extract and recombinant human epithelial growth factor (Ramirez, Sheridan et al. 2004).

Fingerprinting and mycoplasma testing

In order to ensure that the cells lines used in this study were correctly identified initially and throughout, it was necessary to perform DNA fingerprinting. Cells were pelleted and genomic DNA was extracted using a DNeasy kit (Qiagen). Samples were submitted to the UTSW Sequencing Core where they were analyzed by profiling variable number tandem repeats in the samples which are then matched with the known fingerprints of the cell lines.

Mycoplasmas are a type of bacteria that lack a cell wall. This feature makes them resistant to most commonly used antibiotics. While the presence of typical bacterial (or fungal) infections in cell culture can be easily identified by observation, infection by mycoplasma can occur without any obvious changes to the cell culture. Nevertheless, mycoplasma contamination can result in a number of cellular changes including chromosomal alterations, changes in metabolism and cell growth which can significantly impact experimental results and even destroy the cell line. The presence of mycoplasma can be detected using a PCR based assay kit (eMyco Mycoplasma PCR detection Kit, Boca Scientific), in which, if contamination is present, a mycoplasma specific DNA sequence is amplified and visualized on an agarose gel. DNA was extracted from cells using a DNeasy kit. The mycoplasma PCR was performed by Sunny Zachariah or Brenda Timmons.

Cell Viability Assays

MTS

The colorimetric MTS assay was used to screen a large panel of NSCLC cell lines for their dose response to autophagy inhibiting agents, mTOR inhibitors, and well as to standard chemo- and targeted-therapy agents alone or in combinations. On day 0, 500-4000 cells were seeded into columns 2-11 of 96-well plates. On day 1, eight concentrations of each drug in 4-fold dilutions were added to the cells, 1 concentration per column starting with the lowest concentration in column 4 and increasing to the highest concentration in column 11. Columns 2 and 3 were cells only controls. Two replicate plates for each cell line were used for each experiment. For drug response assays using drug combinations, a fixed concentration of drug B was added to the varying doses of drug A in columns 3-11 of the 96 well plates. The dose response of the drugs on each cell line was determined after 4 days of drug treatment. MTS reagent (Promega) was combined with phenazine methosulfate (PMS) at a ratio of 2:0.1 and 20 µl of this indicator solution was added to each well of the plates. After incubation for at 37 degrees C for least 1

hr., the absorbance was read at 490nm. The data were uploaded to in-house database and analytical software, DIVISA, which was used to calculate dose response curves, IC50s, and other measurements of sensitivity or resistance.

Liquid Colony Formation Assays

The effect of drug treatments on the clonogenicity of cancer cells was determined by liquid colony formation assays. Cells were seeded at very low density (500-2000 cells/well) in 6-well dishes in 1 mL of normal growth medium (R5) and allowed to recover overnight. The following day drug dosages were prepared at 2X the desired final test concentrations in R5. 1 ml of each drug dosage was added per well of the 6-well plates without aspirating the media in which the cells were seeded in order to prevent accidental removal of colonies. Colonies were allowed to form by incubating the plates for 1-3 weeks at 37°C in a humidified atmosphere of 5% CO_2. When colonies became visible and contained a minimum of 50 cells, they were fixed and stained with 2% formaldehyde, 0.05% crystal violet, 20% PBS staining solution and counted using Quantity One Software (Bio-Rad).

Xenograft Tumor Formation Assays

Female NOD/SCID mice bearing NSCLC cell line xenografts were used to determine the efficacy of the inhibitors in an *in vivo* setting. Mice were bred and purchased from the UTSW Mouse Breeding Core, and the care and treatment of animals were in accordance with institutional guidelines. After shaving the fur at the injection site, 0.5- 1 million NSCLC cells were suspended in 100uL PBS and injected subcutaneously into the right flank using 27 gauge needles. Tumor volumes were determined by measuring 2 dimensions of the tumor using electronic calipers and then calculated the volume using the following formula: $V_{tumor}=(\pi/6)(d_{large})(d_{small})^2$.

In Vivo Drug Treatments

Tumors were injected and then allowed to grow to a volume of 200 mm^3 before treatments were initiated. AZD8055 was dissolved in Captisol (Cydex) (30% w/v) and given at doses of 10 mg/kg daily by oral gavage in volumes of 0.1 ml/10g of body weight. Erlotinib was dissolved in 6% Captisol and dosed by daily gavage at 25 mg/kg. Mice receiving combination treatments were given both drugs as described above.

Quantitative Real-Time PCR Analysis

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA). The synthesis of cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad). Transcript expression was analyzed by quantitative real-time PCR (qPCR) using TapMan Assay Probes (Applied Biosystems) and a pre-made reaction mixture, iTaq Supermix with Rox (Bio-Rad) which contains iTaq DNA polymerase, buffers, nucleotides and Rox passive protection dye. Samples were run in 96-well qPCR plates on the Applied Biosystems 7300 Real Time PCR system in duplicate. This system's software was utilized to determine Ct values and to calculate ΔCt values normalizing to *GAPDH* expression. Human reference RNA, purchased from Stratagene, was used as a reference to compare expression across non-isogenic samples. Data are displayed as fold expression normalized to the reference RNA sample.

Cell Cycle Analysis

The profile of the cell cycle stages in a population of cells was determined by measuring the DNA content. Cells were fixed in 70% ethanol at -20% overnight. The DNA was stained by incubating the cells buffered staining solution containing 0.05% Triton X-100, 0.1 mg/ml RNase A, and 50ug/ml propidium iodide (PI) in PBS for 30 minutes at 37° C. Cells were briefly spun down and re-suspended in fresh PBS before being analyzed by flow cytometry. The distribution of cells in varying states of DNA replication was determined using FlowJo Software.

Microarray Expression Analysis

Expression data for the NSCLC cell lines have been previously generated using the Illumina (WG6-V2 and V3 BeadChips) array platform. Expression data were analyzed using in-house software developed in the Minna lab by Dr. Luc Girard. This program, MATRIX (MicroArray Transformation in Microsoft Excel), is a Microsoft Visual Basic program that enables the import and analysis of large amounts of microarray expression data. Transcript expression was normalized by the median value across samples, which was then $\log_2$-transformed and color coded. Pearson's correlation analysis was used to determine expression correlations with a continuous variable, such as IC50. To identify the most differentially expressed genes between classes of samples (such as sensitive vs. resistant), two-sample t-tests were performed on the ratio of $\log_2$-transformed signals and filtered for significant expression differences ($p<0.01$).

Gene Silencing

Expression of several individual genes was transiently silenced using siRNAs. siRNAs targeting *Raptor, Rictor, mTOR SGK1, AKT3, and ATG7* were purchased from Qiagen. 20 nM siRNA was reverse transfected into cells using lipid transfection agent RNAiMax (Life Technologies, Inc.). A non-targeting siRNA and a mock transfection using only the transfection agent were used as controls. Knockdown of the gene was confirmed using quantitative PCR or Western blot. When knockdowns were used in conjunction with drug treatment, the drugs were added the day following transfection.

siRNA Screening

In order to identify acquired molecular vulnerabilities related to mTOR signaling in NSCLC, an siRNA "mini-library" screen which would individually knockdown a panel of 55 genes related to this pathway was performed. The siRNA library was purchased from Dharmacon in 96-well format. The

library consisted of 4 plates in which each well contained an siRNA targeting a different transcript, while the corresponding wells on each of the 4 plates contained a unique siRNA targeting the same transcript. The siRNAs from each of the 4 plates was pooled and diluted into a master plate in which each well now contained a pool of 4 different siRNAs all targeting the same transcript. A panel of 25 NSCLC cells lines screened for the effect of each of the knockdowns on viability. As a positive control for reduced viability by siRNA transfection a toxic oligo (TOX), an siRNA targeting the PLK transcript, was used. As a negative control a scrambled PLK siRNA (SCR) that doesn't target any transcript was used. Other controls included non-transfected cells, and cells treated with only lipid transfection agent without siRNA. Cells were transfected with the library by adding lipid transfection agent RNAiMax into the wells, followed by the cells after time was given for lipid/siRNA complexes to form, enabling a reverse transfection. On the fifth day after transfection, the effect of the siRNAs on cell viability was determined using the MTS assay. Cell viability was normalized to the scrambled control. Two measures were used to compare the data: the % viable cells remaining at the end of the assay and the Z-score. The latter measure is a score for each siRNA which is related to the deviation from the average response of a cell line to the panel of siRNAs. It is calculated by the formula: $Z_x=(x-\mu_x)/\sigma_x$, where x is the response of the test siRNA, μ is the average response of a cell line to the siRNAs, and σ is the standard deviation of the siRNA responses of the particular cell line. The majority of the siRNAs will have a Z-score between -1 and 1, meaning the cell line's response to the siRNA is near the mean response. A negative Z-score beyond -1 indicates that the siRNA strongly inhibited growth or caused cell death. A Z-score greater than 1 indicates that the siRNA allowed increased growth beyond that of the scrambled control siRNA.

Detection of Autophagy

In this study, the primary method for determining the level of autophagy/autophagic flux was to determine the level of the autophagosomal membrane bound form of the microtubule-associated

protein 1A/1B-light chain 3 (LC3), LC3-II, by immunoblotting. This form of the protein is phosphatidyl ethanolamine conjugated when autophagy is up-regulated and runs faster on a polyacrylamide gel than the unconjugated (and non-membrane bound) LC3-I form due to hydrophobicity. An increase in the LC3-II band on a western blot correlates with an increased number of autophagosomes in the cells. Such an increase can either indicate that autophagy has been induced and the formation of autophagosomes has increased, or alternatively, that the rate of autophagic flux has been inhibited and the degradation of autophagosomes has decreased, leading to their accumulation. To distinguish between these two possibilities, it is necessary to add inhibitors of lysosomal acidification or of autophagosomal degradation to inhibit autophagic flux in addition to the experimental drug. E64d and pepstatin A are two agents commonly used to this task. Chloroquine is also a known inhibitor of the fusion of autophagosomes and lysosomes and can be used to inhibit autophagic flux. If the addition of these agents leads to a further increase in LC3-II levels compared to the test drug alone, this result indicates that the test drug leads to the up-regulation of autophagic flux. If there is no additional increase in LC3-II levels, it is likely that the test drug was already causing a block in autophagic flux and the additional flux inhibitors have no additional effect (Figure 2.2A).

The number of autophagosomes present in cells can also be determined by ectopically expressing a fluorescently tagged LC3 (GFP-LC3) and following the fluorescence pattern by microscopy (Figure 2.2B). A low level diffuse pattern of fluorescence indicates that autophagy is not active (or active at very low levels) while a punctate pattern indicates that autophagosomes have formed and LC3 has accumulated in them. For these assays it is also necessary to distinguish between up-regulation of autophagy and down-regulation of autophagosomal degradation using inhibitors of lysosomal turnover.

Protein Expression

Protein expression was determined using Immunoblot analysis. Cell lysates were prepared in lysis buffer containing 1% SDS or 1% Triton X-100 followed by boiling. Protein concentration was measured using the Bradford assay to allow for equal amounts of each sample to be analyzed. Standard procedures for immunoblotting were used. Briefly, samples were run on 8,10, or 12% poly-acrylamide gels and then transferred to nitrocellulose. After blocking with 5% milk in TBST, membranes were blotted with antibodies overnight, and washed before being incubated with secondary antibodies followed by detection using a chemiluminescent substrate. The expression of GAPDH was determined as a loading control. Band intensity was quantified using the software ImageJ (NIH).

Determination of Growth Rate

The effect of drug treatments on growth rate was determined using Cell Titer Glo assay in which the number of viable cells was determined daily for 1 week and the growth rate determined. Cells were seeded in 96-well plates and allowed to recover overnight. The following day, drug was added. For each treatment a separate set of samples was used for each time point. The relative number of viable cells was measured by adding 100 µl Cell Titer Glo (Promega) reagent, incubating at room temperature for 10 minutes, and then reading the luminescence output on a fluorescent plate reader.

Glucose Assay

In this study the change in the level of glucose in the media was measured in order to determine the effect of drug treatments on the cell's ability to import glucose. For these experiments cells were grown with and without drug for 0, 1, 8, 24, and 48 hours. For each time point, 100 µl of the media were collected and the number of cells for each sample was counted. The concentration of glucose in the media was measured and normalized to the number of cells for each sample. The level of glucose uptake was determined by subtracting the concentration of glucose remaining in the media at each time point from the concentration at time 0.

The concentration of glucose in a sample can be determined by using an enzymatic reaction that results in a change in absorbance which is proportional to the glucose levels. For these assays a Glucose (HK) Assay kit was purchased from Sigma-Aldrich (Cat# GAHK20), and the assay was performed according to the manufacturer's instructions, with the exception that the reaction volume was reduced to 100 µl total so that 96-well plates could be used. The kit includes the enzyme hexokinase, which phosphorylates the glucose to generate glucose-6-phosphate (G6P). G6P is then oxidized to 6-phospho-gloconate in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this reaction oxidized nicotinamide adenine dinucleotide (NAD) is reduced to NADH. It is this reduction reaction that leads to an increase in absorbance at 340nm. Absorbance measurements were adjusted based on the total blank absorbance, which is the sum of the reagent blank (which included the glucose assay reagent without any sample) and the sample blank (which included the sample without any glucose assay reagent).

Metabolite Extraction and Analysis

The effect of drug treatments on metabolism can be explored by determining the levels of abundant metabolites in cells and analyzing differences in their basal levels in sensitive versus resistant cell lines, or by determining significant changes induced by drug treatments. For these experiments, cells are grown in 6-well plates with triplicate samples per treatment group, plus an extra well to count the number of cells present. To extract the metabolites, the media are washed off the cells and 3 ug of myrisitic acid (0.25 ml of a 12 µg/ml solution) are added to each well as an internal control. Next, to extract the polar metabolites, a 1:1 mixture of methanol and water is added and incubated at room temperature for 15 minutes. Cells are then scraped, collected, and spun down. The polar metabolites are now in the supernatant, which is collected and dried in a speed vac. The pellets are re-suspended in a 3:1 mixture of dichloromethane and methanol to extract the non-polar metabolites. Samples are spun

down and the supernatant is collected and dried overnight in a fume hood (Masson et al., Anal Chem, 2010). The next step is TMS-derivatization of the polar and non-polar fractions. 30 µl of methoxyamine hydrochloride (20 mg/ml in pyridine) are added to each sample and then mixed by vortex for 5 minutes. Next, 30 µl of MSTFA are added, samples are mixed for 1 minute and allowed to react at room temperature for 1 hour. The levels of metabolites in the samples are measured and analyzed using GC-MS (Gullberg at al., Anal Biochem, 2004). For this study, the TMS-derivatization and GC-MS analysis steps were performed by Tzu-Fang "Cherry" Lou and Dr. Hyuntae Yoo at The University of Texas at Dallas.

Figures

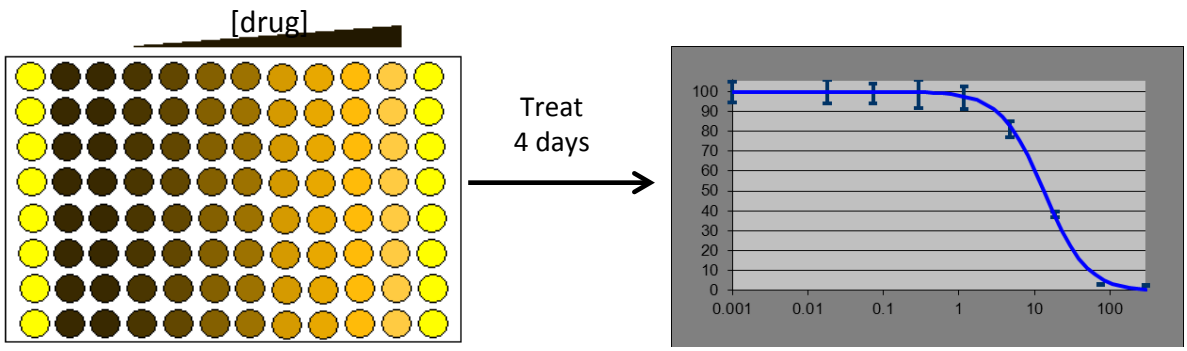


Plate cells Day 0, Add drug Day1

Day 5 Add Reagent, Measure Absorbance, Generate Dose Response Curve

Figure 2.1: Setup of MTS Assay to Measure Dose Response

Drug response screens tested 8 different drug concentrations at 4-fold dilutions each with 8 replicates per plate. 16 replicates per plate were cells only controls and another 16 wells served as blanks. Following 4 days of treatment MTS reagent was added which results in a color change proportional to the number of metabolically active cells. This colorimetric readout is read and converted into dose response curves using DIVISA.

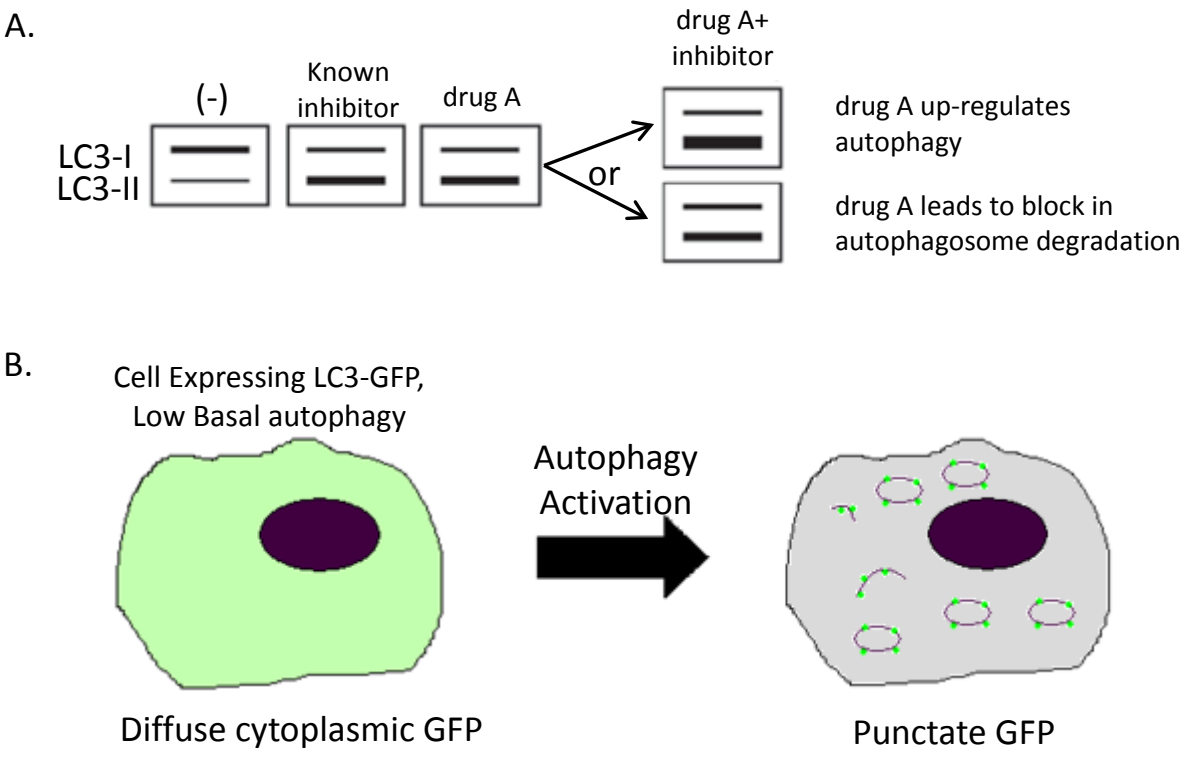


Figure 2.2: Using LC3 to Determine Effect of Drug Treatment on Autophagy
A. The level membrane bound LC3 (LC3-II) seen by immunoblotting correlates to the number of autophagosomes. Known inhibitors of autophagosome degradation, such as chloroquine, lead to an accumulation of LC3-II. The increase of LC3-II caused by drug A could be due to and up-regulation of autophagosomal formation or a block in autophagosomal degradation. Using drug A in combination with a known autophagy inhibitor can help distinguish this difference. B. Exogenously expressed LC3-GFP can be used to monitor autophagic flux. A diffuse GFP pattern indicates low levels of autophagy while a punctate pattern indicates LC3-GFP is being incorporated into newly formed autophagosomal membranes.

Chapter 3: Characterization of mTOR Targeted Therapies for Use in NSCLC

I. Abstract

mTOR is a central regulator of cellular growth and proliferation through the incorporation signals indicating the presence or absence of nutrients, oxygen, and growth factors. While mTOR kinase itself has not been reported to be mutated in cases of NSCLC, its activity is often up regulated through alterations in other components of these signaling pathways. Clinical trials using drugs that target mTOR signaling have been unsuccessful thus far, possibly due to a lack of molecular biomarkers that may identify a subset of tumors that are responsive to mTOR targeted therapy, or possibly due to the inability of first generation mTOR inhibitors to completely inhibit mTOR signaling. As a result, additional pre-clinical evaluation of mTOR inhibitors is necessary to explore possible predictive biomarkers and to characterize the role of mTOR signaling in NSCLC. To this end, in this study we screened a panel of NSCLC lung cancer cell lines with 3 different mTOR inhibitors: the classic allosteric mTOR inhibitor rapamycin, and two novel mTOR kinase inhibitors Torin1 and AZD8055. The NSCLC cell lines panel displayed a wide range of sensitivity to mTOR inhibition, which was not associated with the level or duration of inhibition of mTOR activity. Activation along the EGFR/ERBB2 axis corresponded with sensitivity to mTOR inhibition, while KRAS mutants tended to be resistant. EGFR mutants that were responsive to mTOR inhibitors included those that contain additional EGFR TKI resistance mechanisms such as the second-site T790M mutation or MET amplification. Therefore, mTOR inhibitors may be a useful second line therapy following acquired resistance to TKIs.

II. Introduction

Role of mTOR in Cancer

mTOR is a kinase that exists in two complexes with overlapping and non-overlapping substrates (Laplante and Sabatini 2012). mTORC1 is a central regulator of several pathways that promote tumorigenesis through regulation of protein synthesis and proliferation, as well as several metabolic pathways and the inhibition of autophagy. Recently the role of mTORC2 in cancer has been receiving more attention. It is now known as the kinase that phosphorylates AKT at Ser473, which leads to its full activation and promotes pro-growth signaling (Ikenoue, Inoki et al. 2008). Despite mTOR's potential to promote oncogenesis and deregulated cell growth, mutations are extremely rare in tumors. However, cancer cells frequently incur other changes that ultimately lead to altered regulation of mTOR.

The presence of growth factors signals for activation of mTOR, which leads the up-regulation of pathways that encourage cell growth and proliferation. These pathways are often deregulated in cancer through various mechanisms that activate receptor tyrosine kinase activity, including mutations that allow for constitutive activation and amplification of either wild-type or mutant genes. Mutation and amplification of EGFR, or amplifications of ERBB2 are the most frequently occurring RTK alterations found in lung cancer (Li, Kung et al. 2013). Mutations in the Ras oncogene occur in about 20-30% of NCSLCs, which lead to increased activation of mTOR by signaling through PI3K (Conde, Angulo et al. 2006). Mutations in PI3K or AKT are also found in lung tumors; though they occur at much lower rates (Li, Kung et al. 2013).

In addition to increases in mTOR activation through oncogenic changes, pathways which reduce mTOR activity during conditions of high nutrient availability are frequently lost in cancer cells. PTEN is a phosphatasethat counters PI3K activity, by removing a phosphate to convert PIP_3 back to PIP_2. Mutations which result in loss of PTEN occur in about 5% of lung tumors. STK11/LKB1 is an important

component of an energy sensing pathway. When ATP:ADP ratios are high, STK11 signals through AMPK to inhibit mTOR activity (Hardie 2004). Twenty percent of lung cancers express a truncated form of STK11 which is quickly degraded (Gill, Yang et al. 2011). Additionally the wild-type STK11 allele is also lost, leading to complete loss of STK11 function in the cells. Interestingly, STK11 mutations frequently co-occur with mutations in the KRAS oncogene (Dearden, Stevens et al. 2013). The presence of these co-mutations is thought to have implications for tumor metabolism and drug response phenotypes.

Need for Pre-Clinical Evaluation of Novel mTOR inhibitors

Because so many oncogenic changes occurring in cancer cells seem to converge on mTOR signaling as a means to promote tumor growth and proliferation, mTOR is understandably being evaluated as a target for cancer therapy. To date the majority of mTOR targeted therapies consist of rapamycin and its analogs such as everolimus and temsirolimus (Laplante and Sabatini 2012). These drugs are allosteric inhibitors of mTOR, and are now known to only inhibit partial functions of mTORC1, and to be ineffective at targeting mTORC2 (Thoreen, Kang et al. 2009). While these agents are approved for advanced renal cell carcinoma, they have only shown modest success as mono-therapies in clinical trials of other cancer types (Kwitkowski, Prowell et al. 2010). Response to rapamycin and its analogs may be limited due to reactivation of upstream signaling that results from the release of feedback inhibition of PI3K signaling through mTOR effector S6K (Shah and Hunter 2006).

Next-generation mTOR inhibitors are catalytic inhibitors of mTOR which act by binding to the ATP-binding domain of the kinase. These drugs are more effective at inhibiting functions of mTORC1 that are insensitive to rapamycin treatments, as well as inhibit mTORC2. Such agents are thought to enable more complete inhibition of mTOR signaling and to minimize reactivation of upstream signaling often seen in response to rapamycin (Benjamin, Colombi et al. 2011, De, Miskimins et al. 2013).

Lessons from trials of other targeted therapies stress the benefit of proper patient selection for their successful use and clinical implementation. Therefore, pre-clinical studies are needed in order to identify selection criteria required to identify which patients are candidates for a particular targeted therapy. By screening human lung cancer cell lines for their response to mTOR inhibition, we hope to identify subsets of sensitive and resistant groups in order to examine potential predictive biomarkers. Based on other studies, we hypothesized that dual mTORC1/2 inhibitors would be more effective at inhibiting cancer cell growth due to more complete inhibition of signaling. We also aimed to determine whether alterations through mutation, amplification, or abnormal gene expression would result in dependence on signaling from this pathway and thus sensitivity to its inhibition.

Drugs Used in this Study

Rapamycin

Rapamycin is a bacterially produced macrolide that has broad clinical application as an anti-fungal, immunosuppressant, as well as an anti-cancer drug (Figure 3.1). It inhibits mTOR through an allosteric mechanism in which it binds to its intracellular receptor, FKBP12, which then binds and inhibits mTOR (Brown, Albers et al. 1994, Benjamin, Colombi et al. 2011). Under most conditions, rapamycin only inhibits mTORC1, and complex 2 is often considered the rapamycin resistant complex. However, in some cell types it has been noted that prolonged exposure to rapamycin can also lead to inhibition of mTORC2 activity. This inability to inhibit mTORC2, however, has been speculated as the reason rapamycin has had limited success clinically as an anti-cancer agent. Therefore, the development of novel inhibitors that are able to inhibit both complexes of mTOR has become the primary approach for targeting the mTOR pathway in cancer (Thoreen, Kang et al. 2009).

Torin1

The laboratories of David Sabatini and Nathanael Gray jointly developed a novel ATP competitive mTOR inhibitor named Torin1 (Figure 3.1). The initial compound was first identified in a biochemical screen for inhibitors of mTOR kinase activity. *In vitro* kinase assays using purified mTORC1 or 2 suggested that Torin1 could inhibit both complexes with IC50 values between 2 and 10 nM. In cells the IC50 for mTORC1/2 inhibition was also between 2 and 10 nM. Torin1 is highly selective for mTOR over other related kinases. The cellular IC50 for PI3K was 1.8 uM, showing nearly 1000 fold decrease in potency. Torin1 showed at least 200-fold decrease in potency for other PI3K-like kinases including DNA-damage response kinases ATM and DNA-PK, and class III Pi3K hVps34. Furthermore, 10 μM Torin1 showed no significant effect on a panel of 353 kinases in a screen that measured binding of the target molecule to each kinase (Thoreen, Kang et al. 2009).

Torin1 causes G1/S cell cycle arrest in MEFs, as well as a decrease in cell size more effectively than rapamycin. Interestingly, the improvements of Torin1 over rapamycin do not seem to be the result of increased inhibition of mTORC2 as Torin1 showed similar improvements over rapamycin in MEFs which lack the essential mTORC2 component, Rictor. Under these conditions it would be expected that Torin1 and rapamycin would have the same effect since mTORC2 activity is already eliminated. Therefore, there appeared to be functions of mTORC1 that were disrupted by direct kinase inhibition by Torin1, but not by the indirect inhibition by rapamycin. Torin1 also proved to be a superior inducer of autophagy, stronger inhibitor of cap-dependent translation due to rapamycin's inability to completely inhibit mTORC1-dependent phosphorylation of 4E-BP1 (Thoreen, Kang et al. 2009). Using green fluorescent protein labeled LC3, it was found that Torin1 is more effective at inducing the formation of autophagosomes than rapamycin, due to its ability to inhibit rapamycin resistant functions of mTORC1 rather than through the inhibition of mTORC2.

Pharmacokinetic studies of Torin1 found that the drug has a very short half-life *in vitro* and *in vivo*. In liver microsome stability studies it had a half-life of 4 minutes and in mice following IC administration of 1 mg/kg, oral administration of 10 mg/kg, or IP administration of 10 mg/kg the half-life was 0.5 hr. Torin1 also exhibited low exposure and low bioavailability. Nevertheless, Torin1 was shown to inhibit mTORC1 activity for about 2-3 hours *in vivo* as shown by the suppression of S6 phosphorylation, as well as mTORC2 activity as shown by suppression of AKT Ser473 phosphorylation in the liver. Additionally, mice bearing U87MG xenografts had greater that 99% inhibition of tumor growth when treated with IP injections of Torin1 at 20 mg/kg daily (Liu, Chang et al. 2010).

Due to the poor bioavailability of the initial compound Torin1, further alterations to the drug were made in order to improve the pharmacokinetic properties and enable enhanced efficacy as an anti-cancer agent. The resulting compound, Torin2, exhibits better pharmacokinetic properties and has a 250 pM IC50 for the inhibition of mTOR in cells while largely maintaining selectivity (Liu, Chang et al. 2010). This generation of the mTOR inhibitor does however inhibit of PIKK family kinases, including ATM with an EC50 of 28 nM, ATR with an EC50 of 25 nM, and DNA-PK with an EC50 of 118 nM. Torin2 treatment also prevents the feedback activation of AKT at T308 and inhibited the growth of cancer cells *in vitro*. In a KRAS-driven lung tumor mouse model, Torin2 as a single agent was not very effective. However, combinations of Torin2 with the MEK inhibitor AZD6244 significantly inhibited tumor growth (Liu, Xu et al. 2013).

AZD8055

AZD8055 is an ATP competitive mTOR kinase inhibitor identified from a chemical library screen of compounds based around a pyridopyrimidine scaffold (Figure 3.1). It was shown to inhibit recombinant mTOR enzymatic activity with an IC50 of less than 1 nM and with cellular IC50s of around 25 nM, but does not effectively inhibit other related kinases at 10 μM, and had at least 1000-fold

decrease in potency to closely related class I and III PI3K lipid kinases and PIKK family members ATM and DNA-PK (Chresta, Davies et al. 2010). By observing levels of phosphorylated ribosomal protein S6 at Ser235/236, an indirect substrate of mTORC1, the ability of AZD8055 to inhibit mTORC1 was confirmed in cells. Activity of mTORC2 was also inhibited by AZD8055, as detected by observing the phosphorylation state of AKT at Ser473, a direct mTORC2 substrate. AZD8055 is also greatly more effective at inhibiting another direct substrate of mTORC1, 4EBP1, compared to the classical mTORC1 inhibitor rapamycin. As a result, AZD8055 was able to effectively inhibit cap-dependent translation. Signaling downstream of mTORC2 was also evaluated following AZD8055 treatment. Both AKT and SGK function was impaired. A concentration-dependent decrease of AKT substrates pPRAS40 T246, pTSC2 T1462, and FoxO1 T24 and S256 occurred; however, only a weak decrease in GSK3B phosphorylation of Ser9 was seen. Downstream of SGK, NDRG1 showed a dose-dependent decrease in phosphorylation state by AZD8055. There was no evidence that AZD8055 lead to the induction of feedback activation of AKT on Ser473, which has been shown with rapamycin treatment (Chresta, Davies et al. 2010).

AZD8055 also has a strong effect on cellular proliferation and autophagy. In 2 NSCLC (H838 and A549) and 1 glioblastoma (U87MG) cell lines, drug concentrations of 20-53 nM induced 50% inhibition of proliferation. In these same NSCLC cell lines, AZD8055 was able to show a concentration dependent increase in the number of autophagosomes present after 72 hours, as measured by the number of acridine orange stained punctate structures and the conversion of LC3-I to LC3-II, which was further increased when also treated with lysosomal pH altering agents, E64d/leupeptin (Chresta, Davies et al. 2010). These data strongly suggest that the drug induces autophagy.

AZD8055 has also been successfully used to inhibit tumor growth in xenograft models of a variety of human tumor types. Doses of 10 mg/kg twice a day or 20 mg/kg once a day inhibited tumor

growth by at least 65% and even lead to 25% tumor regression in 1 NSCLC xenograft (Chresta, Davies et al. 2010).

Selection of Cell Lines

The use of human cell lines as a model has enabled the phenomenon of "personalized medicine" to blossom. For these studies a panel of human lung cancer cell lines were used that represent the broad spectrum of oncogenotypes, histotypes, and expression patterns found in actual patients, but are easily grown and analyzed en masse. While nearly 200 human lung cancer cell lines exist, for this project a panel of about 40 cell lines was used (Table 3.1). Cell lines were selected on the basis of a number of parameters. Primarily, cell lines were chosen to allow a variety of common oncogenotypes to be represented. Specific consideration was given to any cell line that contained a mutation or amplification of any gene that would be suspected of leading to activation in mTOR signaling. These included EGFR mutation/amplification, PTEN loss, PI3K mutation/amplification, AKT1 mutation/amplification, and STK11 loss. Cell lines that contained mutations in genes downstream of mTOR signaling were also considered. These mutations and expression characteristics are compared in the context of drug response phenotypes in order to examine potential biomarkers that can be used clinically in order to predict response or non-response to a particular therapy.

III. Results

NSCLC Cell Lines Display a Range of Response to mTOR inhibitors

In order to determine the response of NSCLC cell lines to treatment of mTOR inhibitors we first screened a panel of 39 cell lines for their response to rapamycin, and the dual mTORC1/2 inhibitors Torin1 and AZD8055. Drug response phenotypes for the cancer cell lines were determined by generating dose response curves and using MTS reagent as readout for cell viability. Cells were treated

for 96 hours with 8 different concentrations of drug ranging from 1pM to 300nM. The IC50 was determined as a measure of comparing sensitivity or resistance to the drugs. Figure 3.2A and Table 3.2 summarize the response phenotypes for the cell line panel to each of the three mTOR inhibitors, which were determined to be stable over time (Figure 3.3).

Responses of the cell lines to Torin1 or to AZD8055 were the most significantly correlated, as expected due to their similar mechanism of action and expected targets. Responses to all of the mTOR inhibitors showed significant positive correlation (Figure 3.2B). There was no correlation between responses of the cell lines to the mTOR inhibitors and responses to several other chemo- and targeted therapy agents (Table 3.2). The most notable distinction between the responses to the 3 mTOR inhibitors is the range of IC50s seen across the cell lines panel differs significantly for each drug. The range of response to rapamycin was much broader (3500 fold difference in IC50 between sensitive and resistant) than for the other agents. The Torin1 range of response was quite narrow, with only a 40 fold difference in the IC50 of the most sensitive and the most resistant line. The IC50s of the cell lines varied 300 fold in their response to AZD8055. In a cell line drug screen a broader range of response and/or a bimodal or tri-modal distribution of IC50s is more amenable to finding a statistically significant molecular biomarker.

Catalytic mTOR inhibitors, such as Torin1 and AZD8055, have been developed in order to improve upon the existing mTOR inhibitors, such as rapamycin and its analogs. Catalytic inhibitors are not only better at inhibiting multiple functions of mTORC1 that are resistant to inhibition by rapamycin, but they also inhibit activity from mTORC2, which is expected to enable greater inhibition of AKT signaling due to the function of this complex to phosphorylate and further activate AKT. Interestingly, a subset of four NSCLC cell lines was exquisitely sensitive to rapamycin treatment with IC50s in the sub-nanomolar range (Figure 3.2A, Table 3.2). While these lines were also among the most sensitive to the

dual mTORC1/2 inhibitors, much less rapamycin was required to achieve the same level of inhibition as either Torin1 or AZD8055. This result could indicate there may be a subset of patients that would benefit greater from mTORC1 inhibition only compared to dual inhibition of mTORC1/2.

Inhibition of mTOR Signaling is Equivalent in Both Sensitive and Resistant Cell Lines

In order to determine whether sensitivity to mTOR inhibition was due to superior inhibition of target activity in these lines compared to resistant lines, the levels of mTORC1 activity were evaluated by examining the levels of p-P70S6K by immunoblot, and the levels of mTORC2 activity were evaluated by examining the levels of p-AKT(Ser473) following drug treatment. All three mTOR inhibitors lead to marked reductions in levels of p-P70S6K after 1 hour in both a sensitive, and 2 resistant cell lines (Figure 3.4A, B). After 24 hours these levels returned, indicating either the presence of a mechanism to overcome inhibition, metabolism, or degradation of the drug by this time point. The short duration of inhibition of mTORC1 activity was most notable in the mTOR inhibitor sensitive line Calu-3. Therefore, neither the level nor the duration of mTORC1 inhibition of p-P70S6K can account for the difference in response among NSCLC cell lines. AZD8055 was also shown to reduce mTORC1 activity on P70S6K in a NSCLC sub-cutaneous tumor that was resistant to treatment (Figure 3.5). Torin1 treatment was ineffective at inhibiting levels of p-4EBP1 in resistant cell line, H460, but did reduce them in the sensitive cell line, Calu-3. This result is consistent with the idea that incomplete inhibition of this phosphorylation may be associated with resistance to mTOR inhibition. However, sensitivity to mTOR inhibition does not require a reduction in p-4EBP1 levels, as rapamycin was ineffective at reducing phosphorylation of this protein in either a sensitive or resistant cell line (Figure 3.4C).

Similarly, the 3 mTOR inhibitors showed similar levels of inhibition of mTORC2 activity in both sensitive and resistant cell lines (Figure 3.4A, B). Rapamycin did not reduce the levels of p-AKT at Ser473 as expected due to its known inability to inhibit mTORC2 activity. One hour Torin1 treatment lead to a

reduction in p-AKT(Ser473) levels most notably in resistant cell lines, indicating again that the level of inhibition of target activity does not predict sensitivity to drug treatments. AZD8055 is a known inhibitor of mTORC2 activity, however only resistant cell line H2122 had any measurable reduction in p-AKT levels following 1 hour treatment with AZD8055. Therefore, under the conditions tested, AZD8055 was only minimally effective at inhibiting mTORC2 activity in both sensitive and resistant NSCLC cells.

mTOR Inhibition Reduces the Growth Rate of Cancer Cells, but Does Not Induce Apoptosis

Example dose response curves of a sensitive, intermediate, and resistant cell line for each of the three mTOR inhibitors are shown in Figure 3.6A. An additional assay validated the response phenotypes found through the MTS screen. We determined the effect of mTOR inhibition of the growth rate of cells with differential sensitivities. Cells were plated at low density in 96 well plates and treated with rapamycin, Torin1, AZD8055 or control treatment. The relative number of viable cells was measured every 24 hours over 7 days using a luminescent output from a reaction with Cell Titer Glo reagent. In resistant cell line, H2122, treatment with an mTOR inhibitor had minimal effect on the growth rate of the cells. In a sensitive (Calu-3) and intermediate (H460) cell line, mTOR inhibition significantly reduced the growth rate of the cells compared to the control cells (Figure 3.6B). This assay confirmed the phenotypes in the original screen and suggested that the induction of growth arrest may be responsible for the sensitivity phenotype. Furthermore, KRAS mutant line H460 was also shown to be resistant to AZD8055 in a subcutaneous tumor model (Figure 3.5B).

As further validation that mTOR inhibition slows the rate of growth of sensitive cell lines, a panel of cell lines was screened for the effects of mTOR inhibition on clonogenicity using liquid colony formation assays. mTOR inhibition leads to a reduction in colony forming efficiency and, notably, a reduction in colony size in sensitive cell lines. Colony formation assays also confirm the sensitive/resistance response phenotypes found in MTS viability assays, as the drugs' effects on colony

forming efficiency mimicked the relative dose responses seen in the shorter term assays (Figures 3.6C, 3.7A). In some resistant cell lines, low doses of mTOR inhibitor actually stimulated colony formation efficiency (Figure 3.7B).

mTOR inhibitors are not known as potent inducers of apoptosis, although long term treatment has been shown to lead to apoptotic cell death in some settings (Willems, Chapuis et al. 2012). In NSCLC cells the three mTOR inhibitors did not consistently or broadly induce apoptosis as indicated by only a rare induction of PARP cleavage following treatment. When PARP cleavage was observed, its occurrence did not correlate with sensitivity to the treatment, as it was not seen in sensitive cell line Calu-3, but was after 72 hours of rapamycin or AZD8055 treatment in the much more resistant line H460 (Figure 3.8A).

Previous reports have indicated that rapamycin treatment can induce arrest in the G1 phase of the cell cycle (Decker, Hipp et al. 2003). We wanted to determine whether this was also the case for mTORC1 or mTORC1/2 inhibition in NSCLC cells. Flow cytometric profiling of the DNA content of cells was used to characterize the percent of cells in G0, G1, S, or G2 phases of the cell cycle following 72 hours of treatment with each of the three mTOR inhibitors. No significant changes were seen in the cell cycle profiles of either sensitive or resistant lines compared to untreated controls indicating that cell cycle arrest cannot account for the cell lines' responses to mTOR inhibition (Figure 3.8B). Furthermore, response to mTOR inhibition does not appear to be associated with the growth rate of the cells. When the panel of cell lines was divided into quartiles based on their innate doubling times, there was no difference in response to rapamycin, Torin1, or AZD8055 between these groups (Figure 3.8C).

mTOR Inhibition Response Does Not Correlate with Basal Activation of PI3K-AKT-mTOR Signaling

It has been suggested that high levels of innate signaling activity of the PI3K-AKT pathway (as measured by high levels of p-AKT) is indicative of dependence of signaling through this pathway and

thus susceptibility to pathway inhibition. To determine whether this could be true in NSCLC cells we analyzed a panel of 5 sensitive and 5 resistant cell lines by immunoblotting for levels of phospho-EGFR, mTOR, P70S6K, and AKT relative to total levels of these proteins to examine the basal pathway activation. No significant difference was seen between sensitive and resistant groups for any of the levels of activated components of this pathway (Figure 3.9).

It has also been suggested that the ratio of eIF4E/4E-BP, rather than the individual protein levels, predicts the efficacy of mTOR targeted therapies (Alain, Morita et al. 2012). Therefore we wanted to determine whether the ratio of these 2 proteins could be used to predict the response to mTOR inhibition in NSCLC cell lines. eIF4E was very highly expressed in cancer lines, consistent with previous reports (Graff, Konicek et al. 2008). However, neither the individual level of 4EBP1 or eIF4E (not shown), nor the ratio between them correlated with NSCLC cell line response to mTOR inhibition (Figure 3.9). Therefore, we cannot conclude that this ratio of protein expression will be a useful biomarker for innate resistance to mTOR inhibition in lung cancer cells. It remains possible, however, that changes in expression of these proteins may still be a mechanism that lung cancer cells may use when resistance is acquired in order to maintain levels of protein translation despite treatment with an mTOR inhibitor.

Activation Along the EGFR/ERBB2 Axis Confers Sensitivity to mTOR Inhibitors

Mutations in major oncogenes and tumors suppressors such as Ras, STK11, EGFR, PI3K, and AKT, as well as rare mutations such as in IRS1, TSC1, and Deptor are found in the cell lines at similar frequencies to those found in patients (Table 3.1). All of these mutations potentially alter the activity of mTOR signaling in cancer cells, and the response to mTOR inhibition. We determined whether the presence of a single mutation will be useful as a predictive biomarker for response to mTOR inhibition. KRAS mutant lines tended to be more resistant to rapamycin ($p=0.04$) and AZD8055 ($p=0.03$) than those

with wild-type KRAS (Figure 3.10A). This difference appears to be driven by lines containing EGFR/ERBB2 alterations as this significant difference was lost when lines containing these alterations were excluded (Figure 3.10B). Cell lines that contained either a mutation in EGFR or an amplification of ERBB2 (copy number >3 by qPCR), including those that have additional EGFR TKI resistance mechanisms (i.e. T790M mutations), were more sensitive to rapamycin (p=0.03, 0.03, respectively) and AZD8055 (p=0.02, 0.05, respectively) (Figure 3.10A). These lines were more sensitive to AZD8055 compared to those with either wild-type or mutant KRAS (p=0.05, 0.001, respectively) (Figure 3.10B). The narrower range of response of the NSCLC cell lines to Torin1 likely contributes to the lack of significantly differential responses between these groups for this drug. EGFR mutant cell lines that have additional EGFR TKI resistant mechanisms, including the second-site EGFR T790M mutation and amplification of the MET RTK, were used in this study. Despite having acquired resistance to erlotinib, these lines remained responsive to mTOR inhibition (Figure 3.11).

STK11/LKB1 mutations result in loss of function as well as loss of negative regulation of mTOR through an important energy sensing pathway, and have the potential to influence response to mTOR inhibitors. These mutations tend to co-occur with mutations in KRAS, which are relatively resistant to mTOR inhibitors compared to those with alterations in EGFR or ERRB2. Those lines that have mutations in STK11 without KRAS show similar responses to those that are wild-type for the stated proteins. Though there is a trend to be more sensitive, there also is not a statistically significant difference in response STK11 single mutants compared to KRAS/STK11 co-mutants, possibly due to a low sample size (Figure 3.10C). The differences in mTOR inhibitor response between cell lines with alterations in EGFR/ERBB2 are even more significant when compared to KRAS/STK11 co-mutants than to all KRAS mutant lines (Figure 3.10B, C). The presence of PTEN or PIK3CA mutation was not able to predict response to mTOR inhibition (not shown); however these mutations occur only rarely. Amplifications for FRAP1 (the gene encoding for mTOR, present in 3 of 18 cell lines examined) or KRAS (10/38 lines)

correlated with resistance to rapamycin (p=0.01, 0.02) or AZD8055 (p=0.04. 0.03), however amplifications of PIK3CA, EGFR, ERBB3, or MET did not significantly correlate with mTOR inhibitor response (not shown).

Individual Gene Expression Unlikely to Be Useful as Predictors of mTOR Inhibitor Response

Analysis of microarray expression data was performed in order to determine whether there were any significant differentially expressed genes between mTOR inhibitor sensitive and resistant cell lines. NSCLC cell lines were categorized into sensitive and resistant groups based on the IC50s for each of the three mTOR inhibitors. Log ratios of expression differences on a genome-wide scale between each group were calculated using MATRIX software. Figure 3.12 shows a summary of the results of the analysis of the log ratios for sensitive and resistant cell lines to each of the three mTOR inhibitors. Genes that had a log2 of greater than 2 or lower than -2 (4 fold difference in expression) and a p-value of less than 0.05 were considered significantly differentially expressed between sensitive and resistant lines. The most significant down-regulated genes in sensitive cell lines are represented by the dots in the section of the graph above the horizontal dotted line and to the left of the left-most dotted line. The most significant up-regulated genes in sensitive cell lines are represented in the section of the graph above the horizontal dotted line and to the right of the right-most dotted line.

In rapamycin sensitive lines there were 18 genes that were significantly under-expressed and 22 genes that were significantly over-expressed compared to resistant lines. Torin1 sensitive lines had 15 and 6 genes under- and over-expressed, respectively. AZD8055 sensitive lines had 15 and 16 genes under- and over-expressed, respectively. No single gene was more than 10 fold differentially expressed. Differentially expressed genes and the log 2 fold difference in expression can be found in Table 3.3. Expression of genes related to mTOR signaling, including ERBB family members, PIK3CA, AKT, MTOR among others were not significantly differentially expressed between sensitive and resistant cell lines.

Because all three drugs have the same molecular target and the responses of the cell lines are positively correlated, it might be expected that there would be significant overlap in the most differentially expressed genes between sensitive and resistant cell lines for each drug. In fact, there is very little overlap in the differentially expressed genes. There were no genes in common among the most up-regulated genes for any pair of mTOR inhibitors. For down-regulated genes, 2 were in common (CES1 and TSPAN7) for Torin1 and AZD8055 sensitive lines, 1 was in common (*AKT1C2*) for AZD8055 and Rapamycin, and 2 were in common (*AKR1C3, SOX21*) for Torin1 and Rapamycin. A single gene, *AKR1C4,* was commonly down-regulated in sensitive versus resistant lines for all three mTOR inhibitors.

Several aldo-keto reductase (AKR1C) family members were frequently seen among genes that were down-regulated in mTOR inhibitor sensitive lines. Members of this family are involved in steroid homeostasis, prostaglandin metabolism, and metabolic activation of polycyclic aromatic hydrocarbons. Their expression had been associated with oncogenic potential and drug resistance in GBM, lung and breast cancer; however it is not clear whether there is any association with mTOR (Penning, Jin et al. 2004, Wang, Lin et al. 2007, Le Calvé, Rynkowski et al. 2010). Nevertheless, it is unlikely that the expression of this or any other single gene will be useful in predicting response to mTOR inhibition. Though there is a statistical difference in the average expression of several genes between sensitive and resistant groups, there is also wide variation in expression of each gene within sensitive and resistant subgroups (for examples see Figure 3.13). As a result individual gene expression will be an unreliable biomarker. While it may be possible to generate a multi- gene signature that correlates with drug response phenotypes, we were more interested in looking at genes or pathways that correspond to functional differences between sensitive and resistant groups.

Tumorigenic Progression of HBECs and mTOR Inhibitor Response

An ideal cancer drug will inhibit the growth of tumor cells with minimal effect on normal cells. Targeted therapy agents are intended to target a molecule to which cancer cells have become addicted, and, therefore, will be more sensitive to its inhibition than normal cells. Immortalized human bronchial epithelial cells (HBECs) are an *in vitro* model of normal lung epithelial cells. In order for these cells to grow in culture CDK4 and HTERT must be exogenously expressed (indicated by 'KT' at the end of the cell line name). Despite these alterations, immortalized HBECs are not tumorigenic as they do not form tumors when implanted sub-cutaneously into immuno-compromised mice. In order to determine the potential therapeutic window of mTOR inhibition for inhibiting the growth of lung cancer cells without effecting normal cells, we screened a panel of 6 immortalized HBEC cell lines for their response to Torin1. The HBECs tested were sensitive or had intermediate sensitivity to Torin1, with IC50s ranging from 2-40 nM (Figure 3.14). HBEC30-KT is a normal cell line that has a matched cancer cell line, HCC4017, which was derived from the same patient. This pair had no significant difference in sensitivity between the normal and tumorigenic cell lines as there is a less than 2-fold difference in IC50 between them. These results suggest that mTOR inhibition may not have a very wide therapeutic window in which cancer cells will be preferentially targeted with minimal consequence on surrounding tissues. mTOR inhibitors have been widely used in patients and the toxicities are well characterized and found to be manageable; however, the concentrations of drug that will inhibit growth of the tumor may not be achievable without increasing standard doses and possibly impacting system toxicity.

Several oncogenic manipulations have been added individually to immortalized HBECs as a method to analyze the effects of individual and controlled combinations of alterations on oncogenic progression of lung cancer cells. Examples of such alterations that have been analyzed include the addition of oncogenic KRAS, knockdown of p53, overexpression of MYC, and knockdown of STK11/LKB1. These alterations did not affect response to mTOR inhibition in a consistent manner. In the HBEC30-KT system, the addition of MYC in the background of Ras and p53 had no effect on the sensitivity to

rapamycin. However, in HBEC3-KT, the addition of MYC resulted in a more than 500-fold shift in resistance to rapamycin. MYC overexpression had minimal effect on Torin1 IC50 in either cell line. Knockdown of STK11/LKB1 sensitized HBEC30-RL53 (Ras, p53) to rapamycin, but had no effect on response to Torin1 in either HBEC series. Overall these results suggest that the effects of oncogenic manipulations on response to mTOR inhibition is cell line dependent, possibly due to additional oncogenic changes occurring in response to stresses placed by the oncogenes on the cells.

IV. Discussion

The role of mTOR as a central regulator of several tumorigenic signaling pathways marks it as an attractive target for cancer therapy. However, initial trials that have tested the utility of mTOR inhibitors have only demonstrated mild effects. Analogs of rapamycin, everolimus and temsirolimus, are approved for the treatment of advanced renal cell carcinoma but in other cancer types, including lung cancer, significant therapeutic improvements have not been found. Patient selection using the presence of a biomarker that corresponds with sensitivity to a given targeted agent, or alternately, exclusion of patients whose tumors harbor biomarkers that correspond to resistance to targeted therapy are imperative for the ultimate success of a drug in the clinic. Pre-clinical screens that identify potential biomarkers are required in order to evaluate drug response and potential corresponding biomarkers.

Here we report that NSCLC cell lines display a range of response phenotypes to three mTOR inhibitors, rapamycin, Torin1, and AZD8055. Interestingly, the range of response was broadest for the classical mTORC1 inhibitor rapamycin. A subset of NSCLC cell lines was exquisitely sensitive to rapamycin treatment requiring even less of this drug to reach 50% inhibition than either of the dual mTORC1/2 inhibitors. Rapamycin did not inhibit mTORC2 in these lines, which suggests that these cell lines are more sensitive to inhibition of mTORC1 than to combined inhibition of mTORC1/2.

Nevertheless, these lines were also among the most sensitive of all the cell lines to all three mTOR inhibitors.

The concept of oncogene addiction is now well understood as an "Achilles' heel" for cancer cells and the identification of pathways to which cancer cells are addicted is the new focus for targeted therapies. It is often presumed that signaling pathways that drive cancer growth will be visibly up-regulated as seen through high levels of expression and active forms of signaling proteins. Previously it has been suggested that high levels of phospho-AKT corresponds to sensitivity to mTOR inhibitors (Wendel, De Stanchina et al. 2004, Macaskill, Bartlett et al. 2011). In NSCLC cell lines examined for this study, there was no correlation in the levels of basal pathway activation and sensitivity to mTOR inhibition. Similarly, the ratio of eIF4E to 4EBP1 has been explored as a potential biomarker for mTOR inhibitor response. In Alain et al. (Alain, Morita et al. 2012) researchers found that down-regulation of 4EBPs and increased availability of eIF4e resulted in acquired resistance of active-site mTOR inhibitors due to the failure of these inhibitors to prevent translation of eIF4e sensitive mRNAs in cells with high eIF4e/4eBP1 ratios. They also found that resistance was not associated with differences in ribosomal protein S6 or AKT phoshphorylation. Consistent with their observation that up-regulation of eIF4e is common in tumorigenic cells, we also found that in NSCLC cell lines eIF4E was regularly highly expressed, and S6 and AKT phosphorylation were similarly affected in sensitive and resistant lines. However, we quantified the ratio of eIF4e/4EBP1 and found no correlation with response to the mTOR inhibitors used in this study. Researchers in Alain et al. utilized an isogenic cell model system in which Ras-transformed and p53 negative MEFs that were 4EBP1/2 WT or 4EBP1/2$^{-/-}$, or where eIF4E was exogenously expressed were used to compare sensitivity to mTOR inhibition. In cells derived from human tumors a full constellation of mutations and expression irregularities are present which may not be fully reflected in the isogenic MEF system. The complexity of alterations found in lung cancer may lead to additional mechanisms which result in resistance or sensitivity to pathway inhibition.

The range of response to mTOR inhibition could not be explained by differences in the level or the duration of pathway inhibition. mTORC1 activity, measured by the level of p-P70S6K, was equivalently inhibited in both sensitive and resistant cell lines. Previously, the reduction of p-P70S6K levels in response to rapamycin treatment was suggested as a marker of drug response (Nozawa, Watanabe et al. 2007). The level of mTORC2 inhibition was more variable among the cell lines using the different inhibitors, however, once again the level of mTORC2 inhibition did not correlate with response, as mTORC2 inhibition was often more effective in resistant cell lines compared to sensitive ones. Similarly, the duration of response did not determine the sensitivity of the cell lines to the drugs. In both sensitive and resistant lines both mTORC1 and mTORC2 activity could be observed at higher levels by 24 hours of treatment.

The role of 4EBP1 in response to mTOR inhibition in NSCLC cells is still in question. While we have already shown that levels of 4EBP1 protein expression individually and in a ratio of eIF4E/4EBP1 do not correlate with response, it has also been suggested that the ability of a drug to inhibit the phosphorylation of 4EBP1 determines the drug's ability to affect growth (Ducker, Atreya et al. 2013). Similarly, rapamycin has been shown to be ineffective at inhibiting phosphorylation of 4EBP1, while ATP competitive mTOR inhibitors, such as Torin1, have been shown to be more effective at inhibiting this function of mTORC1 (Thoreen, Kang et al. 2009). Consistently, over 72 hours of treatment, rapamycin was unable to inhibit 4EBP1 phosphorylation in either the sensitive line Calu-3, or the resistant line H460. Torin1 was able to reduce levels of p-4EBP1 only in the sensitive line. While the response to Torin1 corresponds with its ability to inhibit p-4EBP1 in Calu-3, this cell line is also very sensitive to treatment with rapamycin, which is unable to prevent this phosphorylation. This result indicates that inhibition of this activity of mTORC1 is not necessarily required to affect growth in lines that respond to mTOR inhibition, but may still play a role in innate resistance.

Several biomarkers in cancer patients have been previously identified which enable clinicians to select targeted therapies that will be more effective. For example, in patients with metastatic melanoma harboring BRAFV600E mutations, treatment with a BRAF inhibitor, vemurafenib, is an effective strategy. Furthermore in lung cancer, mutations in the EGFR suggest sensitivity to EGFR tyrosine kinase inhibitors, erlotinib or gefitinib, while rearrangements in ALK suggest sensitivity to ALK inhibitor, crizotinib. Responses to therapies targeting mTOR are less understood, because unlike EGFR and ALK, mTOR is not mutated in lung tumors.

Two primary oncogenes, KRAS and EGFR, are frequently mutated in cancer (Kris MG, Johnsen BE et al. 2011), lie upstream of mTOR, and have the potential to alter its signaling. Amplifications of EGFR and its family members, including ERBB2, are other common mechanisms to activate downstream signaling (Pao and Girard 2011). Interestingly, concurrent mutations in KRAS with those along the EGFR/ERBB2 axis are exceedingly rare (Stella, Scabini et al. 2013). Despite having similar presumed effects on downstream mTOR activity, cells with each of these mutations have very different responses to mTOR inhibitors. KRAS mutants are relatively resistant to mTOR inhibition, while those with EGFR or ERBB2 alterations are among the most sensitive. This suggests that activation of RTK signaling results in greater dependence on mTOR. One line, H1155, contains both a mutation in KRAS, and a small amplification of ERBB2 (copy number=4). As this line is sensitive to rapamycin and Torin1, and intermediate to AZD8055 it behaves most similarly to the other EGFR/ERBB2 altered lines. Additional validation will be required to determine whether this dominance of EGFR/ERBB2 alteration over KRAS in determining mTOR inhibitor response is a general phenomenon.

Our results coincide with a recent study that reported that ERBB2 amplifications may be a potential biomarker to predict sensitivity to AZD8055 in uterine cancer cell lines (English, Roque et al. 2013). In this study, researchers detected gene amplification using FISH and found that the sensitivity in

ERBB2 amplified lines was associated with a greater block in the G0/G1 cell cycle phase and a greater decrease in S6 phosphorylation with drug treatments, though these physiological changes also occurred to a lesser extent in non-amplified lines (English, Roque et al. 2013). Our study demonstrates that EGFR mutant cell lines that were sensitive to mTOR inhibition include those that have additional mechanisms that enable them to be resistant to EGFR targeted therapies, such as the secondary T790M mutation in EGFR and amplification of MET. It has been reported that resistance to EGFR TKIs is associated with persistent activation of PI3K-AKT signaling in the face of EGFR inhibition (Guix, Faber et al. 2008). Furthermore, EGFR TKI-resistant cells have been reported to have higher levels of mTORC2 activity and to be sensitive to mTOR kinase inhibitor ku-0063794 (Fei, Zhang et al. 2013). Therefore, it is not surprising that EGFR mutant cell lines are sensitive to the inhibition of mTOR, which is downstream of this aberrant pathway that permits their survival. These results suggest mTOR inhibition may be a useful strategy for treating patients whose tumors no longer respond to EGFR or ERBB2 inhibition, or as a strategy for delaying or preventing acquired resistance, and are currently under investigation as such (Mayer 2013).

Relative to cells with EGFR/ERBB2 activations, KRAS mutant cells, and KRAS/STK11 co-mutant cells tended to be more resistant to mTOR inhibition, most significantly to AZD8055. There is no difference however in response to KRAS mutant lines and lines that are wild-type for both KRAS and EGFR/ERBB2. KRAS mutations are frequently associated with drug resistance including mTOR inhibitors (Ducker, Atreya et al. 2013). A recent report found that while KRAS mutant NSCLC cell lines are more vulnerable to MEK and Raf inhibition compared to their wild-type counterparts, while PI3K, AKT, and mTOR inhibitors (including AZD8055 and rapalogs) did not show selective toxicities between these two groups (Molina-Arcas, Hancock et al. 2013). Despite the role of mutant KRAS to aberrantly activate PI3K-AKT-mTOR signaling, it does not appear that inhibition of this pathway alone is sufficient to reduce growth of cells containing this oncogene. More likely, simultaneous inhibition of multiple pathways

including MAPK and RTK signaling will be necessary to target KRAS mutant tumors. In support of this Molina-Arcas et al. found that combined inhibition of IGF1R and MEK had a significant effect on viability of KRAS mutant cell lines through combined inhibition of the PI3K, MEK/ERK, and mTORC1 pathways (Molina-Arcas, Hancock et al. 2013).

Also from this drug screen we found that KRAS and STK11 mutations individually or when they co-occur are resistant to mTOR inhibition relative to cell lines with alterations along the EGFR/ERBB2 axis, most significantly to AZD8055 treatment. While KRAS mutations are frequently associated with drug resistance, including to mTOR inhibitors (Ducker, Atreya et al. 2013), previous reports have also suggested that cells that are deficient in STK11/LKB1, with or without co-occurring KRAS mutations are more sensitive to agents that inhibit MTORC1, opposing the results from this screen. Notably, Shackelford et al. (Shackelford, Abt et al. 2013) found that NSCLC cell lines and mouse tumor models deficient in STK11 are more sensitive to the diabetes drug phenphormin. This drug inhibits Complex I of mitochondria and leads to metabolic stress. Upon drug treatment, downstream target of STK11, AMPK, becomes activated and subsequently inhibits mTOR. The authors of this study suggest that STK11 deficiency makes the cells unable to activate AMPK induced mitophagy and leads to accumulation of defective mitochondria, increased levels of reactive oxygen species (ROS), and apoptosis. Furthermore, experiments performed at UTSW in the labs of Drs. R. DeBerardinis and M. White have found that KRAS/STK11 mutant cell lines use less non-glucose derived carbons to feed into the TCA cycle. It seems that this combination of mutations drives an alternate metabolic phenotype which makes them more sensitive to inhibition of glycolysis by 2-DG. mTOR inhibitors would also be expected to inhibit glycolysis and glucose uptake, but KRAS/STK11 mutant cell lines do not respond to mTOR inhibiting agents. Since mTOR is inhibited to a similar extent in cell lines with these co-mutations, it is possible that they have enough metabolic flexibility that makes mTOR inhibition insufficient to impact the cells' metabolic requirements (R. DeBerardinis, personal communication).

Another study (Liang, Ma et al. 2010) found that KRAS/STK11 mutant tumors were non-responsive to rapamycin, supporting the results of the present study. It was suggested by the authors of Shackelford et al. that STK11 loss leads to alterations in multiple pathways in addition to mTOR signaling. Therefore, the opposing drug response phenotypes between phenphormin and rapamycin may be due to the effects of these additional pathways altered in STK11 deficient tumors which continue to fuel tumorigenesis when mTORC1 is suppressed, but are sensitive to metabolic stresses induced by phenphormin.

Interestingly, mutation or amplification of *PIK3CA* did not determine response to mTOR inhibition in our study. Previously, mutation in the catalytic subunit *PIK3CA* was a significant genetic marker indicating sensitivity to an ATP-competitive mTOR inhibitor PP242 based on a screen of over 600 human cancer cell lines, including those from lung (Ducker, Atreya et al. 2013). Mutations in PIK3CA are not particularly common in NSCLC, occurring in less than 10% of patients (Reungwetwattana, Weroha et al. 2012). Only 4 of the cell lines used in this study had mutations in this gene. Eight of the cell lines reported here had amplifications of the *PIK3CA* gene, but this amplification did not correlate with response to mTOR inhibition. PTEN mutations also had no correlation response with mTOR inhibition, though they have been previously reported to be associated with sensitivity (Neshat, Mellinghoff et al. 2001). Both mutation and amplification of *FRAP1*, the gene encoding for mTOR seem to be very rare in NSCLC. Nineteen cell lines were tested previously by qPCR for the number of copies of the *FRAP1* gene and only 3 had 3-4 copies of the gene. Of these, 2 cell lines (H1355 and H2122) were tested for their response to mTOR inhibition and were intermediate and resistant, respectively. This low sample number was not enough to find a significant correlation with drug response and gene copy number. Mutations and amplifications of *AKT1* are similarly rare and their presence fails to significantly correlate with drug response.

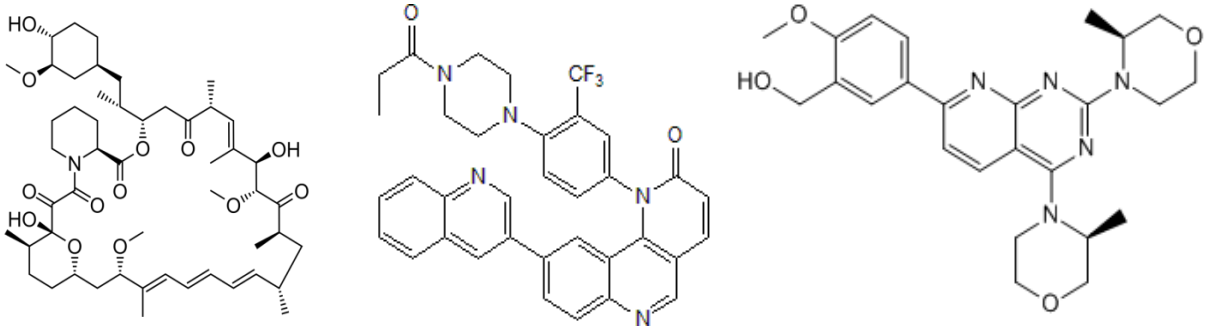
In addition to oncogenic changes affecting mTOR described above, TSC1/2 loss also potentially leads to aberrant mTORC1 signaling. Such alterations have been described in NSCLC, but are very rare. Liang et al. (Liang, Ma et al. 2010) found that TSC1 loss and KRAS activation confers sensitivity to rapamycin. The exact role the TSC1/2 complex is playing in response to mTORC1/2 inhibition is unclear. This complex has a well-defined role as an inhibitor of mTORC1 activity through its inhibition of GTPase Rheb which activates mTORC1 signaling (Pan, Dong et al. 2004, Kwiatkowski and Manning 2005). However, Rheb does not activate mTORC2 signaling (Yang, Inoki et al. 2006) and TSC1/2 was found to be required for proper activation of mTORC2 independent of its activity toward Rheb (Huang, Dibble et al. 2008). Two NSCLC cell lines, H2347 and HCC4017, have mutations in TSC1. H2347 also seems to have lost a copy of *TSC2*. This cell line was sensitive to dual mTORC1/2 inhibition by Torin1 and AZD8055, but resistant to rapamycin. HCC4017 has a small amplification of the *TSC2* gene, and is resistant to mTOR inhibition. Overall, response to rapamycin does not correlate with *TSC1/2* copy number, and the effect of the presence of a mutation in either of these genes on mTOR inhibitor response is inconclusive.

Recently, a golgi protein GOLPH3 was identified as a potent oncogene that is commonly amplified in human tumors and leads to activation of mTOR signaling and sensitivity to rapamycin (Scott, Kabbarah et al. 2009). Amplification (CN>3) of the *GOLPH3* gene is seen in approximately 30% of NSCLC cell lines. However, gene amplification as an individual biomarker was not associated with response to mTOR inhibition by rapamycin in NSCLC cells (not shown).

Based on the results of the mTOR inhibitor screen performed in this study, we conclude that aberrant RTK activation, including EGFR mutation or amplification, or ERBB2 amplification are the best predictors of sensitivity to mTOR inhibition. As the presence of RTK alterations is already frequently screened in lung cancer patients, it will not be such a great leap to begin using these biomarkers as molecular diagnostics to predict mTOR inhibitor drug response in a clinical setting. Other alterations

that modify mTOR signaling and which may be expected to predict mTOR inhibitor response, including mutation and/or amplification of *PIK3CA, FRAP1, PTEN,* or *AKT* did not correlate with drug response. Overall, dual mTORC1/2 inhibition through the use of ATP-competitive mTOR inhibitors was not a superior treatment strategy compared to mTORC1 inhibition by rapamycin. In fact, a subset of cell lines had a stronger response to rapamycin than they did to either Torin1 or AZD8055. Interference with feedback loops resulting from mTORC1/2 inhibition may play a role the efficacy of these agents. Chapter 5 will explore the effect of interfering with mTOR regulated feedback mechanisms and drug combination strategies as an approach to improve response to the single agents.

V. Figures



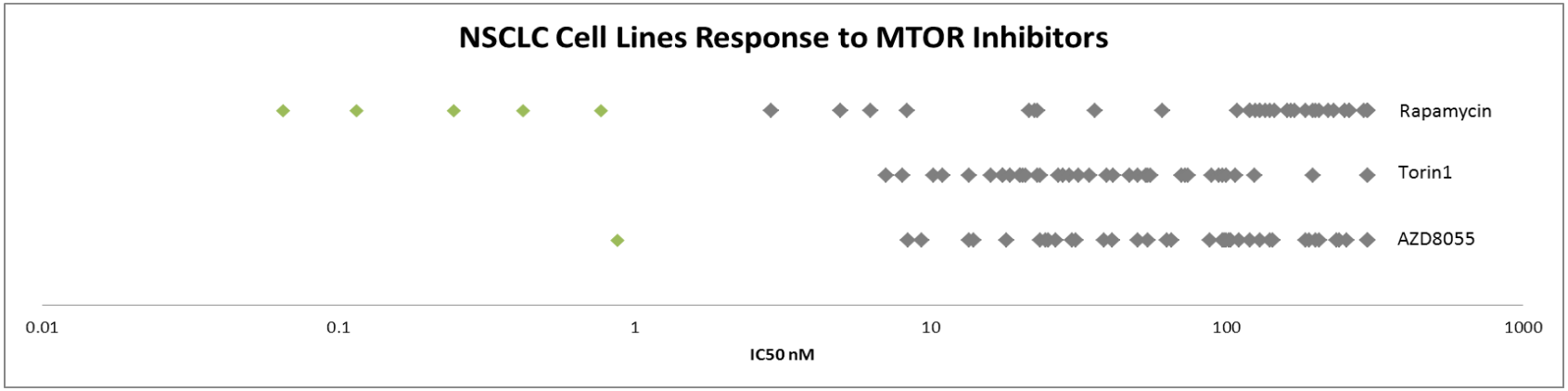
Rapamycin Torin1 AZD8055

Figure 3.1: Structures of mTOR Inhibitors Used in This Study

Table 3.1: mTOR Pathway Mutations Found In NSCLC Cell Lines

	p53	KRAS	STK11	PTEN	PIK3CA	EGFR	AKT1	IRS1	TSC1	PREX1	SLC2A4	SGK3	GCK	SGK2	SCNN1A	SLC2A10	SLC5A1	ULK1	DEPTOR	GYS1	RRAGA	SREBF1	PC	RPS6KB1	PLD1	SLC6A15	SLC5A2
A549	WT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
Calu-3	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1155	MUT	MUT	WT	MUT	WT	WT	WT			MUT								MUT						MUT			MUT
H1299	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT
H1355	MUT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1395	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H157	MUT	MUT	MUT	MUT	WT	WT	WT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT
H1650	MUT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1693	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1781	MUT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1819	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1975	MUT	WT	WT	WT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H1993	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H2009	MUT	MUT	MUT	WT	WT	WT	WT	WT	WT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	MUT	MUT	WT	WT	WT	WT	WT
H2052	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H2073	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H2087	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H2122	MUT	MUT	MUT	WT	WT	WT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H2126	MUT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H23	MUT	MUT	MUT	MUT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT		MUT	WT	WT
H2347	MUT	MUT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT
H322	MUT	WT	MUT	WT	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H3255	MUT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H358	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H441	MUT	MUT	WT	WT	WT	WT	WT																				
H460	WT	MUT	MUT	WT	MUT	WT	WT																				
H596	MUT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H661	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
H820	MUT	WT	WT	WT	WT	MUT	WT																				
H920	MUT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC2374	MUT	MUT	WT	WT	WT	WT	MUT																				
HCC2450	MUT	WT	WT	WT	MUT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT
HCC2935	MUT	WT	WT	MUT	WT	MUT	WT																				
HCC366	MUT	MUT	MUT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC4006	MUT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC4017	MUT	MUT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	MUT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC44	MUT	MUT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC827	MUT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
HCC95	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

A.



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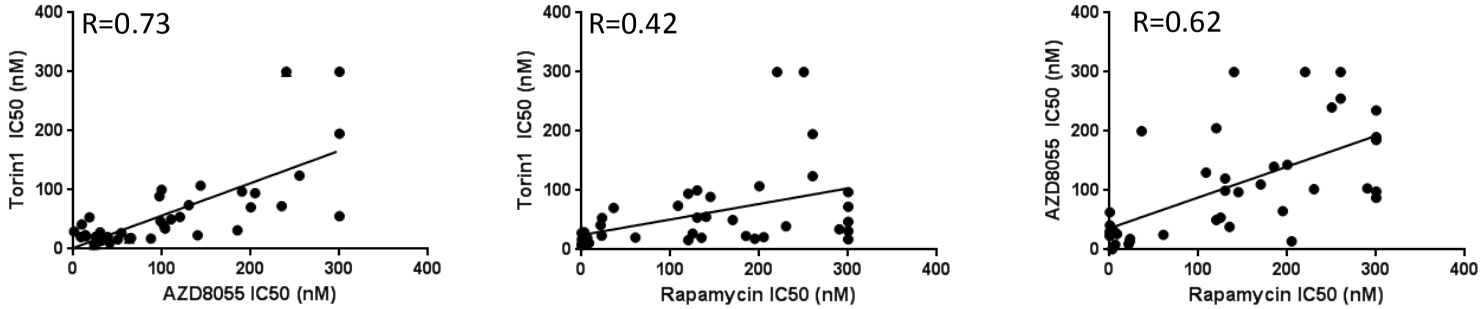


Figure 3.2: NSCLC Cell Lines Display a Range of Response to mTOR Inhibitors
A. Each dot represents the IC50 of a NSCLC cell line. Green indicates sensitivity to the inhibitor. B. Correlation of responses between the three mTOR inhibitors.

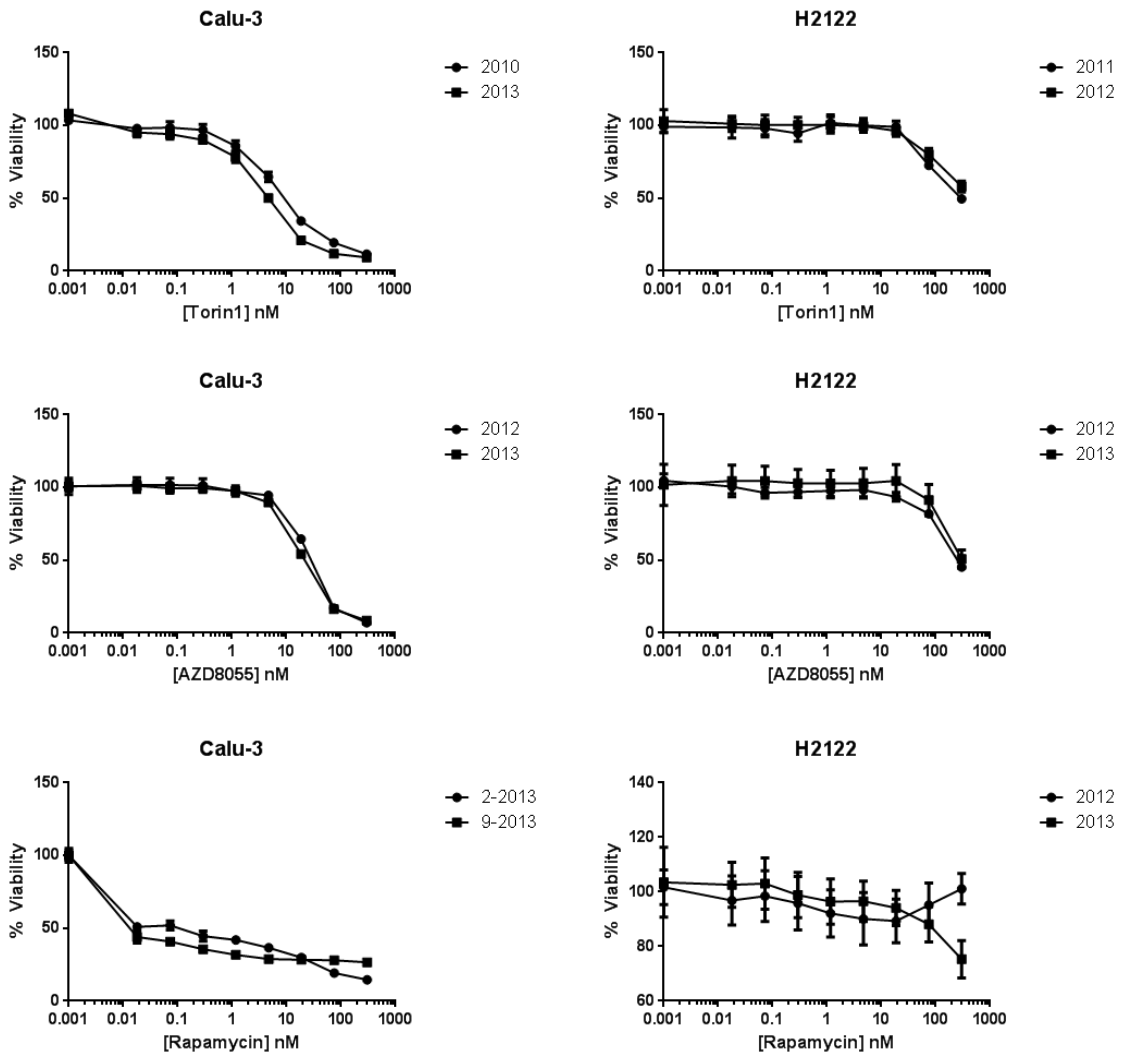


Figure 3.3: NSCLC Responses to mTOR Inhibition are Stable Over Time
mTOR inhibitor dose response curves for a sensitive (Calu-3)and resistant (H2122) cell line in replicate assays performed several months apart indicate the phenotypes are stable.

	Rapamycin		Torin1		AZD8055	
	IC50	stdev	IC50	stdev	IC50	stdev
A549	250	83.02	300	139.40	240	10.00
Calu-3	0.115	0.04	7.05	2.09	23.5	3.20
H1155	0.245	0.46	17.5	15.25	63	4.12
H1299	130	84.64	54	45.36	120	76.69
H1355	300	0.00	47	10.07	98	2.83
H1395	135	35.69	20	3.89	38.5	4.27
H157	185	65.32	23	2.97	140	22.17
H1650	200	122.79	107	84.82	143.5	64.12
H1693	4.95	10.74	13.5	8.04	31	4.12
H1781	145	7.07	89	126.52	97	61.33
H1819	0.065	0.17	28	15.48	30	13.39
H1975	125	91.74	27	25.92	54	15.52
H1993	120	19.31	16	9.10	50	6.24
H2009	108.5	83.60	74	77.10	130	10.33
H2052	0.77	3.90	8.05	1.36	24.5	2.75
H2073	6.25	3.53	20	6.75	8.4	6.58
H2087	260	46.19	195	117.13	300	0.00
H2122	260	75.77	124	102.51	255	64.32
H2126	36	8.08	70.5	13.96	200	117.53
H23	0.42	0.17	10.2	1.13	41	1.41
H2347	205	21.21	21	16.72	14	3.24
H322	220	60.24	300	135.56	300	0.00
H3255	22.5	0.71	23.5	10.97	13.5	0.71
H358	300	0.00	17.5	0.71	87.5	2.12
H441	290	47.61	34.5	19.55	103.5	35.54
H460	130	94.20	100	57.34	99.5	67.08
H596	60.5	7.78	20.5	0.71	25	4.24
H661	300	85.73	97	92.73	190	127.31
H820	8.35	0.21	11	3.62	26.5	0.96
H920	300	0.00	72.5	3.54	235	21.21
HCC2374	300	0.00	31.5	13.44	185	7.07
HCC2450	195	94.16	18.5	3.54	65	7.07
HCC2935	23	4.24	53.5	23.92	18	1.41
HCC366	140	90.50	55.5	11.08	300	0.00
HCC4006	21.5	2.12	41.5	22.11	9.35	0.92
HCC4017	170	65.28	50	78.41	110	16.00
HCC44	120	97.58	94.5	112.09	205	114.19
HCC827	2.9	0.42	29.5	8.67	0.875	0.11
HCC95	230	98.88	39.5	26.83	102	28.23

	Cisplatin	Gemcitabine	Paclitaxel/ Carboplatin	Pemetrexed	Erlotinib
Rapamycin	-0.10	0.13	-0.11	0.19	0.34
Torin1	0.15	0.20	0.01	0.26	0.09
AZD8055	-0.06	0.17	0.10	0.37	0.19

Table 3.2: mTOR Inhibitor Responses and Oncogenotypes

Median IC50 and standard deviation from 2-6 replicate experiments and correlation coefficients for the responses of mTOR inhibitors and other standard chemo- and target-therapy agents.

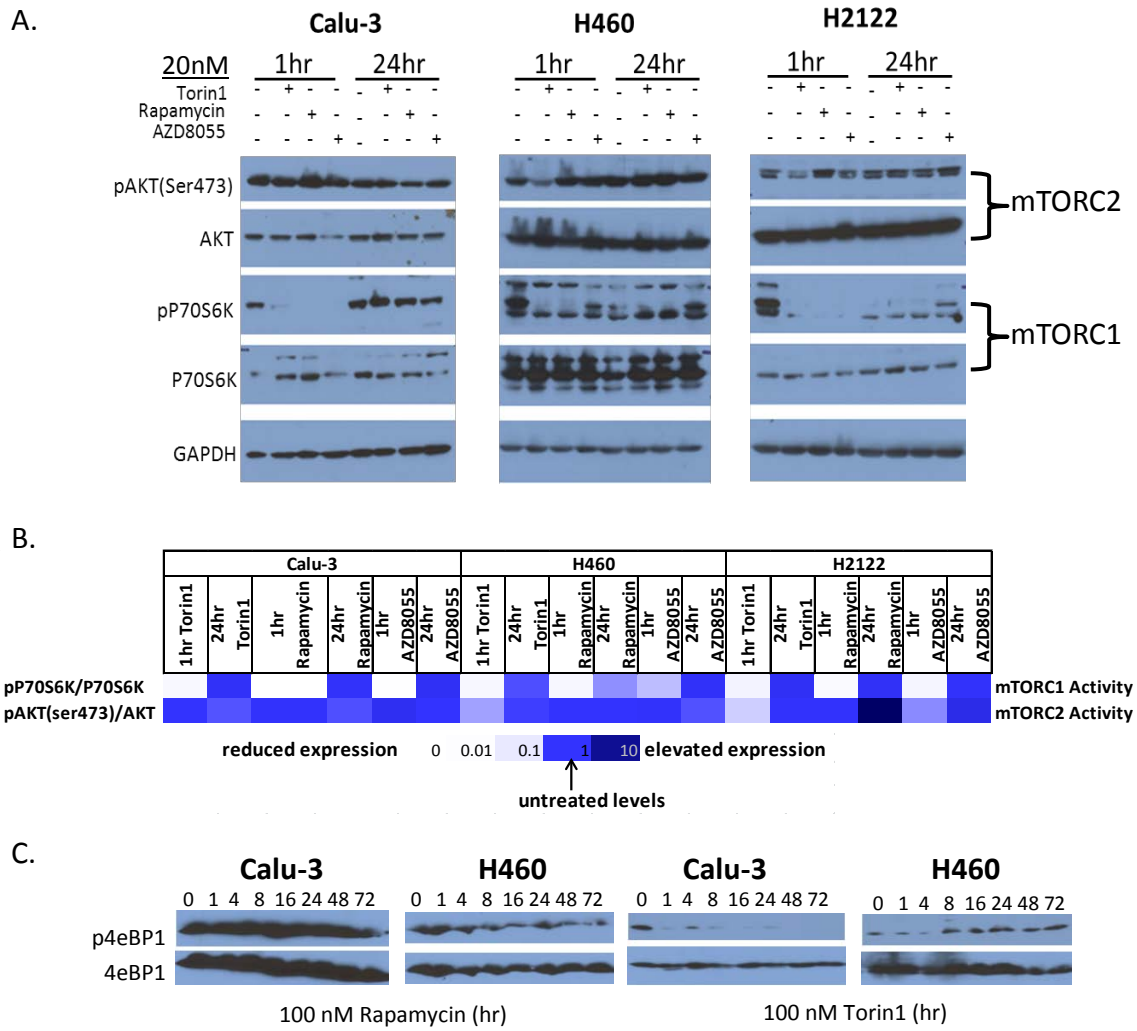


Figure 3.4: Effect of Drug Treatments on mTOR Activity A. Immunoblot of pAKT (ser473) as a readout of mTORC2 activity and pP70S6K as a readout of mTORC1 activity in response to mTOR inhibitor treatment. B. Quantification of band intensities of the ratio of phospho- to total-P70S6K and AKT. C. Immunoblot for phospho- and total 4eBP1 following drug treatment.

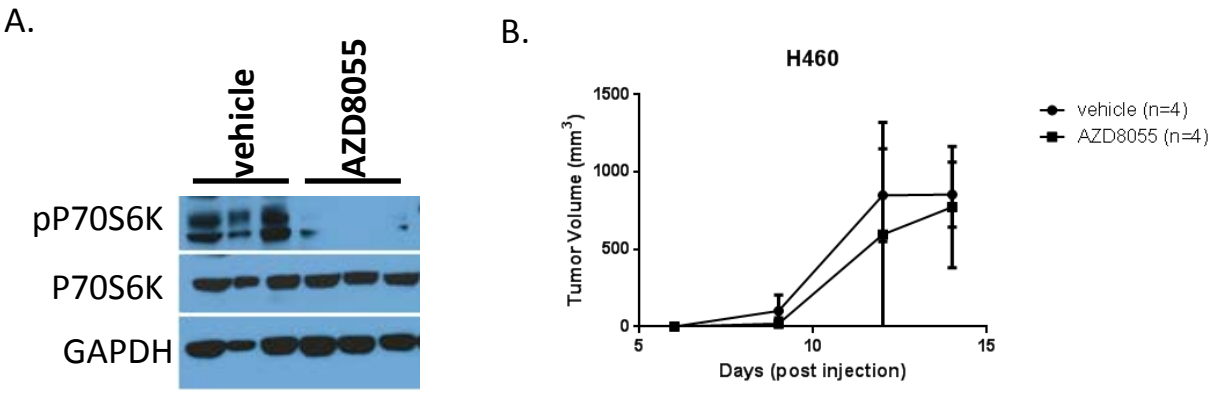


Figure 3.5: *In vivo* Efficacy of AZD8055 in NSCLC Sub-Cutaneous Tumors
A. AZD8055 Inhibits mTORC1 Activity of P70S6K in resistant sub-cutaneous tumors (H460). B. Tumor Volume of H460 sub-cutaneous tumor in mice treated with 10mg/kg/day AZD8055.

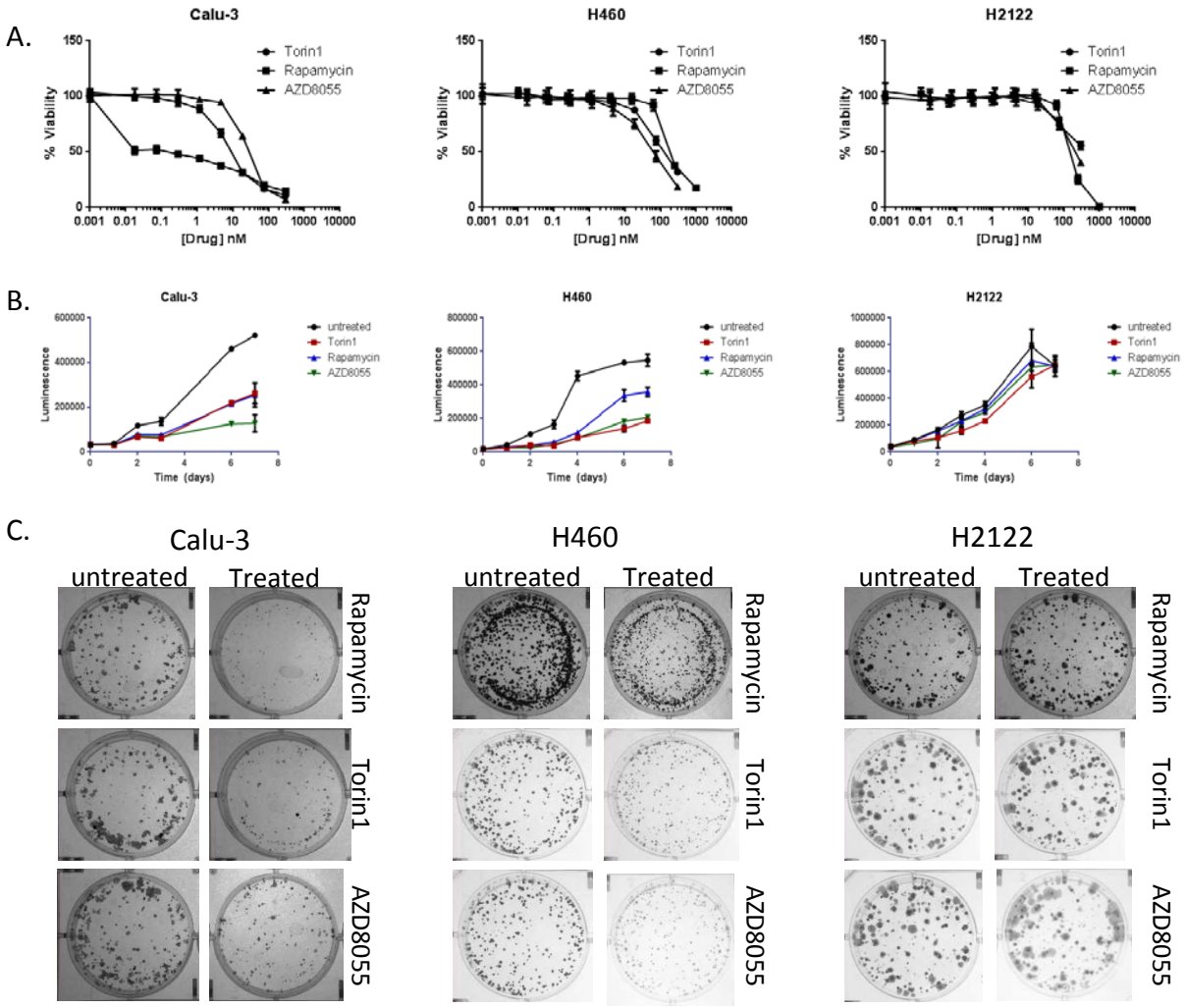


Figure 3.6: mTOR Inhibition Reduces Growth Rate and Colony Size in Sensitive Cell Lines
A. Dose response curves for a sensitive (Calu-3), intermediate (H460), and resistant (H2122) cell line to three mTOR inhibitors. B. Measurement of growth rate of cell lines untreated and treated with mTOR inhibitors C. Effect of each mTOR inhibitor (4.7nM Rapamycin and Torin1, 18.8nM AZD8055) on colony size.

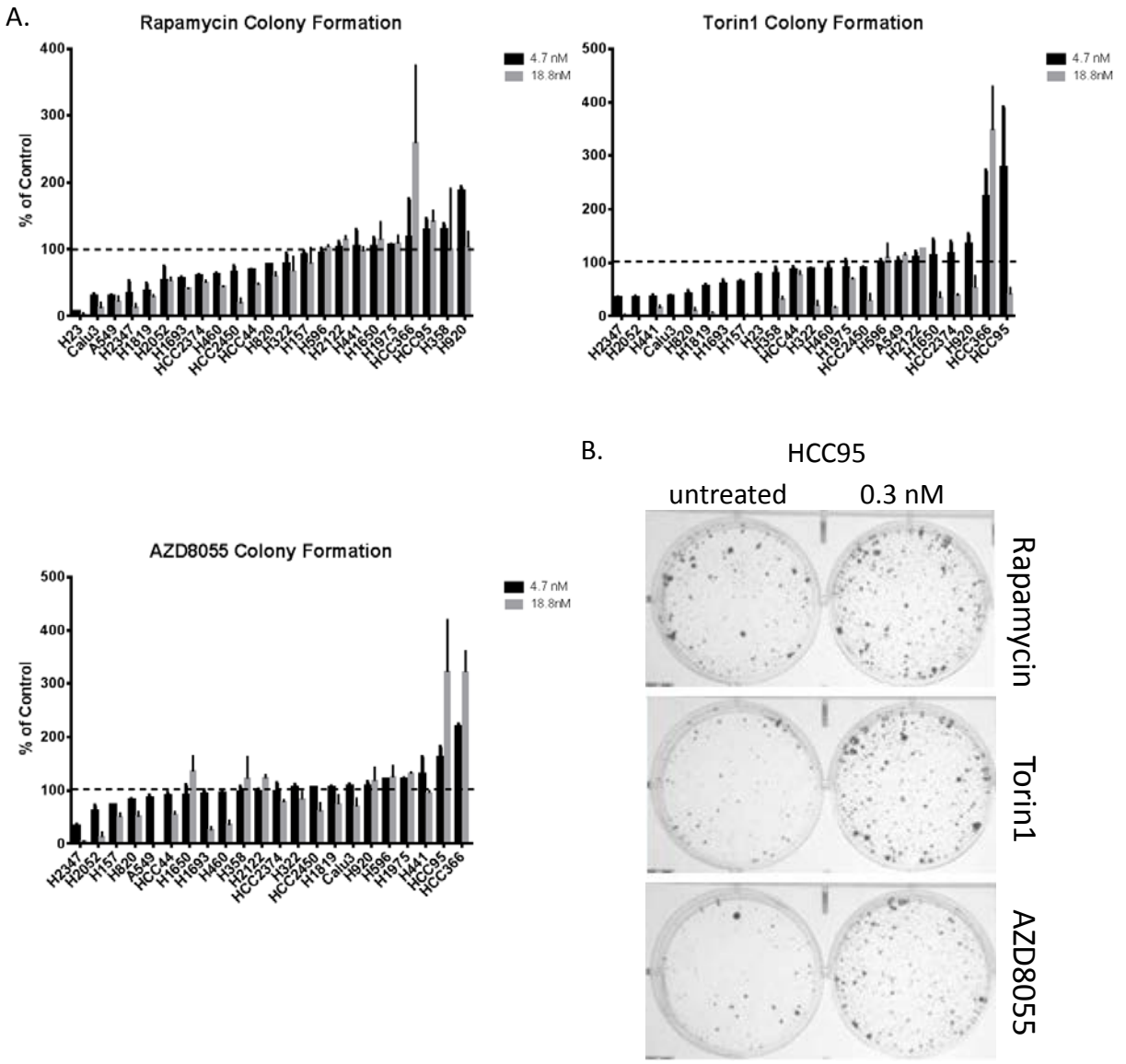


Figure 3.7: Effect of mTOR Inhibitors on Colony Formation in NSCLC
A. Number of large colonies of NSCLC cell lines treated with 4.7 and 18.9nM mTOR inhibitor relative to untreated control B. In some resistant cell lines (HCC95 shown), low doses of mTOR inhibitors stimulated growth.

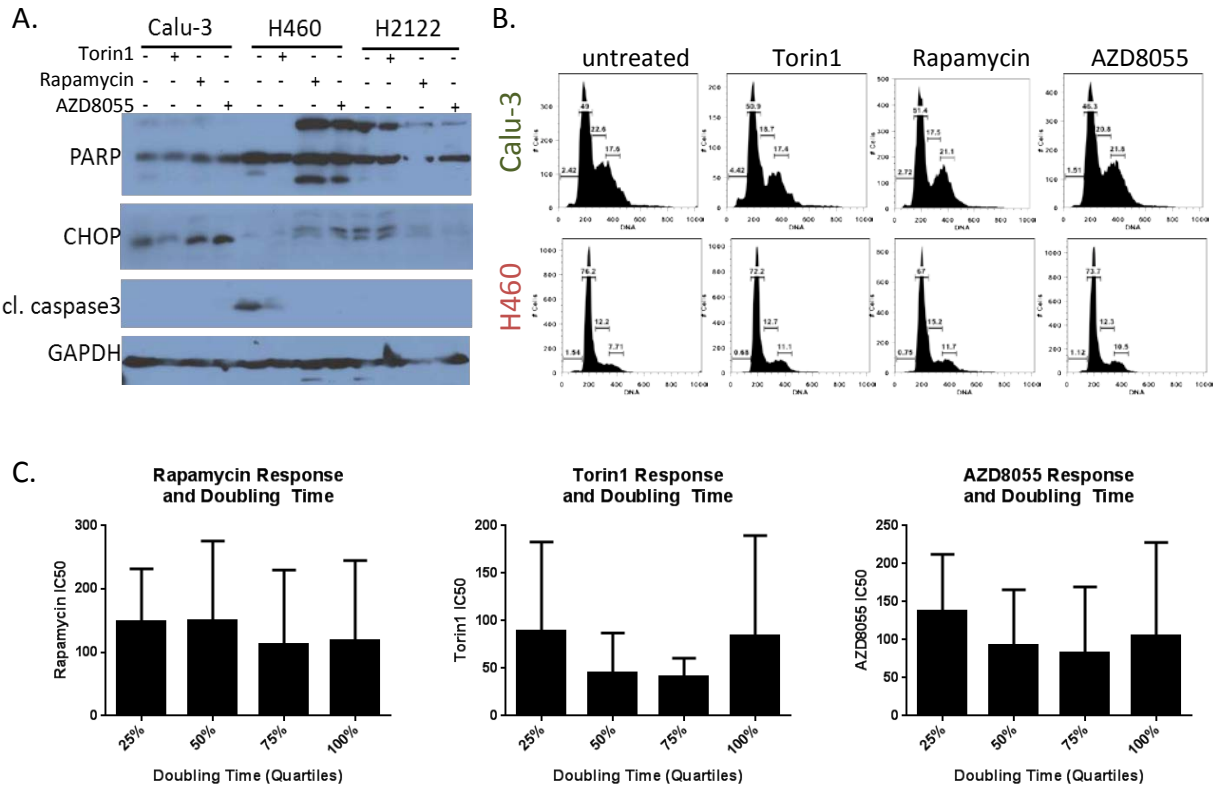


Figure 3.8: mTOR Inhibition Does Not Significantly Induce Apoptosis, Alter the Cell Cycle or Correlate with Growth Rate

A. Immunoblot for apoptotic markers. B. Determination of cell cycle profile using flow cytometry to measure DNA content. C. Doubling time for each cell line was determined and divided into quartiles. There was no relationship between growth rate and response to mTOR inhibitors.

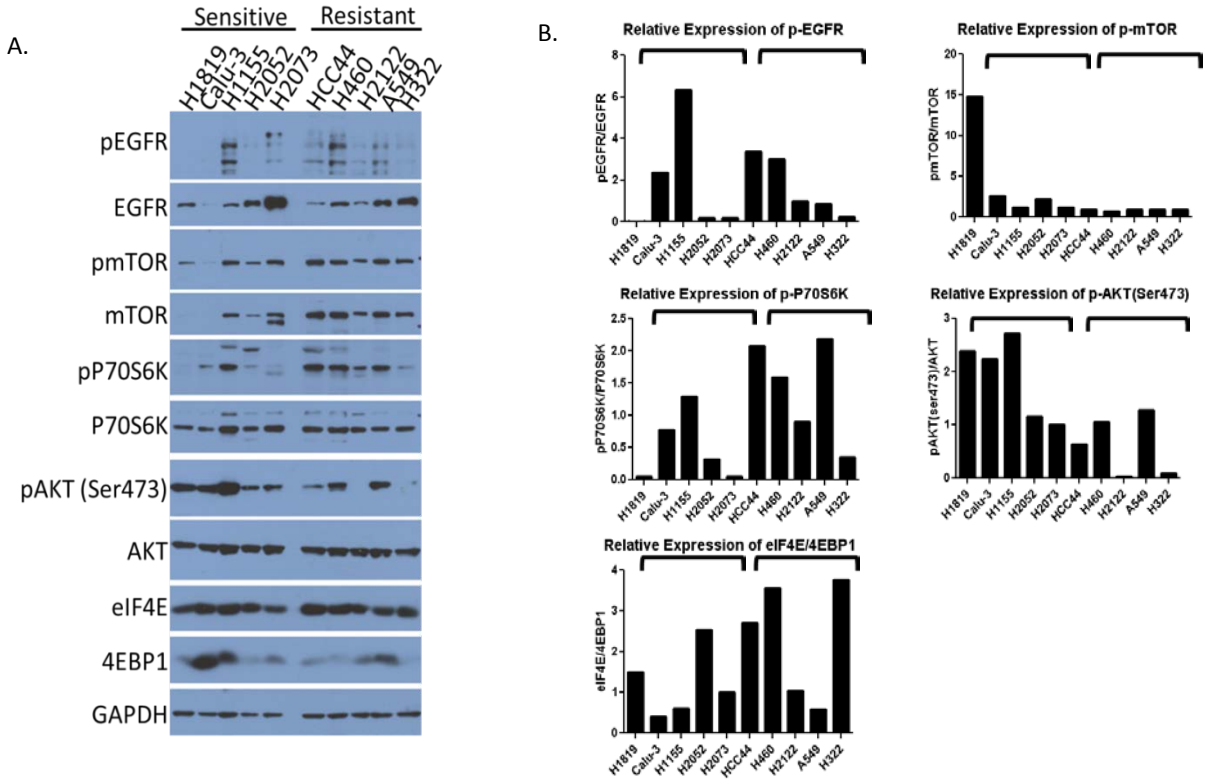


Figure 3.9: Basal Levels of Activation of mTOR Pathway Signaling Does Not Correlate with Response to mTOR Inhibition A. Immunobot for phosphorylated and total EGFR, mTOR, P70S6K, AKT, and eIF4E and 4EBP1. B. Quantification of band intensity for the ratio of phospho to total EGFR, mTOR, P70S6K, AKT, and eIF4E/4EBP1

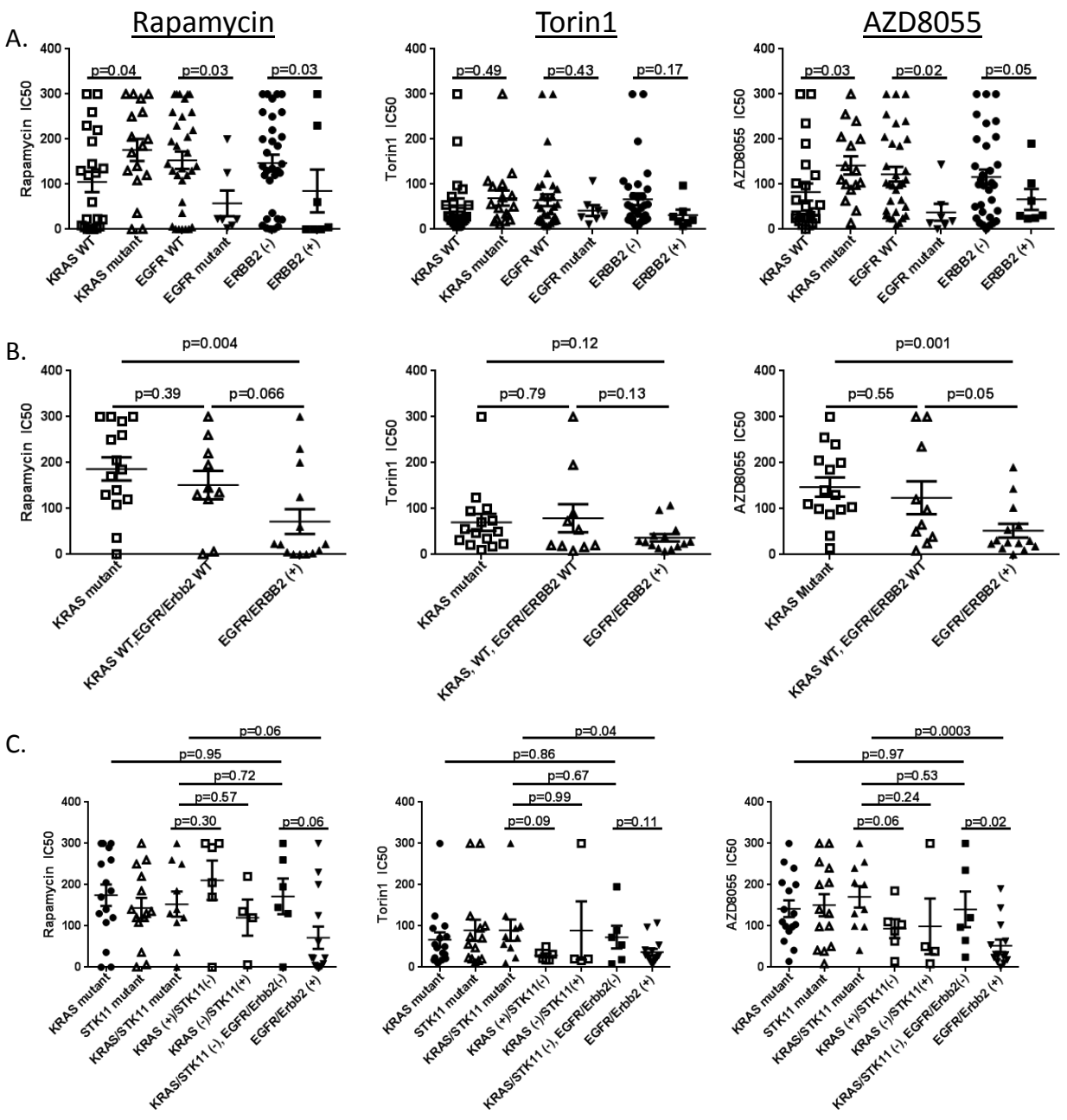


Figure 3.10: Comparison of Oncogenotypes, Copy Number, and Drug Response
A. Comparison of IC50s of Kras, EGFR mutants vs wild-type (WT) and lines with (+) and without (-) ERBB2 amplifications. B. Comparison of IC50s of KRAS mutants, EGFR/ERBB2 altered lines vs those WT for these alterations. C. Comparison of IC50s of lines with KRAS, STK11, EGFR/ERBB2 alterations.

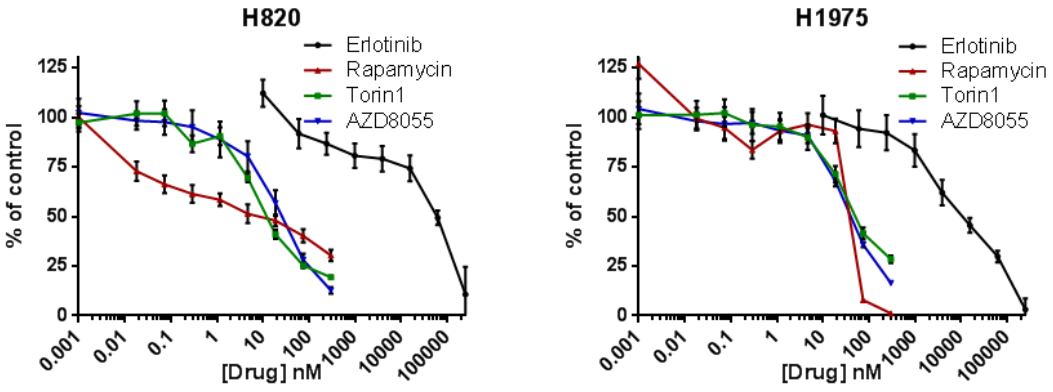


Figure 3.11: Erlotinib Resistant EGFR Mutant Cell Lines Remain Sensitive to mTOR Inhibition
Dose response curves for H820 (T790M, MET amplification) and H1975 (T790M) to erlotinib, rapamycin, Torin1, and AZD8055.

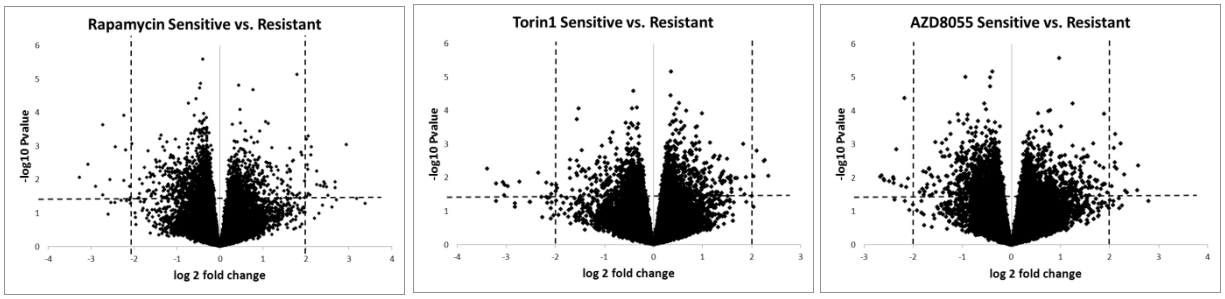


Figure 3.12: Differences in Gene Expression Between mTOR Inhibitor Sensitive and Resistant Cell Lines
Volcano plots of log2 ratio of expression differences between mTOR inhibitor sensitive and resistant cell lines and –log10 of the p-values. Dots in the upper left-most and right-most represent genes which are significantly down- and up-regulated in sensitive vs. resistant lines, respectively.

Table 3.3: Log Ratios of Gene Expression Between Sensitive and Resistant Cell Lines

Rapamycin Sensitive vs Resistant			
Gene ID	Symbol	log ratio	T-test P value
10153	PTGES	-3.27	0.009
19879	AKR1C4	-3.07	0.004
34303	AKR1C2	-2.90	0.016
16756	SOX21	-2.73	0.000
46883	AKR1C3	-2.73	0.030
35529	ALDH3A1	-2.56	0.010
40398	C10orf116	-2.54	0.050
2092	ABCC3	-2.45	0.050
16383	COL7A1	-2.44	0.001
12000	PTGS2	-2.30	0.042
6169	RUNX2	-2.24	0.000
24566		-2.24	0.011
2579	CFH	-2.20	0.001
36128	IRX3	-2.17	0.043
25437	S100A4	-2.10	0.016
33178	SOX2	-2.10	0.041
13897	MLPH	-2.08	0.035
21425	CFH	-2.05	0.001
15946	HOXB7	2.01	0.001
46935	IQCA1	2.02	0.032
47816	DNAJC22	2.03	0.004
1120	ARHGAP44	2.04	0.005
10776	ID2	2.06	0.001
21999	ARL14	2.06	0.026
616	CPVL	2.10	0.029
26468	VANGL2	2.12	0.002
8180	ID2	2.13	0.001
46209	TRNP1	2.25	0.014
30625	LRCH2	2.25	0.006
33631	TRO	2.34	0.015
27139	TMSB15B	2.41	0.016
857	TMSB15B	2.42	0.012
4643	CLIP3	2.44	0.014
12848	TMSB15B	2.48	0.017
9983	TRO	2.51	0.030
46988	CXCR4	2.68	0.012
11345	TMSB15A	2.68	0.018
43285	KCNS1	2.71	0.041
36747	C12orf75	2.94	0.001
3364	ALDH1A2	3.18	0.038

Torin1 Sensitive vs. Resistant			
Gene ID	Symbol	log ratio	T-test P value
42233	ALDH1A1	-3.39	0.006
19879	AKR1C4	-3.21	0.015
17608	CES1	-3.07	0.014
34236	AKR1B10	-3.04	0.034
46883	AKR1C3	-3.03	0.017
7136	ALDH1A1	-2.97	0.018
18440	AKR1B10	-2.73	0.014
2111	COL4A5	-2.35	0.007
3012	TSPAN7	-2.28	0.039
42203	BTBD11	-2.18	0.014
23353		-2.18	0.039
36973	CALB2	-2.16	0.041
16756	SOX21	-2.12	0.039
2081		-2.08	0.032
6223	NR0B1	-2.07	0.016
11704	C2orf55	2.00	0.036
12313	MAGEA4	2.04	0.009
22887	CCND2	2.10	0.002
4424	CX3CL1	2.25	0.003
30779	ISL1	2.27	0.003
41772	CADM1	2.34	0.009

AZD8055 Sensitive vs Resistant			
Gene ID	Symbol	log ratio	T-test P value
31366	GAGE12I	-2.67	0.009
6906	GAGE7	-2.65	0.008
15248	GAGE2B	-2.60	0.011
6821	GAGE6	-2.59	0.012
19879	AKR1C4	-2.49	0.013
12745	GAGE7	-2.41	0.009
44677	GAGE7	-2.40	0.014
48303	GAGE6	-2.40	0.011
34303	AKR1C2	-2.38	0.045
3012	TSPAN7	-2.34	0.001
440	GAGE7	-2.24	0.017
9794	POPDC3	-2.18	0.000
24006	CSAG1	-2.17	0.050
44690	CSAG2	-2.14	0.019
17608	CES1	-2.11	0.011
47950	PRKCDBP	2.01	0.047
42396	SLC2A10	2.04	0.018
6200	ERP27	2.08	0.049
24895	ITGA2	2.09	0.001
27599	RASSF10	2.11	0.003
12085	C19orf46	2.11	0.005
12490	CTSH	2.12	0.000
3971	COBL	2.14	0.005
46757	PRSS8	2.19	0.041
46894	LIPG	2.23	0.001
41902	DNAJA4	2.30	0.008
7531	LAD1	2.32	0.024
15716	SFTA2	2.35	0.028
24300	SPOCK2	2.56	0.024
14612	SPINK1	2.58	0.004
47581	CEACAM6	2.79	0.049

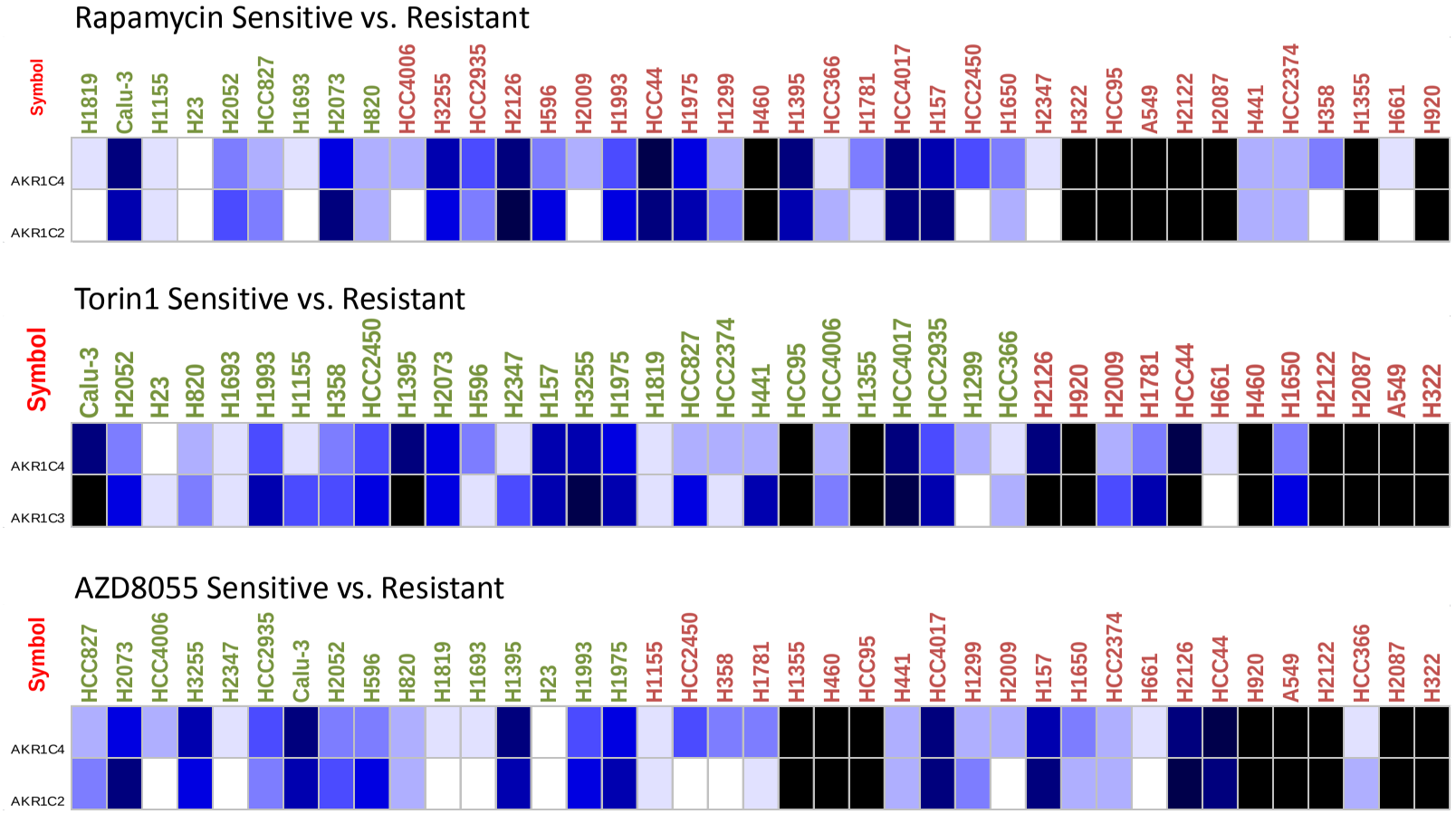


Figure 3.13 : Relative Expression of Most Differentially Expressed Genes Green text indicates sensitivity to the mTOR inhibitor, while red text indicates resistance. The darker the blue color correlates with higher relative expression of the indicated genes. In these examples, the average expression is significantly higher in resistant lines, however there is significant variation of expression in both sensitive and resistant cell lines.

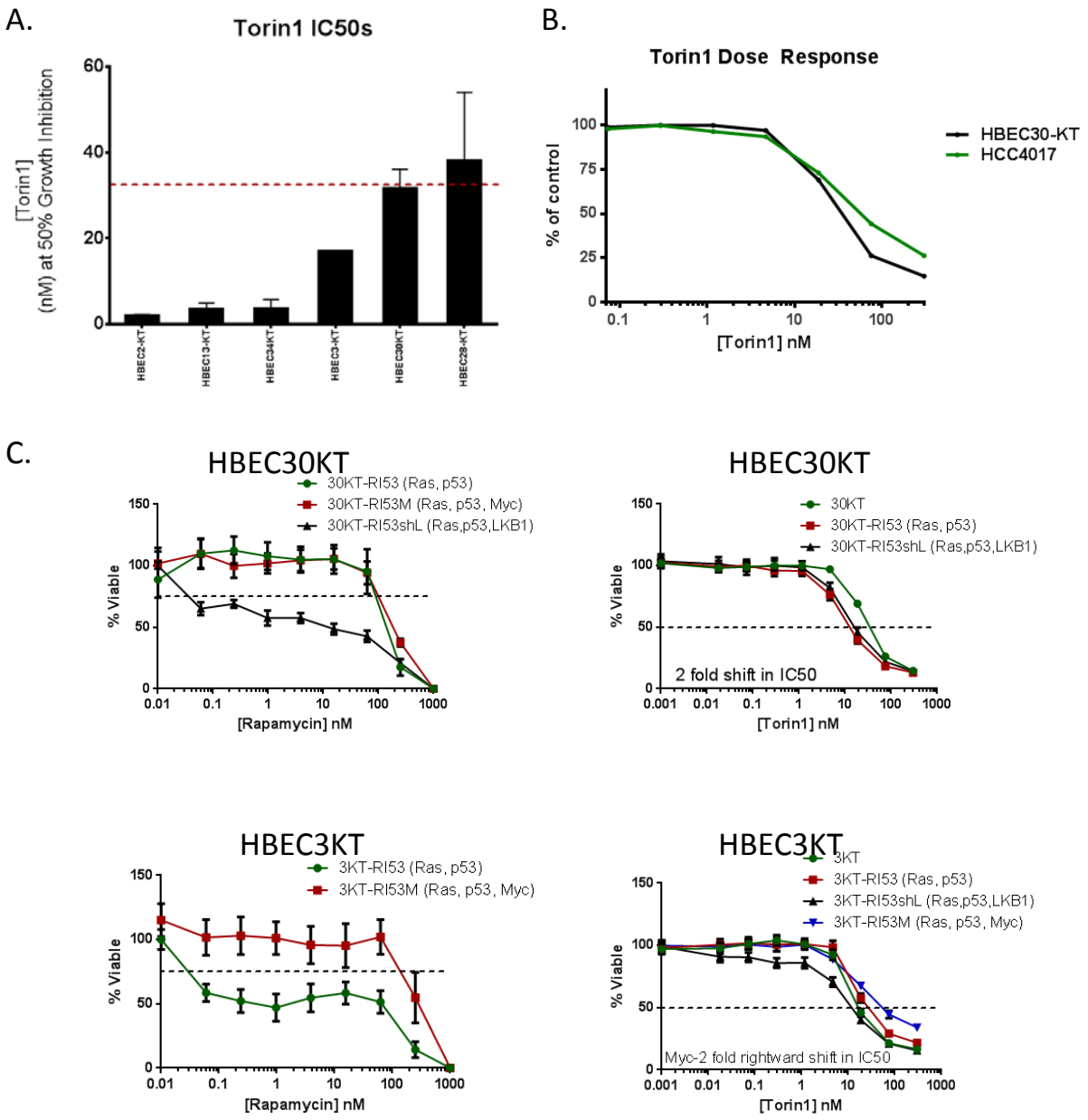


Figure 3.14: Response of Normal and Progressed HBECs to mTOR Inhibition
A. IC50 of HBECs to TorIn1. Dashed red line indicates the median IC50 of NSCLC Cell Lines. B. Comparison of dose response of HBEC-30KT and the matched tumor cell line HCC4017. C. Dose responses of HBEC30-KT and 3-KT with various oncogenic manipulations.

Chapter 4: Genetic Screen for mTOR Related Vulnerabilities

I. Abstract

While the presence of a mutation and/or gene amplification is often associated with dependence on expression of the altered gene, it is also possible that cancer cells could be dependent on expression of genes which are not easily identified by genetic abnormalities or expression differences. Furthermore, oncogenic mutations may result in synthetic lethality with certain gene knockdowns. In order to identify acquired vulnerabilities to components of mTOR signaling, we performed a genetic screen composed of a library of siRNAs designed to target various components of the mTOR pathway, autophagy genes, and glucose metabolism. A set of 54 genes were knocked down individually in a panel of 26 NSCLC cell lines, and the effect on viability determined after 5 days. These siRNAs produced a heterogeneous response in the NSCLC cell lines. There was not a significant correlation with response to siRNA knockdown and pharmacologic inhibition of the same targets. Only in a limited number of cases did the presence of a mutation in an oncogene or a tumor suppressor correlate with a kill phenotype for a knockdown. Off-target effects of siRNAs have always been a caveat of RNAi technology and necessitate additional validation methods including verifying the knockdown, validating the phenotype using multiple independent siRNAs and rescue experiments. These additional validation methods are still required in order to validate the hits in the screen.

II. Introduction

Genetic Probe to Assess Vulnerabilities Related to mTOR Signaling

The previous Chapter described a pharmacologic screen to characterize NSCLC cell vulnerabilities to mTOR inhibition. mTOR kinase is central to a vast network of oncogenic signals, a number of components of which may be amenable to targeting with drugs. In order to expand the characterization

of vulnerabilities of NSCLC cells related to a variety of components of mTOR signaling, including metabolism and autophagy, we decided to perform a genetic RNA interference screen to individually knockdown mTOR related genes.

Gene silencing using RNA interference is also being employed to identify potential gene-specific therapeutic opportunities. This implementation of the technology is of particular importance for cancer targeted therapy development. RNAi screens can be used to identify additional cancer drivers that may be exploited for cancer therapy. Often these acquired vulnerabilities are associated with the presence of a mutation or gene amplification of the oncogene, typically an activated kinase that transduces survival signals or suppresses anti-proliferative signals. Cancer cells become dependent on the continued expression of these oncogenes because they lose the ability to activate compensatory signaling, and therefore, experience oncogene addiction (Sharma and Settleman 2010). A number of molecular vulnerabilities acquired by cancer cells but not surrounding normal cells have already been identified, and are being targeted using pharmacologic agents in the clinic. Understanding the effects on oncogene silencing on the proliferation rate and survival in cancer cells enables researchers to identify potential new drug targets.

RNA Interference and Gene Silencing Mechanism

RNA interference (RNAi) is a mechanism for the inhibition of gene expression. This mechanism is an evolutionarily conserved process used in primitive organisms to protect the genome against viruses and to regulate developmental processes (Dykxhoorn and Lieberman 2005). In RNAi, gene expression is silenced by cleaving mRNAs in a sequence specific manner. The target mRNAs contain homologous or identical sequences to short oligonucleotides that are introduced into the cell. Dykxhoorn and Lieberman describe the silencing mechanism in RNAi which was elucidated in Drosophila, and is summarized here (Dykxhoorn and Lieberman 2005). Short interfering RNAs (siRNAs) are RNAi effector

molecules of about 21-24 nucleotides (nt) of double stranded RNA (dsRNA). Naturally occurring siRNAs are produced by the cleavage of long dsRNAs by the Dicer family of RNase III-like enzymes yielding a 21-23 nt dsRNA duplex with symmetric 2- to 3-nt 3' overhangs. These or chemically synthesized siRNAs introduced into the cells get incorporated into the multi-subunit RNA-induced silencing complex (RISC). The duplexed siRNA is unwound starting at the 5' terminus and the anti-sense or guide strand is taken up by activated RISC (holoRISC) and initiates recognition of the target mRNA. A component of the RISC, Argonaute 2 (Ago2 or Slicer), acts as the endonuclease that cleaves the mRNA when the RNA in the RISC is complementary to a sequence in the target RNA. In addition to chemically synthesized siRNAs, target genes can be silenced using DNA-based vector systems that encode short hairpin RNAs (shRNAs). These are introduced into the cells using viral transduction, become incorporated into the host genome, and express siRNA precursors that are processed into competent siRNAs and allow for stable suppression of gene expression (Paddison, Caudy et al. 2002).

Gene Silencing as a Tool for Understanding Gene Function and Development of Gene-Specific Therapeutics

In 2001 Elbashir et al. reported for the first time that siRNA mediated gene silencing could be used in mammalian cells (Elbashir, Harborth et al. 2001). Thus, the potential for this technology as a tool for biomedical research and therapy was propelled into the spotlight. Knockdown of the expression of a specific gene of interest is a valuable tool for reverse genetics studies. The function of the target gene can be characterized based on the effects of its disruption. This technology, in combination with sequencing of the human genome, allows researchers the potential to silence any gene in the genome. Knockdown of genes encoding tubulin, DNA methyltransferases, lamin A, Cadherin, Cdc20 and Cdk2 are some early examples where RNAi-based gene silencing was used to probe for gene function in mammalian cells (reviewed in (Lieberman, Song et al. 2003).

Similar to the concept of oncogene addiction is that of "lineage addiction." This model explains the requirement of lineage specific genes in tumor cells from a particular cell lineage for proliferation, but which cause differentiation in normal cells (Sharma and Settleman 2010). Microphthalmia-associated transcription factor (MITF) is the best described lineage oncogene. MITF is amplified in a subset of melanomas and together with oncogenic BRAF regulates melanoma proliferation. Overexpression of MITF in primary melanocytes induces cell cycle arrest, while it induces proliferation in melanoma (Wellbrock and Marais 2005). Inhibition of MITF and BRAF significantly reduces melanoma cell proliferation (Kido, Sumimoto et al. 2009). RNAi screens will be useful in identifying this class of oncogene addiction and hopefully isolate targetable vulnerabilities of subsets of cancer.

Occasionally the knockdown of a gene may only be lethal to the cancer cell in the presence of a mutated oncogene. This effect is known as synthetic lethality. Synthetic lethality refers to the phenomenon where the mutation of either one of two genes has no effect on cell viability, while simultaneous mutation/inhibition results in lethality (Kaelin 2005). Exploiting synthetic lethal effects by identifying which genes are synthetically lethal to cancer-causing mutations will make it possible to target these genes to specifically kill the cancer cells that harbor the mutations without affecting normal cells. Furthermore, targeting a gene that is synthetically lethal with the loss of a tumor suppressor is an approach that allows for the exploitation of loss of function mutations frequently found in cancer that are not amenable to direct targeting with a drug (Kaelin 2005). RNAi screens can identify synthetic lethal interactions with known oncogenes when screened with cancer cell line panels where the driving oncogenes and mutated tumor suppressors are already characterized. Cell lines can be classified into subsets that contain matching oncogenotypes and then classified based on their response to particular gene knockdowns. Any knockdown that appears to specifically inhibit viability in these subsets may be synthetically lethal. However, additional validation will be required to confirm the synthetic lethal effect and its potential therapeutic implications.

Cancer cells also have the potential to be addicted to the expression of a gene that is not considered an oncogene. This is referred to as "non-oncogene addiction" and may also be useful to identify potential therapeutic targets. A non-oncogene could be anything that is hyperactive in cancer cells and supports their growth, including genes involved in DNA damage, replication, metabolic and oxidative stress, angiogenesis, stromal signaling and many more (Luo, Solimini et al. 2009, Sharma and Settleman 2010). These non-oncogenes do not undergo oncogenic mutation or genomic alteration in cancer cells and are difficult to identify without functionally characterizing the effect of their knockdown.

While RNAi screening is vital for the identification of cancer acquired vulnerabilities and potential drug targets, there is also the possibility of using RNA-based gene silencing itself as a new class of small molecule therapeutics. The ability to use RNAi as a therapeutic has unlimited potential as all cells are thought to contain the machinery to employ RNAi and any gene is a potential target (Dykxhoorn and Lieberman 2005). In addition to pervasive applicability, ease of synthesis and low cost of production make this class of therapeutics very attractive. A primary hurdle for RNAi-based therapies however is the difficulty in delivery of the therapeutic into the tumor cells (Dykxhoorn and Lieberman 2005). siRNAs are unable to cross the mammalian cell membrane without the addition of transfection reagents. Strategies to deliver siRNAs *in vivo* continue to be under investigation but thus far have not lead to a successful solution.

Goals of siRNA Screen

In the present study we describe an RNAi screen designed to identify acquired vulnerabilities of genes related to mTOR signaling, autophagy, and glucose metabolism. We screened a panel of NSCLC cell lines for the effect on growth and viability of individual gene silencing using a library of siRNAs targeting 54 genes related to these pathways. This genetic screen is an additional method to probe for cancer cell dependence on mTOR signaling. Knockdown of mTOR or various components of mTORC1

and 2 will allow for characterization of varying dependencies on either of these two complexes, separately or together. The effect of the knockdowns of these complexes can be compared to pharmacologic inhibition using mTORC1 or dual mTORC1/2 targeting drugs examined in Chapter 3.

While a few known mutated oncogenes were silenced in the screen, the majority of targets are not frequently mutated in cancer. The goal of this screen was to ascertain whether any of these genes represented non-oncogene addictions in subsets of lung cancer cell lines, which may signify novel drug targets. We also sought to characterize these subsets of lung cancer lines with similar siRNA responses based on additional molecular and phenotypic classifications including gene expression, mutation, or drug responses, which may help identify potential biomarkers that predict response to knockdown of a particular gene. By characterizing the siRNA response phenotype of cell lines that share a specific oncogenotype, we may also be able to find genes whose knockdown results in a synthetic lethal effect with a particular oncogenic mutation.

Setup of Screen and Analysis of Results

The RNAi screen used in this study individually knocked down a panel of 54 genes related to mTOR signaling, autophagy, or glucose metabolism (see Figure 4.1). Pools of 4 siRNAs with unique sequences but designed to target the same mRNA were used to knockdown each gene (validated siRNAs purchased from Dharmacon). Pooled siRNAs were used in order to ensure successful mRNA targeting and loss of target gene expression. The final concentration of siRNA in each reaction was 20nM. Each siRNA pool was contained in 1 well of a 96-well plate. The siRNAs were incorporated into the cells using reverse transfection mediated by a lipid transfection reagent (Lipofectamine2000, Invitrogen). On the fifth day following the transfection, the effect of the knockdown on viability was determined by measuring the relative number of viable cells using the spectrophotometric MTS assay described in the Methods section. A positive control siRNA (targeting PLK) was used to validate a successful transfection

with a known toxic effect. As a negative control, a scrambled version of the toxic oligonucleotide was used. This siRNA doesn't target any sequence found in the genome and should have no phenotypic effect on viability. The viability following knockdown of the test siRNAs were normalized to the negative control (scrambled) siRNA.

Optimal transfection conditions vary between the cell lines. In order to obtain the greatest knockdown, these optimal transfection conditions were determined prior to performing the screen. Variables including the amount of transfection reagent and the number of cells were tested in transfection optimization assays. The majority of NSCLC cell lines were optimized by Ryan Carstens for a previous RNAi screen. A few additional cell lines were optimized for this study.

Two measures were calculated to reveal "hits" in the screen. The first measure is a simple calculation of the percent viability following each knockdown relative to the negative control. A reduction in viability greater than 40% is considered a significant growth inhibitory effect, while a relative viability of greater than 120% can be considered a significant growth stimulatory effect. Secondly, a Z-score is calculated for each siRNA pool. This value related to the deviation from the average responses to the siRNA panel of a particular cell line, based on a normal distribution of responses. The Z-score is calculated using the formula,

$$Z_x = \frac{x - \mu_x}{\sigma_x}$$

Where x is the siRNA value, μ is the mean response, and σ is the standard deviation. The Z-score for most siRNAs will fall in the range of -1 to 1, indicating that the cell line response to their knockdown was close to the average response. siRNAs that produce a Z-score less than -1 for a particular cell line indicate that there was a significant growth inhibitory effect. A positive Z-score greater than 1 indicates that there was a growth stimulatory effect.

This chapter will summarize the preliminary results from the siRNA screen. The methods described here provide a valuable tool to characterize NSCLC cell lines for their functional response to knockdown of genes related to mTOR signaling, autophagy, or glucose metabolism. Additional studies will be required in order to determine whether any hits described here will be useful therapeutic targets.

III. Results

NSCLC Cell Lines Display a Heterogeneous Response to Knockdown of 54 mTOR/Autophagy/Metabolism Related Genes

Knockdown of the 54 genes related to mTOR signaling, autophagy, or metabolism yielded a heterogeneous effect on growth in the NSCLC cell line panel (Figure 4.1). With the exception of the positive control (toxic oligo, TOX), no siRNA pool inhibited the growth of all the cancer cell lines tested. Not surprisingly, knockdown of two metabolic genes essential for energy production, glutaminase (GLS) and hexokinase2 (HK2) produced a broad growth inhibitory phenotype in the majority of the lung cancer cell lines with a significantly negative Z-score in 84% and 70% of cell lines, respectively. Among the other broad growth inhibitors were siRNAs targeting MAPKAP1, G6PC3, SLC2a1/4, IRS1 and AKT3. The majority of the siRNAs in the screen divided the cell lines into three groups, those that were growth inhibited, those that had no effect on viability following knockdown, and those that had a growth stimulatory effect following the same knockdown. siRNAs targeting SGK1, DEPTOR, SLC5a2, and GSK3β stimulate growth. DEPTOR is a negative regulator of the mTOR complexes. Its knockdown is expected to relieve the inhibition of mTOR kinase activity and thus permit the propagation of mTOR dependent pro-growth signals. Therefore the broad growth phenotype seen with its knockdown is anticipated. GSK3β negatively regulates glucose homeostasis and Wnt signaling by phosphorylating a number of

substrates. The growth phenotype produced by its knockdown can be explained due to the release of inhibition of these important signaling pathways. The broad growth stimulatory effects observed with the other siRNAs are not as easily explained.

Cluster analysis was performed to determine the similarity of growth responses in the cell lines among the panel of siRNA pools (Figure 4.2). In general the response pattern for each siRNA target was unique. Even among siRNAs targeting genes whose products would be expected to have related downstream effects there was very little similarity in responses. The closest similarity in response of the cell lines was to the siRNA pools targeting GTPase Rheb, and ubiquitin ligase NEDD4L. It is unclear why these knockdowns would behave the most similarly in the cell line panel. These two proteins are quite dissimilar in their cellular functions. Rheb activates the kinase activity of mTORC1, while NEDD4L inhibits the cell surface localization of several glucose transporters downstream of mTORC2. Therefore the fact that these siRNAs cluster together is not likely to be due to functional similarity of their knockdown in the cells.

The cell lines were also analyzed by cluster analysis to determine how similarly they responded to the siRNA knockdowns and whether any predictable subsets of cell lines clustered together. Again, each cell line responded uniquely to the siRNA panel. Similarity in common oncogenotypes did not result in similar response to siRNA knockdown. Two isogenic cell lines pairs were tested for their responses to the siRNAs in the screen. H1693 and H1819 are cell lines derived at separate times from the same patient, the former from a lymph node metastasis prior to treatment, while the latter is also derived from a lymph node metastasis after the patient was given chemotherapy. This pair of cell lines clustered together based on their similarity of response to the knockdowns. The other pair of cell lines consists of H1993, which was derived from a lymph node metastasis prior to treatment, and H2073, which was derived from the primary lung tumor following chemotherapy. This pair of cell lines did not

display similar response phenotypes to the siRNA knockdowns, possibly due to variations in vulnerabilities acquired during metastatic progression.

Knockdown of Glucose Metabolism Genes

Ralph DeBerardinis' laboratory has characterized the NSCLC cell lines according to the activity of these metabolic pathways and has determined that various subsets of metabolic phenotypes exist. In the present study, we wanted to determine whether there were subsets of cancer cell lines that were dependent on various components of these metabolic pathways. Eleven siRNA pools in the screen targeted genes involved in glucose and glutamine metabolism. As mentioned previously, siRNA pools targeting GLS, HK2, and G6PC3 were broadly toxic in the majority of NSCLC cell lines. GLS (glutaminase) catalyzes the hydrolysis of glutamine to glutamate and ammonia and has an essential role for generating energy metabolism (Gallagher, Kettunen et al. 2008). Knockdown of this gene failed to meet the cutoff ($Z<-0.7$) for a significant growth inhibitory effect in 4 of the NSCLC cell lines tested. However, in 1 cell line the Z-score just barely missed the cutoff with a Z-score of -0.69, and two other cell lines were significantly growth inhibited by knockdown of the related gene GLS2. HK2 is responsible for the first step in most glucose metabolism pathways which is to phosphorylate glucose to produce glucose-6-phosphate (Robey and Hay 2006). It has been suggested that this gene is involved the increased rate of glycolysis in cancer cells (Moreno-Sanchez, Marin-Hernandez et al. 2014). Two of the 8 cell lines that weren't inhibited by knockdown of this gene were affected by knockdown of the related protein GCK. G6PC3 encodes the catalytic subunit of glucose-6-phosphatase that reverses glucose phosphorylation. This enzyme catalyzes the hydrolysis of glucose-6-phosphate to glucose in the last step of gluconeogenesis (Guionie, Clottes et al. 2003). These metabolic genes are important genes for a variety of tissues, and therefore may not be ideal targets due to the possibility of broad effects in normal cells.

However, additional tests comparing the growth effect on cancer versus normal cells may clarify the extent of this potential problem.

The siRNA pools designed to target GPI, GCK, GYS1, GLS2 and PC were toxic in about 30% of the NSCLC cell lines tested. There was some, but not complete overlap in the cell lines that were growth inhibited by knockdown of these metabolism genes. The siRNA pools targeting PKM2, GSK3β, and PGLS were not very toxic to the NSCLC cell lines (Figure 4.1).

Knockdown of Autophagy Genes

We also wanted to know whether there were subsets of cancer cell lines that were vulnerable to knockdown of autophagy related genes. Three siRNA pools were tested against genes important for autophagy activation and autophagosome formation (ATG7, ULK1, and BECN1). NSCLC cell lines were not sensitive to the siRNA pools designed to target these genes. In only a single cell line, Calu-3, the siRNA pool targeting ATG7 was toxic (Figure 4.2). However, this cell line was not sensitive to knockdown of the other autophagy genes. These results suggest that autophagy is not required in NSCLC cell lines to maintain viability.

Sensitivity to Knockdown of mTORC1 vs. mTORC2

Another goal of this siRNA screen was to determine whether NSCLC cell lines exhibited variation in their dependence on mTORC1 signaling versus mTORC2 signaling or the combination by examining the differences in response to raptor, rictor, or mTOR knockdown. Two cell lines (H1395 and H1819) were significantly growth inhibited following knockdown of raptor but had no effect following knockdown of rictor, suggesting a dependence on mTORC1 but not mTORC2. Two other cell lines (H1975 and H1693) were significantly growth inhibited by knockdown or rictor but not raptor, indicating dependence on mTORC2 but not mTORC1. In total, only five cell lines (Calu-3, H2347, H1975, H1385,

and H1693) were significantly growth inhibited by knockdown of mTOR. The first two of these (Calu-3 and H2347) were only sensitive to mTOR knockdown, but not raptor or rictor, suggesting that the function of either of the two mTOR complexes is sufficient to maintain growth, but losing both together leads to growth inhibition in a synthetically lethal manner. In H1819, raptor knockdown inhibited growth of the cells, but mTOR knockdown did not. The expectation is that where either raptor or rictor knockdown have a phenotypic effect, the response to mTOR knockdown would match the phenotype. Additional validation experiments will be required to verify this result in H1819. If the phenotype proves to be reproducible, it could indicate a situation where the growth inhibitory effect caused by mTORC1 is reversed with the concurrent knockdown of mTORC2, or dependence on mTOR independent functions of raptor.

Fifty percent of the cell lines tested had no change in growth with knockdown of either mTORC1, mTORC2, or both together. One cell line, A549, was significantly growth stimulated (Z-score greater than 1.3) following knockdown of mTORC1 by siRNA targeting raptor. Five cell lines (H2073, HCC44, HCC4017, H1993, and HCC366) were significantly growth stimulated following rictor knockdown, and two (HCC366, and H2122) were significantly growth stimulated following knockdown of mTOR.

Examination of Synthetic Lethality with Known Oncogenes

Next we wanted to determine whether a growth inhibitory phenotype was due to a synthetic lethal effect with the presence of a known oncogene (Figure 4.3). We compared the siRNA responses of mutant versus wild-type for several common mutations in NSCLC. Three siRNA pools significantly ($p<0.05$) reduced the growth of KRAS mutant lines compared to KRAS WT lines, those designed to target SLC2A1, RRAGA, and TSC1. LKB1/STK11 mutant cell lines were more significantly growth inhibited by siRNAs targeting SLC2A4 and GLS2 compared to wild-type cell lines. The siRNAs designed to target SLC2A1 and Rbx1 were significantly more toxic to cells with co-occurring mutations in both KRAS and

LKB1. None of these knockdowns however were exclusively toxic to mutant cell lines as all also produced growth inhibitory effects in a subset of the WT cell lines. Furthermore, some cell lines containing these mutations were not affected by the knockdown, suggesting there is not a true synthetic lethal effect, or these cell lines have additional alterations that enable their survival despite gene depletion.

Knockdown vs. Pharmacologic Inhibition

Logically, genetic inhibition and pharmacologic inhibition of the same target should result in a similar phenotypic effect, and any cell line that is sensitive to mTOR inhibition using a drug ought to be similarly sensitive to mTOR knockdown. Rapamycin is an allosteric mTORC1 inhibitor described in Chapter 3. Theoretically, NSCLC cell line response to rapamycin would match the response to knockdown of the essential mTORC1 component, raptor. However, results from the siRNA screen indicate this is not the case, as there is no correlation between the response to raptor knockdown and rapamycin treatment. Torin1 and AZD8055 are mTOR kinase inhibitors. Responses to these drugs would be expected to be replicated by knockdown of mTOR. There was a significant positive correlation between drug response and mTOR knockdown response for both of these drugs, which was stronger for AZD8055 ($R= 0.68$, $p=0.006$ vs. 0.48, $p=6E-5$ for Torin1). These drugs also exhibited a stronger, but still very weak, correlation with mTORC1 knockdown compared to knockdown of mTORC2 as indicated by a positive correlation with drug response and raptor knockdown, and no significant correlation with rictor knockdown and drug response (Figure 4.4).

In addition to mTOR targeting drugs, many NSCLC cell lines have been previously screened for their response to drugs targeting other members of the pathway that are represented in the siRNA screen. These include drugs that target EGFR (erlotinib), AKT (MK2206), or PI3K (BEZ235, also inhibits

mTOR). However, response to these agents does not correlate with response to the siRNA pool with the same target (not shown).

Mutation of an mTOR Pathway Gene Does Not Correlate with Response to its Knockdown

Based on the current mutation data available on the NSCLC cell lines, 21 of the genes targeted by siRNAs used in this screen are mutated in at least 1 of the cell lines used in the screen (see Table 3.1). Mutation could indicate a loss of regulation and potentially lead to dependence on continued activity of the affected protein. Alternately, mutation could indicate loss of function, in which case siRNA knockdown would have no or minimal effect. The presence of a mutation of an mTOR pathway gene does not generally correlate with response to the siRNA pool targeting that gene. This includes known oncogenes PIK3CA and EGFR. Two cell lines tested have mutations in PIK3CA (H460, and H1975), but neither had any growth effect following knockdown of this gene. EGFR mutations were also represented by two cell lines, H1650 and H1975. Knockdown of EGFR only inhibited the growth of H1975. One cell line, HCC366 is known to have a mutation in IRS1 and was vulnerable to its knockdown. Twelve other cell lines were also vulnerable to knockdown of IRS1, half of which have wild-type IRS1, while the remaining 6 have not been evaluated for the presence of a mutation in this gene.

Response to Knockdown Does Not Correlate with Expression of the Target Gene

Next, we determined whether gene expression correlated with response to siRNA knockdown. We used microarray expression data from the NSCLC cell lines to determine whether response to siRNA knockdown correlated with the expression of the gene target. There were two examples of siRNAs where the growth inhibition correlated with lower expression, mTOR and TSC2. Though statistically significant, the correlation was not very strong ($R= 0.49$, $p=0.01$ and 0.45, $p=0.02$, respectively). In the case of mTOR, there is very minimal variation of expression across the panel of cell lines. There was only

one gene, PC, where high expression correlated with a growth inhibitory phenotype. Two microarray probes significantly correlated with siRNA response but the correlation coefficients were only -0.54, p=0.004 and -0.49, p=0.01 (Figure 4.5).

IV. Discussion

RNAi screens are a valuable tool for identifying potential new drug targets for the treatment of cancer. As a functional screen, these methods have the potential to identify non-oncogene addictions that would not be readily identifiable based on mutation or gene expression data. Synthetic lethal effects with known oncogenes may also be identified. Here we described the preliminary results of an siRNA screen intended to identify acquired vulnerabilities related to mTOR signaling, autophagy, and glucose metabolism in NSCLC. The heterogeneous responses to the siRNAs in the NSCLC cell lines indicate that subsets of lung cancers have varying acquired vulnerabilities, which may be identified and exploited using novel treatment strategies. Additional validation of promising targets determined by the screen may help to identify novel drug targets for subsets of tumors. The siRNA pools that inhibit growth in the majority of cancer cell lines or an identifiable subset of cell lines without affecting the growth of normal HBEC lines will be the most interesting for follow-up studies.

In siRNA screening studies, ideally each siRNA would efficiently knockdown its intended target in a specific manner, without affecting the expression of genes other than the target of interest. However, since the experimental implementation of RNAi screening, it has been a challenge to combine high specificity with high efficacy. RNAi screens have significant potential to yield false negative and false positive results, the former due to ineffective knockdown and the latter due to non-specific siRNAs (Jackson and Linsley 2004). More efficient siRNAs are now currently available which have been experimentally validated to knockdown the intended target during production, and optimistically reduce

the rate of false negative results. Overcoming the hurdle of identifying and eliminating false positives results is perhaps an even greater challenge.

It is now understood that siRNAs can regulate gene expression in a broader manner using a mechanism similar to that of microRNAs (miRNA). Nucleotides 2-7 of each siRNA strand are referred to as the "seed region". When the seed sequence is complementary to the 3'UTR of an off-target mRNA, the expression of this unintended target can be reduced. Variability in the frequency of the seed complements in the 3' UTR transcriptome likely determine the number of off-target mRNAs affected by seed-sequence based off-target effects, but it is likely that each siRNA could be affecting hundreds of unintended targets. As a result of these unexpected changes in gene expression, it will not be clear whether the observed phenotype of the knockdown is due to reduction of the intended target, an off-target gene, or the combined effects of a blend of on- and off-target genes.

Additional siRNA screens performed in our lab have indicated that false positives due to seed-based off-target effects are a prevalent problem. While many of the observed phenotypes may be due to off-target rather than on-target knockdowns, the RNAi screens have still succeeded in functionally classifying differences in vulnerabilities in subsets of NSCLC and other cell lines. With advances in *in vivo* delivery of RNAi agents, it may be possible in the future to exploit these functional differences for therapeutic benefit. This strategy however is not currently feasible in human patients. Alternately, it may be possible to further characterize the affected on- and off- target mRNAs and de-convolute the true cause of the phenotype to identify the actual potential drug target.

In the screen described in this study, each knockdown was performed using a pool of 4 unique siRNAs designed to reduce the expression of the same target gene. Since each siRNA is double stranded, up to eight unique seed sequences may be present, each with confounding off-target effects on gene expression. As a first step to try and deconvolute the observed phenotype each siRNA pool that was a

hit in the screen can be re-tested using individual siRNAs. This will reduce the number of seed sequences in each reaction to two. If the phenotype is recapitulated using multiple individual siRNAs that each have been confirmed to knockdown the intended target, this raises the likelihood that it is the result of an on-target effect. In the situation that only a single siRNA recapitulates the phenotype when all of the individual siRNAs efficiently knock down the target, the seeds present in the phenotype driving siRNA can be identified and tested in a number of ways.

A new method to further validate that the phenotype is due to a seed based off-target effect rather than an on-target knockdown, C911 mismatched controls, has recently been described (Buehler, Chen et al. 2012). These are siRNAs that are identical to the experimental siRNA being examined for on- and off-target based effects, except that bases 9-11 have been replaced with their complement. These bases seem to be the most important for regulating expression of on-target mRNAs; however any seed-based off-target effects should remain intact. Therefore, by testing a siRNA concurrently with its C911 mismatched control the true and false positives can be distinguished. These controls can also help identify the precise seed sequence from the double-stranded RNA that is responsible for the positive result. However, even once the seed sequence in question is identified, discovering the target or targets of the seed sequence that induces the growth inhibitory phenotype in the cancer cells will still be very difficult and time consuming. Microarray expression studies before and after introduction of the siRNA may help narrow down potential target genes by reducing it to only those genes whose expression is significantly reduced following transfection. Online databases that can predict potential targets by comparing complementarity to the 3'UTR of known mRNAs will also be helpful in reducing the number of potential targets. In the end however, the phenotype may still be the result of a combination of gene expression changes induced by the miRNA like effects of the seed sequences and will not easily be replicated by pharmacologic inhibition. For this reason development of improved delivery systems for novel RNAi based therapeutics are highly desirable.

Therapeutic application of RNAi classes of drugs will be enormously promising. Thus far, a number of barriers have prevented successful implementation of RNAi based therapeutics, including low stability of siRNAs, lack of efficient delivery to target tissue, unintended off-target silencing, and activation of an undesirable immune response (Gavrilov and Saltzman 2012). Chemical modifications of siRNAs such as modifications to sugar moieties or the RNA backbone can increase half-life and cellular uptake (Gavrilov and Saltzman 2012). Liposomes are phospholipid vesicles that have been used to deliver RNAi drugs to target cells by loading siRNAs into the inner aqueous compartment (Kowalski, Leus et al. 2011). Similarly, nanoparticles made of biodegradable materials such as chitosan, cyclodextrin and other materials are being investigated as delivery vehicles (Diaz and Vivas-Mejia 2013). In order to target the delivery vehicles to the desired tissue they can be conjugated to ligands such as antibodies, aptamers, small molecules or peptides that are expressed on the surface of the desired cell population, such as a tumor (Gavrilov and Saltzman 2012). While these technologies are making progress toward improving RNAi based therapies, additional improvements are needed to make them a reality.

Alternately or in addition to uncovering the targets of seed-based effects, these off target effects may also be eliminated simply by excluding them from the analysis and focusing on hits that are more likely to be on-target. As mentioned previously, a pool of siRNAs that produces a desirable phenotype can be tested separately using the individual siRNAs. If multiple individual siRNAs are able efficiently reduce expression of the intended target gene, replicate the phenotype observed in the pool, and the C911 mismatched control does not reproduce the phenotype, then the result is more likely due to the on-target effect. As a final validation of the effect, genetic rescue experiments can be performed in which exogenous expression of the target gene (optimally a mutated version that remains functional but is not targeted by the siRNA) is used to demonstrate that its continued expression is able to prevent the siRNA induced growth inhibition. Once validated, the gene identified in the siRNA screen represents a novel drug target for cancer therapy.

Functional RNAi screens are a valuable tool for identifying potentially targetable vulnerabilities in cancer cells. Screens such as these may identify non-oncogene addictions and synthetic lethal targets that are not obvious solely from mutation and expression data. However, in order to avoid publication of inaccurate results, and to save time and money on unnecessary follow-up experiments, RNAi screens need to be carefully designed, and the results prudently scrutinized in order to distinguish between true results and false positives due to off-target effects of siRNA knockdown.

V. Figures

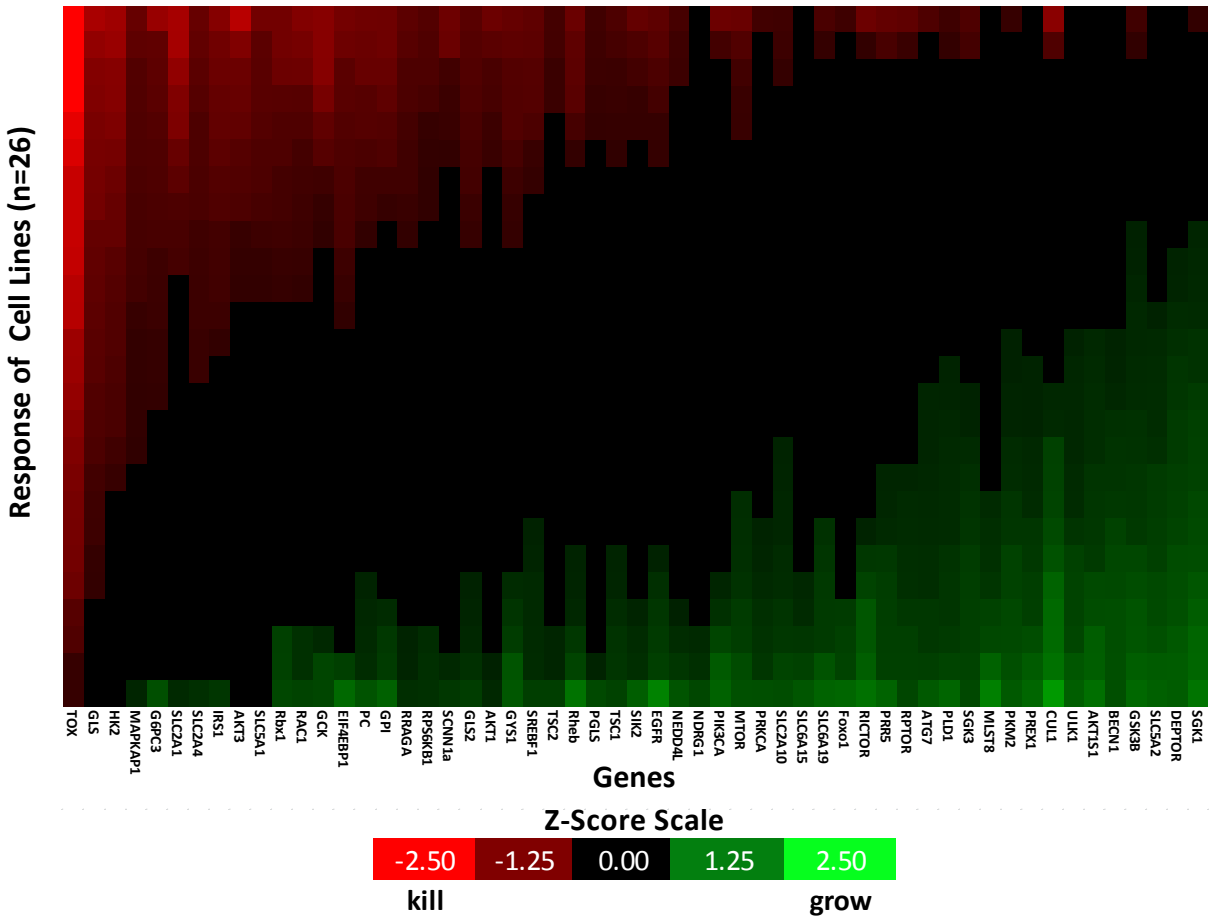


Figure 4.1: Knockdowns Produces a Heterogeneous Response in NSCLC Cell Lines Each square represents the response of a cell line to a pool of siRNAs targeting the indicated gene, sorted from strongest growth inhibitory phenotype at the top, to strongest growth stimulatory response at the bottom.

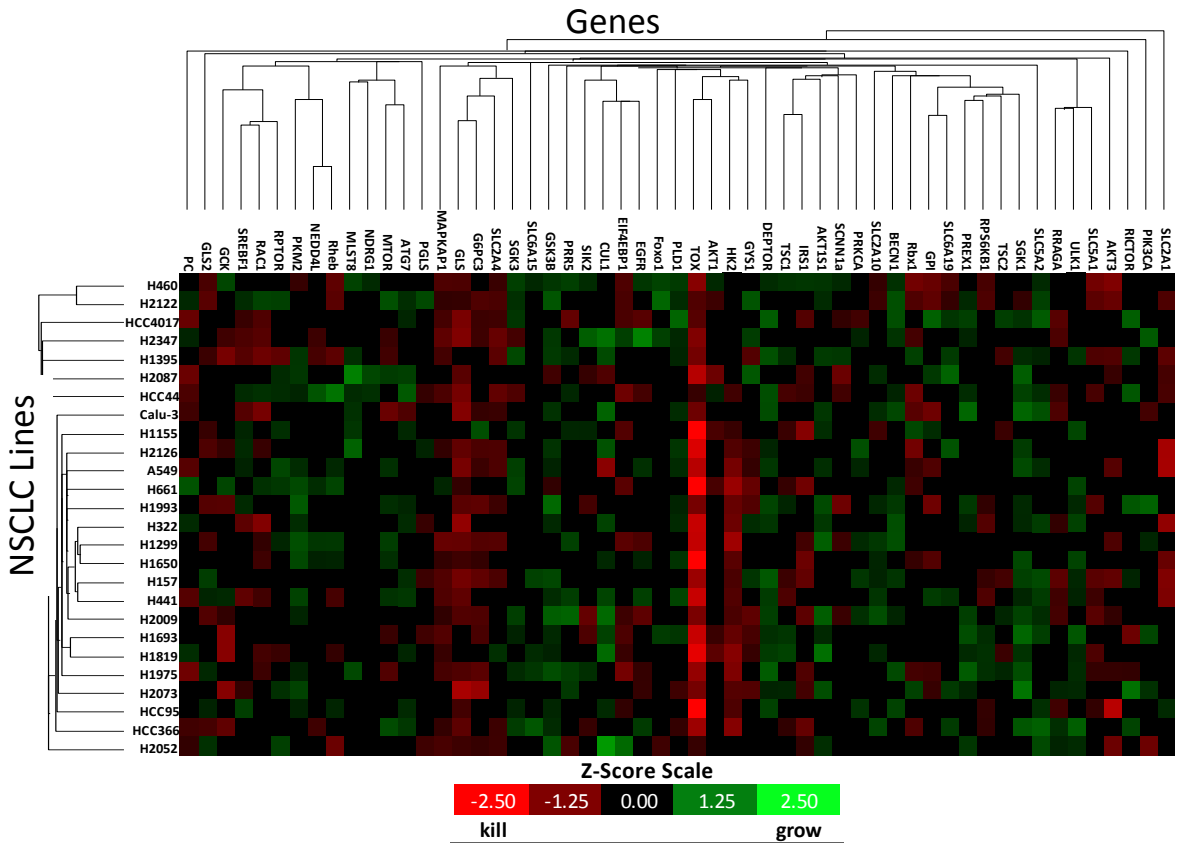


Figure 4.2: Cluster Analysis of NSCLC Cell Lines Responses to Knockdowns Cell lines and genes are clustered according to the similarity of responses.

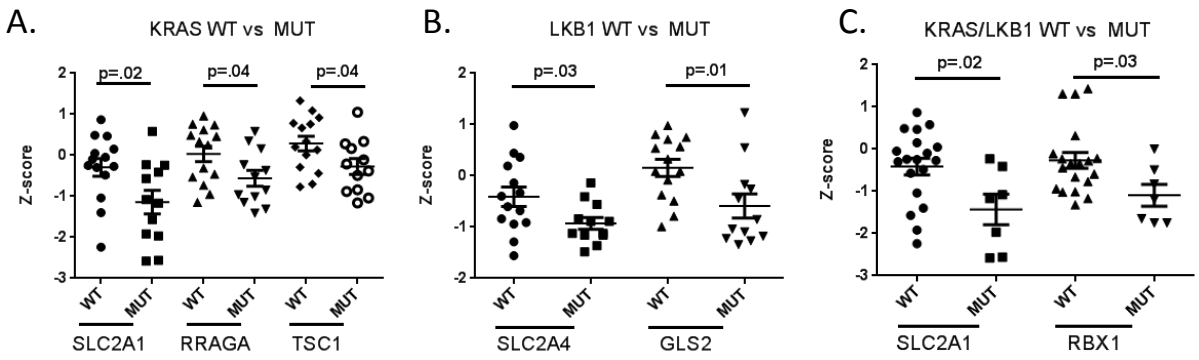


Figure 4.3: Synthetic Lethality with Known Oncogenes/Tumor Suppressors Comparison of Z-Score of most significantly different responses to siRNAs between mutant and wild-type KRAS (A), LKB1 (B), or KRAS/LKB1 co-mutation (C).

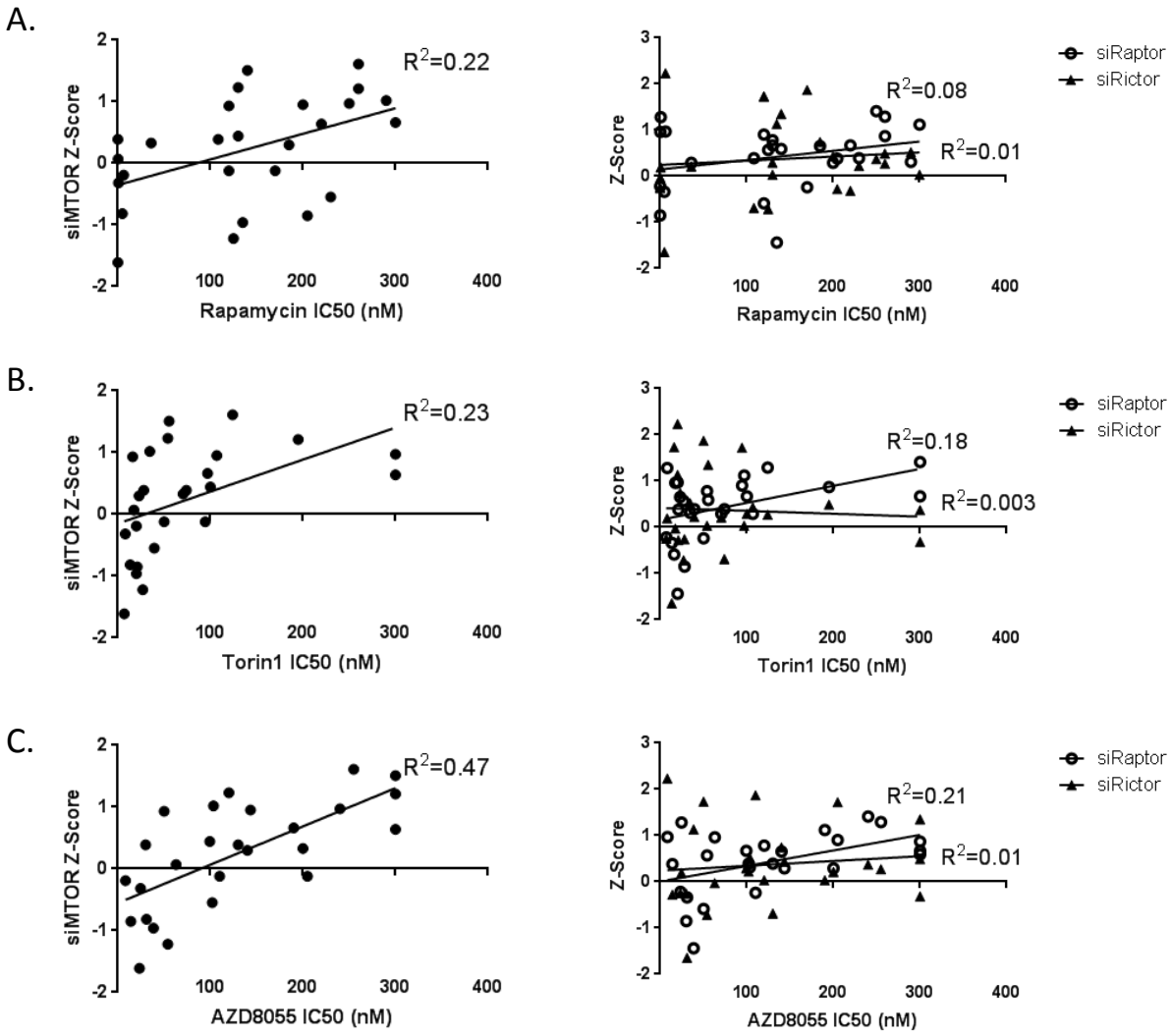


Figure 4.4: Correlation of Targeted Therapies and mTORC1/2 Knockdown A. Rapamycin, B. Torin1, C. AZD8055 IC50s plotted against Z-scores for siMTOR (left panels), siRaptor, and siRictor (right panels).

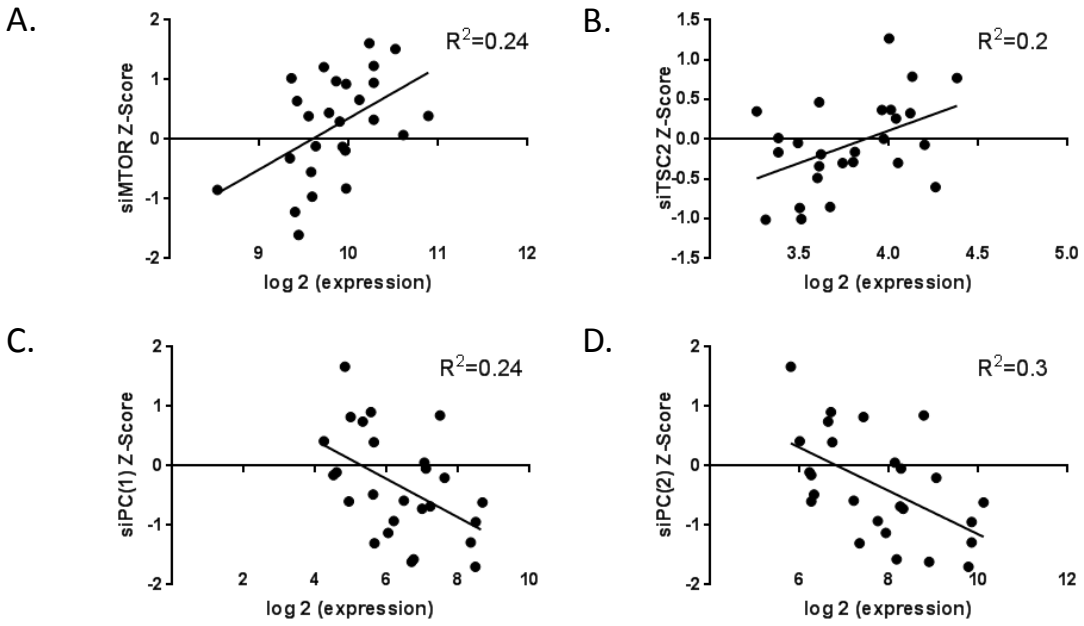


Figure 4.5: Correlation of Gene Expression and Response to Knockdown Log 2 expression of A. mTOR, B. TSC2, C. PC (probe1) and D. PC (probe 2) plotted against the Z-scores for the respective siRNA pool.

Chapter 5: mTOR Inhibitors in Drug Combinations

I. Abstract

The use of mTOR inhibitors as single agents for the treatment of cancer has had only limited success. Reactivation of upstream signaling through interference of feedback loops by mTOR inhibition is a primary mechanism of resistance to mTOR targeted therapies. As a result, mTOR inhibitors may have more clinical value in combination with other chemo- and targeted- therapy agents. A combination approach would enable improved inhibition of oncogenic signaling and/or a synthetic lethal effect. To determine the utility of mTOR inhibition in combination therapies, we combined a fixed concentration of each of the three mTOR inhibitors, rapamycin, Torin1, and AZD8055 with varying doses of the standard targeted therapy, erlotinib, or the standard chemotherapy doublet, paclitaxel/carboplatin. NSCLC cell lines were sensitized to the standard agents with the addition of an mTOR inhibitor in either an additive or synergistic manner. Interestingly, many of the cell lines that responded synergistically to the combination of erlotinib with an mTOR inhibitor had mutations in KRAS and LKB1. Sensitization to erlotinib was associated with improved inhibition of signaling through the PI3K-AKT pathway. Preliminary data suggested that the subset of cell lines that responded synergistically to the erlotinib+Torin1 combination may be more susceptible to inhibition of glucose transport resulting from the combined inhibition of EGFR and mTOR, which might explain the synergistic response. However, follow-up studies indicated that such a mechanism is not likely the main reason for the synergistic responses, as the use of glucose uptake inhibitors did not phenocopy the erlotinib and Torin1 response and the reduction in cell viability by this combination was not able to be rescued by the addition of an alternate energy source. Nevertheless, mTOR inhibitors do show promise in their ability to improve standard chemo- and targeted- therapies, even in tumors that are resistant to single agent therapy.

II. Introduction

Single Agent mTOR Inhibitors Have Had Minimal Clinical Success

The role of mTOR as a central regulator of several signaling pathways that promote cell growth and proliferation makes it an attractive target for anti-cancer therapeutics. Based on initial pre-clinical studies using rapamycin and its analogs, such as everolimus and temsirolimus, the use of these agents as targeted cancer therapy agents was expected to be very promising. Unfortunately, clinical success of these agents has been limited. Only a few cancer types responded to mTOR inhibition including mantle cell lymphoma, renal cell carcinoma, and endometrial cancer (Dancey 2010). While the exact reason these rare cancers respond while others do not is unknown, it has been suggested that perhaps these agents are more effective at inhibiting mTORC2 in the responsive cancers (Dancey 2010). This idea and new information about the role of mTORC2 in oncogenic signaling have paved the way for the development of improved mTOR targeting agents, including mTOR catalytic inhibitors. While these agents have the potential to further inhibit mTOR dependent cell growth, we have shown that catalytic inhibition of mTOR does not necessarily result in greater sensitivity of human lung cancer cells.

mTOR Inhibition Interferes with Feedback Mechanisms That Can Result in Innate Resistance

Greater understanding of the complexity of regulation of mTOR related signaling has uncovered a number of feedback mechanisms that are at play. Interference of these feedback mechanisms is now understood as a potential cause of the limited efficacy of mTOR inhibitors in cancer therapy. When mTOR is active, it phosphorylates its downstream target S6K1, which is part of an inhibitory feedback loop that targets upstream insulin signaling. In this pathway, mTORC1 and S6K1 promote the depletion of IRS1 through phosphorylation of several serine residues associated with insulin resistance (Shah and Hunter 2006). mTOR has also recently been shown to regulate insulin signaling through Grb10. As a result of these feedback loops, inhibition of mTOR using rapamycin leads to the activation of IRS1 and downstream signaling, including the activation of PI3K and AKT. Rapamycin also increases tyrosine

phosphorylation of EGFR and activates ERK1/2 signaling and increases cell survival (Chaturvedi, Gao et al. 2009). Although the simultaneous blockade of mTORC1 and 2 was presumed lead to improved inhibition of mTOR signaling and reduce AKT activity, mTOR kinase inhibitors also interfere with feedback mechanisms downstream of mTOR. Such selective ATP-competitive mTOR inhibitors have been shown to induce receptor tyrosine kinase activation and expression (Rodrik-Outmezguine, Chandarlapaty et al. 2011).

Rationale for Using mTOR Inhibitors in Combination Therapy

Overall, inhibition of mTOR does leads to aberrant activation of several pro-oncogenic components. This paradoxical effect on signaling has severely impaired the clinical utility of mTOR inhibitors for treatment of cancer. While the use of mTOR inhibitors as single agents may not be a successful therapeutic strategy, combinations of these agents with other standard targeted and chemotherapeutics are currently being explored. The goal of a combination approach is to overcome mechanisms of resistance in order to improve outcomes by amplifying anti-tumor activity.

Since the activation of several receptor tyrosine kinases has been reported as resulting from treatment with mTOR inhibitors, the combined inhibition of mTOR with the use of a tyrosine kinase inhibitor is a rational strategy to improve the efficacy of each. Similar combinations have been previously reported to be beneficial for treatment in several cancer types. For example, a dual blockade of EGFR and mTOR signaling was shown to improve survival in biliary tract cancer cells (Herberger, Berger et al. 2009). Combinations of erlotinib with RAD001 in SCLC yielded synergistic effects on signaling at the molecular level, and on cell viability, proliferation, and autophagy (Schmid, Bago-Horvath et al. 2010). Furthermore, resistance to EGFR targeted therapies has been associated with continued signaling through PI3K-AKT-mTOR despite EGFR inhibition. Therefore, the addition of a second downstream inhibitor in combination with erlotinib may improve inhibition of signaling in EGFR

TKI resistant cancer cells. Consistent with this hypothesis, when everolimus was combined with gefitinib in a panel of NSCLC cell lines, it resulted in a significant decrease in MAPK and mTOR signaling and a growth-inhibitory effect (La Monica, Galetti et al. 2009). A similar study in breast cancer found that combinations of lapatinib and ATP competitive mTOR inhibitor, INK-128, resulted in significant inhibition of both the PI3K/AKT/mTOR and ERK pathways and synergistic cell death in ERBB2-postive, but lapatinib refractory cells (García-García, Ibrahim et al. 2012).

Single agent therapy using mTOR inhibitors is limited due to the ineffectiveness of these agents as inducers of cell death. Rapamycin and its analogs are rather poor inducers of apoptosis and tend to have a cytostatic rather than cytotoxic effect. The use of mTOR inhibitors in combination with cytotoxic drugs such as standard chemotherapeutics including platinum agents may enhance the cell death phenotype (Liu, Thoreen et al. 2009). Rapamycin interacted synergistically in combinations with chemotherapy agents, paclitaxel, carboplatin, and vinorelbine in breast cancer cells. Rapamycin dramatically increased apoptosis in combination with paclitaxel or carboplatin (Mondesire, Jian et al. 2004).

Current targeted therapies that are in use for lung cancer include EGFR tyrosine kinase inhibitors as first line therapies in patients with activating mutations in EGFR, and second-line therapies for chemotherapy non-responsive tumors (Favoni and Alama 2013). Chemotherapy doublets such as paclitaxel/carboplatin are standard of care as first line therapy for the majority of patients (Favoni and Alama 2013). In this study, we aimed to determine the efficacy of three mTOR inhibitors, rapamycin, Torin1, and AZD8055 in combination with erlotinib or paclitaxel/carboplatin doublet therapy as a potential strategy to improve the response to these standard agents. We find that NSCLC cell lines respond synergistically or additively to combinations of erlotinib or paclitaxel/carboplatin with mTOR inhibitors. Strategies that combine mTOR inhibitors with standard chemo- and targeted agents have the

potential to improve upon current treatments and enhance responses of tumors that are resistant to single agents.

III. Results

mTOR Inhibition Leads to Activation of Upstream Signaling

Previous reports have demonstrated that mTOR inhibition can lead to the activation of upstream signaling especially the activation of receptor tyrosine kinases. As a result the use of mTOR inhibitors as single agents may be limited because signaling becomes re-activated. We examined the level of phosphorylated (activated) EGFR in NSCLC cell lines treated with mTOR inhibitors and found that rapamycin and Torin1 lead to a significant increase in p-EGFR in both sensitive and resistant cell lines (Figure 5.1). This result suggests that combining mTOR inhibitors with an EGFR tyrosine kinase may be an effective strategy to treat cancer cells by allowing for more complete and prolonged inhibition of pro-growth signaling.

NSCLC Cell Lines Respond to Erlotinib+mTOR Inhibitor Combinations in a Synergistic or Additive Manner

In order to determine the utility of combining an mTOR inhibitor with an EGFR TKI we performed dose response assays using varying doses of erlotinib alone or in combination with a fixed dose of an mTOR inhibitor. All three mTOR inhibitors, rapamycin, Torin1, and AZD8055 were tested in combinations with erlotinib on a panel of 28 NSCLC cell lines. Two measures were analyzed to determine the utility of such combination therapies. First, we compared the IC50s from the dose response curves when erlotinib was used alone, compared to when the drug was combined with a fixed concentration of mTOR inhibitor. In all cell lines, the IC50 was either not significantly affected or was

more sensitive to the combination than to erlotinib alone. No cell line was more resistant to the combination that to the single agents. For the erlotinib+Torin1 combination, 19/28 cell lines had a more than 4-fold sensitization ($IC50_{Erlotinib}/IC50_{Combination}$) to erlotinib by adding 20nM Torin1. The most significant difference in IC50s between erlotinib and the erlotinib/Torin1 combination was over 2500-fold. Rapamycin sensitized 22/28 cell lines more than 4-fold to erlotinib, with the greatest sensitization being almost 900-fold, while AZD8055 sensitized 27/28 cell lines to erlotinib 4-fold or more with the greatest sensitization at 1500-fold (Figure 5.2A, Table 5.1).

Combination indices (CI) were also calculated for the combination treatment in order to determine whether the responses were synergistic, additive, or even antagonistic (Table 5.1). A CI value under 1 indicates a synergistic response, equal to one indicates an additive response, and greater than 1 indicates an antagonistic response. Ten, 18, and 8 cell lines responded synergistically to combinations with erlotinib and Torin1, rapamycin, and AZD8055, respectively, using a strict CI cutoff of below 0.6. In a few cell lines (0, 5, and 3, for Torin1, rapamycin, and AZD8055 combinations, respectively), the CI value indicated an antagonistic response to the combination. However, the antagonism is presumed to be an artifact of the assay and of the calculation because these cell lines are sensitive to both erlotinib and mTOR inhibitors as single agents and at the concentrations tested would be expected to reduce viability more than 100% if additive, which is, of course, impossible.

Combinations of mTOR Inhibitors with Paclitaxel/Carboplatin Doublet Chemotherapy

While the presence of feedback mechanisms upon inhibition of mTOR that activate RTK signaling suggest combination of mTOR inhibitors with TKIs such as erlotinib by be beneficial, such a combination will be difficult to employ clinically due to realistic concerns about using targeted agents to treat patients that have biomarkers that predict resistance to the individual agents. It may be more realistic to attempt to combine mTOR inhibitors with some of the cytotoxic chemotherapy agents that

are still preferred for a larger majority of patients. A common treatment strategy used for lung cancer consists of doublet chemotherapy using a platinum agent and taxane. Since these agents are considered the standard of care for NSCLC patients, novel therapeutics are often tested in combination with them with the hope that the combination can improve upon the standard of care. However, there is also an unfortunate possibility that a novel targeted agent may lead to an antagonistic response, either due to incompatible responses within the tumor or because of increased toxicities to the patient. Pre-clinical assessments of targeted therapies in combination with standard chemotherapy are needed in order to determine possible benefits or drawbacks to such novel combinations.

We combined the three mTOR inhibitors, rapamycin, Torin1, and AZD8055 with paclitaxel/carboplatin doublet chemotherapy to test whether such a combination strategy may be useful in all or a subset of NSCLC cells. Similar to the combination screen with erlotinib, 28 cell lines were screened with varying doses of paclitaxel/carboplatin (at a 2/3 ratio), with a fixed dose of each of the three mTOR inhibitors (Figure 5.2B, Table 5.2). In all cell lines, the IC50 was either not significantly affected or was more sensitive to the combination than to paclitaxel/carboplatin alone. No cell line was more resistant to the combination than to the single agents. For this combination, 21/28 cell lines had a more than 4-fold sensitization ($IC50_{Pac/Carb}/IC50_{Combination}$) to paclitaxel/carboplatin by adding 20nM Torin1. The most significant difference in IC50s between paclitaxel/carboplatin and the paclitaxel/carboplatin + Torin1 combination was 900-fold. Rapamycin sensitized 15/28 cell lines more than 4-fold to paclitaxel/carboplatin, with the greatest sensitization being 500-fold, while AZD8055 sensitized 24/28 cell lines to paclitaxel/carboplatin 4-fold or more with the greatest sensitization at 1600-fold.

Combination indices (CI) were also calculated for the combination treatment in order to determine whether the responses were synergistic, additive, or even antagonistic (Table 5.2). Four, 18,

and 4 cell lines responded synergistically to combinations with paclitaxel/carboplatin and Torin1, rapamycin, and AZD8055, respectively, using a strict CI cutoff of below 0.6. In a few cell lines (1, 5, and 2, for Torin1, rapamycin, and AZD8055 combinations, respectively) the CI value indicated an antagonistic response to the combination.

Cell Lines That Respond Synergistically are Enriched in KRAS, LKB1 Co-Mutants

We next wanted to determine whether certain oncogenotypes influence the level of synergistic response to the drug combinations. In general, cell lines that are already sensitive to erlotinib were not further sensitized when combined with an mTOR inhibitor, indicating that there is only a minimal increase in efficacy in lines that already respond to erlotinib. Interestingly, there was an enrichment of cell lines with co-mutations in KRAS and LKB1 among those that had the most synergistic response to combinations with erlotinib. These lines are among the most resistant to both erlotinib and mTOR inhibitors as single agents. Eight cells lines that had co-mutations in KRAS and LKB1 were tested. Combinations of erlotinib with Torin1 or rapamycin resulted in a synergistic response in 6 of these co-mutant lines each, while 4 co-mutants had a synergistic response to erlotinib + AZD8055. Therefore, such combination therapies may be a valuable treatment option for tumors of this oncogenotype.

Torin1 Does Not Further Sensitize Erlotinib Sensitive, EGFR Mutant Lines

Cell lines and patient tumors that harbor mutations in the EGFR are known to respond very strongly to EGFR inhibition by erlotinib. We wanted to determine whether response to erlotinib could be improved any further in EGFR mutant (and erlotinib sensitive) cell lines by adding an mTOR inhibitor. These assays required the use of reduced doses of erlotinib than those used in the original screen in order to generate a dose response curve in which the IC50 fell at about the median concentration tested, rather than less than the lowest dose tested. We found that the addition of 20nM Torin1 did not

significantly affect these sensitive cell lines responses to erlotinib (Figure 5.3). These cells were not tested in combinations with rapamycin or AZD8055.

EGFR mutant lines that have acquired resistance to erlotinib through second site T790M mutations, MET amplification, or other mechanisms were not generally re-sensitized to erlotinib by adding Torin1, with one exception. The response to erlotinib in H820, which has a T790M mutation in EGFR and amplification of MET, was sensitized more than 2000 fold by adding Torin1. However, this cell line is one of the most sensitive to Torin1 as a single agent, and therefore the response to the combination is additive and not synergistic (Table 5.1). The erlotinib resistant EGFR mutant lines were more significantly sensitized to erlotinib using the other two mTOR inhibitors, rapamycin and AZD8055. H1650 had a synergistic response to both of these combinations and a 13 and 15-fold sensitization to erlotinib for each. H1975 responded synergistically to erlotinib+rapamycin treatments and a 4 and 90-fold sensitization to erlotinib for rapamycin and AZD8055, respectively (Table 5.1).

HBECs Are Only Minimally Sensitized to Erlotinib by Adding Torin1

We also determined whether normal lung epithelial cells may be vulnerable to combined inhibition of EGFR and mTOR in order to define possible toxicities. A panel of six immortalized HBECs was tested for their response to combinations of erlotinib and Torin1. These “normal” cells were only moderately sensitized to erlotinib by the addition of Torin1. Shifts in IC50s ranged from 4- to just over 15-fold (Figure 5.4). These shifts are insignificant compared to the greater than 1000-fold sensitization seen in some of the cancer cell lines. Still, *in vivo* toxicity studies and early phase clinical trials will be required to determine the safety of using similar drug combinations in patients.

Erlotinib+Torin1 Does Not Lead to Increased Levels of Apoptosis or Changes in Cell Cycle Profiles

In order to determine whether the increased sensitivity to erlotinib with the combination therapies was due to an increase in apoptosis, we examined the levels of cleaved PARP in cells treated with erlotinib, Torin1, or their combination. In only 2 of the cell lines tested was there an increase in cleaved PARP levels with the drug combination. Overall, in the cell lines tested the drug combination did not lead to a significant increase in cleaved PARP compared to the single agents, or to untreated cells (Figure 5.5A).

Flow cytometric profiling of the DNA content of cells was used to characterize the percent of cells in G0, G1, S, or G2 phases of the cell cycle following 72 hours of treatment with Torin1, erlotinib, and their combination. No significant changes were seen in the cell cycle profiles compared to untreated controls indicating that cell cycle arrest cannot account for the cell lines' sensitization to erlotinib (Figure 5.5B).

Erlotinib+mTOR Inhibitor Combinations Lead to Improved Inhibition of mTOR Activity

Previous reports have indicated that when erlotinib is ineffective, continued signaling through PI3K-AKT-mTOR is present, despite inhibition of EGFR. We have shown that the mTOR inhibitors are effective at inhibiting mTORC1 activity and that Torin1 and AZD8055 can also inhibit mTORC2 activity. Therefore, we wanted to know how mTOR activity was affected by the drug combinations with erlotinib and paclitaxel/carboplatin. We determined the levels of p-P70S6K and p-AKT(Ser473) as a readout of mTORC1 and mTORC2 activity, respectively. We find that mTORC1 activity is completely abolished by 1 hour with combinations of erlotinib or paclitaxel/carboplatin with either Torin1 or AZD8055. Similarly, mTORC2 activity is significantly reduced with these combinations, though slightly more p-AKT(Ser473) remains following Erlotinib+AZD8055 combination than for the others (Figure 5.6). In an mTOR inhibitor resistant xenograft, combinations of erlotinib and AZD8055 resulted in inhibition of relative tumor growth (Figure 5.7A). While AZD8055 alone was able to reduce mTORC1 activity on P70S6K in this *in*

vivo model, mTORC1 activity on 4EBP1 and mTORC2 activity on AKT were only significantly inhibited when AZD8055 was combined with erlotinib (Figure 5.7B, C).

Investigating Possible Mechanisms that Result in a Synergistic Response

A number of approaches were analyzed in order to determine whether any mechanisms in addition to more complete inhibition of growth signaling were playing a role in determining how certain cell lines responded synergistically to the erlotinib and Torin1 combinations, rather than simply having an additive response. These analyses were performed following an initial combination screen on a smaller panel of NSCLC cell lines, which used a slightly different determination of response than in the final screen. As a result, a few lines that were determined to have a synergistic response in the final screen were not initially considered to have such in the initial screen and were considered erlotinib+torin1 "non-sensitized" lines for these further analyses.

Gene Expression Differences Are Not Predictive of Synergistic vs. Additive Response

Similar analyses of microarray expression data as those used to evaluate differences in gene expression in mTOR inhibitors sensitive and resistant groups were performed on groups of cell lines that responded synergistically to a drug combination versus those that did not. Groups of synergistic versus non-synergistic lines for each drug combination were analyzed separately, and log ratios of expression differences of the transcriptome were calculated using MATRIX software as before. Figure 5.8 shows a summary of the results of these analyses. Genes that had a log2 of greater than 2 or lower than -2 (4 fold difference in expression) and a p-value of less than 0.05 were considered significantly differentially expressed between sensitive and resistant lines.

In erlotinib + Torin1 synergistic lines there were 7 genes that were significantly under-expressed and 1 gene that was significantly over-expressed compared to non-synergistic lines. Erlotinib +

rapamycin synergistic lines had 18 and 15 genes under- and over-expressed, respectively, and erlotinib + AZD8055 synergistic lines had 6 and 3 genes under-and over-expressed, respectively (Table 5.3). For the combinations with paclitaxel/carboplatin, 17, 17, and 34 genes were under-expressed in Torin1, rapamycin, and AZD8055 synergistic lines, respectively, and 4, 3, and 16 genes over-expressed in Torin1, rapamycin, and AZD8055 synergistic lines, respectively (Table 5.4). Most differentially expressed genes have a less than 16-fold average difference in expression between synergistic and non-synergistic lines. There is also wide variation of expression within each group (synergistic or non-synergistic) reducing the likelihood that expression of any single gene will be a useful biomarker.

The genes that are differentially expressed between synergistic and non-synergistic responding cell lines do not include those that are involved in the mTOR pathway. It is unclear whether the differentially expressed genes have any biological relationship with the responses to drug combinations, as there is no apparent rationale for the genes to confer a sensitivity phenotype. Furthermore, there is only minimal overlap in the differentially expressed genes in the synergistic cell lines for the combinations with the different mTOR inhibitors. Therefore, gene expression differences are not likely to be useful as biomarkers to predict a synergistic response to these drug combinations.

mTOR Inhibition Reduces Glucose Uptake

mTOR is a central regulator of several metabolic pathways in response to growth factor and energy sensing pathways. Therefore we sought to determine how mTOR inhibition may be affecting the levels of various metabolites in order to get an idea of what metabolic pathways may be most important in determining response to Torin1 alone and in combination with erlotinib. The changes in global metabolite levels that occurred in response to Torin1 were determined using mass spectrometry experiments. We speculated that the levels of a large number of metabolites would be significantly altered following Torin1 treatments; however, surprisingly only 2 changes were seen in H460 cells.

Levels of intracellular glucose were reduced 3-fold, and levels of intracellular lactic acid were reduced 5-fold upon treatment with Torin1 (Figure 5.9A). Reduction in glucose levels could indicate an increase in glucose utilization; however the concurrent decrease in lactic acid suggested that the rate of glycolysis was also reduced. Therefore, we surmised that the uptake of glucose into the cell had been reduced due to Torin1 treatments.

In order verify that glucose uptake was being affected, we measured the levels of glucose being absorbed by the cells from the growth media over time in untreated, Torin1, erlotinib, and erlotinib + Torin1 treated cells. This assay uses an enzymatic reaction in which the glucose in the sample is converted to glucose-6-phosphate by hexokinase and subsequently oxidized (while NAD is reduced) to form 6-phosphogluconate and NADH. NADH accumulation leads to a change in absorbance proportional to the concentration of glucose in the sample. In H460 cells, Torin1 treatment reduced the amount of glucose that was absorbed from the media (normalized to the total number of cells present in untreated and treated samples) over the course of 48 hours. Erlotinib alone had no measurable impact on the level of glucose uptake in these cells and had no additional impact on the reduction of glucose uptake in combination with Torin1 (Figure 5.9B).

Synergistic Responders are Uniquely Vulnerable to Knockdown of Several Metabolic Genes

A genome-wide siRNA screen performed on 12 cell lines and 1 HBEC line that was performed in the laboratory of Dr. Michael White served as a source for potential insight into unique vulnerabilities present in erlotinib + Torin1 synergistic lines compared to non-synergistic lines. By determining what gene knockdowns killed/growth inhibited the synergistic subset, while not affecting the other lines, we hoped to identify players in mTOR/EGFR signaling that may be affected by the drug combinations and may explain improved sensitization. One synergistic cell line, H1299, was not used in the siRNA screen

and therefore did not play into the analysis of unique siRNA hits. A hit was defined as siRNAs having a Z-score less than -3.

Thirteen genes related to metabolism were determined to be unique hits for the erlotinib + Torin1 synergistic lines (Table 5.5). Two of these (AKT3 and SGK3) were particularly intriguing because they are direct targets of mTORC2 signaling. While AKT1 is the more well known of the AKT isoforms, and has a well-defined role in cancer signaling, AKT3 can also mediate PI3K signaling and regulation of growth and glucose metabolism. mTORC2 phosphorylates AKT3 on Ser472 in the hydrophobic motif in a manner similar to AKT1. SGK3 is a member of a family of serum and glucocorticoid related kinases, which are similar to AKT. mTORC2 has been shown to phosphorylate and activate SGK (García-Martínez and Alessi 2008).

Both AKT3 and SGK3 play a role in glucose homeostasis through their regulation of glucose transporters at the cell surface. The transcription and localization of the glucose transporter Glut1 is regulated by AKT signaling. SGK regulates a sodium dependent glucose transporter SGLT1 (SLC5A1) by inhibiting NEDD4-2, an ubiquitin kinase that targets SGLT1 for degradation (Dieter, Palmada et al. 2004). Furthermore, EGFR has been shown to interact with and stabilize SGLT1 in order to promote survival in irradiated A549 cells (Huber, Misovic et al. 2012). These details led us to hypothesize that the synergistic response to the erlotinib + Torin1 combination could be a result of a synthetic lethal effect on a number of glucose transporters to which the sensitized subset of cells was uniquely vulnerable.

Investigating the Role of Glucose Transporters in Determining Erlotinib + Torin1 Response

We began testing this hypothesis by first validating the siRNA knockdowns and their effects on the viability of NSCLC cell lines. Consistent with the results of the siRNA screen, independent siRNAs targeting SGK3 reduced viability of synergistic lines H460, H2009 and H2122, while having minimal effect on non-synergistic line, H1155 (Figure 5.10A). Knockdown of the SGK3 protein was confirmed both in

cell lines where viability was and was not affected (Figure 5.10B). Knockdown of AKT3 also reduced the viability of a subset of NSCLC cell lines, however without making a clear distinction between synergistic versus non-synergistic responders seen in the screen results (Figure 5.10A).

We then wanted to test whether Torin1 and erlotinib treatments affected the activation of SGK3 and AKT3. In H460 cells, the levels of phospho- and total-SGK3 were reduced following 24 hour treatment with Torin1 alone and in combination with erlotinib. Erlotinib alone had no effect on p-SGK3. H2009 and H2122 had the most significant decrease in p-SKG3 levels when treated with the combination. In another synergistically responding cell line, H1299, p-SGK3 levels were not affected by any of the drug treatments, and similarly in a non-synergistic line, H1155, p-SKG3 was unaffected (Figure 5.10C). These results verify that at least in a subset of cell lines, SGK3 is a target of mTOR whose activation can be reduced by mTOR inhibition. Rapamycin treatment did not affect p-SGK3 in H460 in accordance with previous reports that this phosphorylation is rapamycin insensitive and a target of mTORC2 rather than mTORC1 (not shown). Torin1 treatments also reduced levels of phospho- and total-AKT3 in H460 cells (Figure 5.10D).

In order to determine whether the drug combination may be inhibiting glucose uptake though transporters controlled by mTOR signaling, the expression of glucose transporters SGLT1 and Glut1 was determined by immunoblot following drug treatment. Of the cell lines tested, treatment of only 1 cell line, H460, had any effect on the expression of SGLT1 or Glut1. In this cell line, erlotinib+Torin1 reduced levels of SGLT1, and Torin1 alone and in combination with erlotinib reduced expression of Glut1 (Figure 5.11). Interestingly, the addition of the autophagy inhibitor chloroquine with these drug treatments rescued the loss of expression. This result suggests that following treatment in H460, these transporters are degraded using autophagy (Figure 5.11).

Glucose Transport Inhibitors Do Not Phenocopy the Erlotinib + Torin1 Synergy

As a further measure to examine whether the synergistic responses to the erlotinib + Torin1 combination resulted from combined inhibition of several glucose transporters, we tested known inhibitors of glucose transporters in combination with erlotinib to determine whether NSCLC cell lines responded to this combination in a manner similar to the combination with Torin1. Phloridzen dihydrate and Fasentin are inhibitors of SGLT1 and Glut1, respectively. High doses of these agents will lead to growth arrest in NSCLC cell lines. Concentrations of phloridzen dihydrate greater than 1mM are required in order to reach an IC50 in the cell lines tested, while 30 μM fasentin inhibits lung cancer cell growth by 50% (not shown). At concentrations previously shown to inhibit glucose transport, these inhibitors were not able to sensitize any of the cell lines to erlotinib, indicating that inhibiting these transporters is not sufficient for the synergistic response seen with erlotinib and Torin1 (Figure 5.12A).

Finally, if the glucose deprivation from the down-regulation of glucose transport was the mechanism of action for the synergistic erlotinib and Torin1 response, this phenotype should be rescued with the addition of an alternate energy source. Pyruvate generated by the glycolytic pathway is fed into the tri-carboxylic acid cycle (TCA cycle) to generate energy and other metabolic precursors. Adding a membrane permeable form of pyruvate, methyl-pyruvate, should rescue the growth inhibition phenotype caused by any deficiency in energy pre-cursors into the TCA cycle that may result from erlotinib and Torin1 drug treatments. However, the addition of methyl-pyruvate was not sufficient to rescue the growth inhibition caused by erlotinib + Torin1 combination treatments in any of the cell lines tested (Figure 5.12B). This result indicates that glucose deprivation is not likely an important mechanism for determining the response of combined EGFR and mTOR inhibition.

IV. Discussion

mTOR inhibitors have not had broad clinical efficacy as single agents. Interference with feedback mechanisms, a cytostatic rather than a cytotoxic effect, and incomplete inhibition of signaling contribute to innate resistance to mTOR inhibitors. As a result, using mTOR inhibitors in combination with other chemo- and targeted-therapy agents is likely to be a more effective therapeutic strategy. In order to test the efficacy of combination therapies using mTOR inhibitors, we screened a panel of NSCLC cell lines for their response to combinations of erlotinib or paclitaxel/carboplatin with the mTORC1 inhibitor rapamycin, or dual mTORC1/2 inhibitors Torin1, and AZD8055. NSCLC cell lines responded additively or synergistically to combinations of erlotinib or paclitaxel/carboplatin with the mTOR inhibitors. The improved response to the combinations is associated with improved inhibition of signaling through the PI3K-AKT-mTOR pathway, but not necessarily an increase in the levels of apoptosis or the effect on the cell cycle.

Resistance to mTOR inhibition is associated with re-activation of upstream signaling including increased levels of p-EGFR and p-AKT. Similarly, resistance to EGFR targeted therapies is associated with continued activation of signaling through PI3K-AKT-mTOR (Guix, Faber et al. 2008). Therefore, the combination of mTOR inhibitors with EGFR TKIs is a rational approach to improve the efficacy of both classes of agents by more completely inhibiting signaling than either agent alone. This strategy has already been tested in other cancer types, described in the introduction. Another recent report indicated that this combination strategy resulted in improved inhibition of signaling through the pathway. In human colorectal cell lines combination treatment with erlotinib and an mTOR kinase inhibitor, PP242, resulted in complete inhibition of mTORC1 and 2 signaling, a reduction in colony forming efficiency, and increase in apoptosis, and a reduction in growth of xenografts (Wang, Wei et al. 2013).

Presence of a mutation in the Ras oncogene is a validated biomarker for resistance to EGFR targeted therapies. Ras is a downstream effector of EGFR. When constitutively active due to an activating mutation, inhibition of an upstream receptor tyrosine kinase has no effect on pathway inhibition downstream of Ras, thus limiting its efficacy in these tumors. Nevertheless, combination therapeutic strategies including TKIs may still be effective in Ras mutant tumors due to greater inhibition of signaling. In this study, we show that the addition of an mTOR inhibitor to the EGFR inhibitor erlotinib is able to sensitize Ras mutant NSCLC cell lines to EGFR TKI. A similar phenomenon has been observed recently in colorectal cancer cells. Li et al. report that combinations with erlotinib and rapamycin inhibit downstream signaling and inhibit cell growth in both KRAS wild-type and mutant cells (Li, Gao et al. 2012). Also, a multi- BCR/Abl and Src family TKI, dasatinib, was able to sensitize KRAS mutant colorectal tumors to cetuximab (Dunn, Iida et al. 2011). However, another study examined combinations with erlotinib/gefitinib and a rapamycin or a PI3K inhibitor, ZST474, in primary ovarian cancer cell cultures. Only 1/9 samples in this set had a mutation in KRAS, but this tumor had only an additive effect for combinations of EGFR inhibitors with PI3K inhibition, and with erlotinib + rapamycin, but an antagonistic effect (CI calculated at 50% cell death) with a gefitinib + rapamycin combination (Glaysher, Bolton et al. 2013).

Another promising combination targeted therapy is the dual inhibition of the MAPK and PI3K signaling pathways. In a colorectal cancer patient derived xenograft model, MEK inhibition using AZD6244 combined with PI3K/mTOR inhibition with BEZ235 inhibited tumor growth more effectively than monotherapy, and triple therapy with cetuximab further enhanced response (Migliardi, Sassi et al. 2012). Similar results were found using a variety of MEK inhibitors with PI3K or mTOR inhibitors in a murine lung cancer model (Engelman, Chen et al. 2008), BRAF and KRAS mutant human cancer cell lines and xenografts (Hoeflich, Merchant et al. 2012), and colorectal cells (Martinelli, Troiani et al. 2013). However, in the present study only a minimal increase in sensitivity was seen in NSCLC cell line with

combinations of MEK inhibitor, AZD6244, and Torin1 (not shown) and this combination was less effective than combinations with erlotinib.

Most patients with NSCLC will receive a standard chemotherapy doublet regimen, such as paclitaxel/carboplatin doublet therapy. However, innate or acquired resistance to these drugs occurs frequently and tumor recurrence is common even when initial response is seen. Therefore, we wanted to test the potential efficacy of mTOR inhibition in combination with this standard chemotherapy regimen, especially since novel targeted therapies are often initially applied in a clinical setting in combination with the current standard of care. Additionally, single agent mTOR inhibitors tend to have a cytostatic rather than cytotoxic effect. Combinations with chemotherapy may enhance tumor cell death and promote tumor regression to improve upon tumor growth inhibition seen with the single agents. In a panel of cancer cell lines from a variety of cancer types, everolimus was shown to enhance cisplatin induced apoptosis in a p53 dependent manner by inhibiting the expression of p21 (Beuvink, Boulay et al. 2005). Temsirolimus in combination with cisplatin was shown to be synergistic in human melanoma cell lines and xenografts (Thallinger, Poeppl et al. 2007), and in small-cell and NSC lung cancer models that were either selected for *in vitro* resistance to cisplatin, or derived from a cisplatin resistant patient tumor (Wu, Wangpaichitr et al. 2005). The mTORC2 component, rictor, was recently shown to suppress cisplatin induced apoptosis in ovarian cancer cell lines by activating and stabilizing AKT. Suppression of mTORC2 by siRNA knockdown of rictor enhanced cisplatin induced apoptosis in a p53 dependent manner (Im-Aram, Farrand et al. 2013). In this study, we found that NSCLC cell lines respond to combinations with paclitaxel/carboplatin and mTOR inhibitors in an additive or synergistic manner. Interestingly, combinations with rapamycin had more frequent synergy compared to combinations with the dual mTORC1/2 inhibitors, suggesting that mTORC2 is not necessarily primarily responsible for chemotherapy resistance in these cells. The sensitization was also not associated with any particular oncogenotype.

Phase I clinical trials will be very important in assessing the feasibility and safety of drug combinations such as those proposed here. Not only does the possibility of improved efficacy need to be addressed, but the effects of these combinations on normal physiology and identifying overlapping or unexpected toxicities need to be carefully evaluated. Previous trials have determined maximum tolerated doses of erlotinib and everolimus ranging from erlotinib 100 mg/day plus everolimus 2.5 mg/day to erlotinib 150 mg/day plus everolimus 5 mg/day or 50 mg/week (Park, Davis et al. 2013). Another study found that weekly 25 mg/m^2 cisplatin and 60gy radiation therapy in combination with 2 mg/day rapamycin was well tolerated in lung cancer patients (Sarkaria, Schwingler et al. 2007). Additional clinical trials examining the safety and efficacy of combination therapies using mTOR targeting agents are underway and will be discussed further in Chapter 7.

Additional experiments were performed in this study to try and develop a molecular understanding for the synergistic effect on growth inhibition observed in a subset of NSCLC cell lines with combinations of erlotinib and Torin1. Since this subset was enriched in lines with KRAS mutations, which are known to be resistant to erlotinib monotherapy, it would be intriguing to understand the mechanism for the sensitization in these cells in order to further exploit this vulnerability that seemed to occur as a synthetic lethal effect in the drug combination. As the PI3K-AKT-mTOR pathway promotes cell growth and regulates metabolism in response to growth factors, and the AMPK-mTOR pathway regulates metabolic pathways in response to energy levels, we sought to determine whether Torin1 was affecting certain metabolic pathways which then made the cells more vulnerable to EGFR signaling.

Preliminary experiments analyzing changes in the relative levels of abundant metabolites in Torin1 treated versus untreated cells indicated that glucose transport may be inhibited following mTOR inhibition, as the main metabolite changes were reduced lactic acid and glucose. This finding was further validated by determining that Torin1 treatment led to a reduction in the amount of glucose the

cells took up from the growth media. Finally, results from a genome-wide siRNA screen indicated that cell lines that responded synergistically to the combination were vulnerable to knockdown of two downstream targets of mTORC2, SGK and AKT3, which play a role in the regulation of glucose transporters. EGFR also has been previously reported to stabilize a glucose transporter SGLT1 (Weihua, Tsan et al. 2008). These data led us to the hypothesis that the synergy observed to the erlotinib plus Torin1 combination in a subset of NSCLC cell lines was the result of a synthetic lethal effect on pathways affecting glucose transport, the inhibition of which caused this subset of cell lines to be vulnerable.

Contrary to the proposed hypothesis, we did not find that this synthetic lethal effect on inhibition of glucose transporters played a broad mechanism to promote synergy. Expression of glucose transporters was only affected in a single cell line, H460, and inhibitors of glucose transport did not mimic the synthetic lethal effect of the erlotinib plus Torin1 combination. Furthermore, the growth inhibitory effect could not be rescued by supplementation of a membrane permeable nutrient, which should replace the need to import additional glucose into the cell. Therefore, the mechanism that allows this subset of cells to respond synergistically to this drug combination remains to be elucidated.

Another explanation for the changes in metabolite levels is that Torin1 may initiate a metabolic switch from glycolysis to another pathway that utilizes glucose without increasing lactic acid levels. Unique metabolic pathways in various subsets of NSCLC cells are currently being investigated using a variety of techniques in the lab of Ralph DeBerardinis. These include metabolic flux analysis, as well as nutrient utilization and dependence screens. They have already determined that KRAS and LKB1 co-mutation appears to drive a unique metabolic phenotype in the cancer cells. Thus far, however, no significant associations can be found with these metabolic phenotypes and synergistic response to combined EGFR and mTOR drug responses (not shown).

Overall, we have found that drug combination approaches using mTOR inhibitors together with standard chemotherapy doublet paclitaxel/carboplatin or targeted therapy erlotinib is a promising approach to improve response to these monotherapies. NSCLC cell lines responded to these combinations in a synergistic or additive manner. Cell lines that responded synergistically were enriched in KRAS mutations indicating that these combination strategies may also be effective in patients that would be predicted to be resistant to monotherapy. Additional pre-clinical and clinical studies will determine the actual utility of these drug combinations in patient therapy.

V. Figures

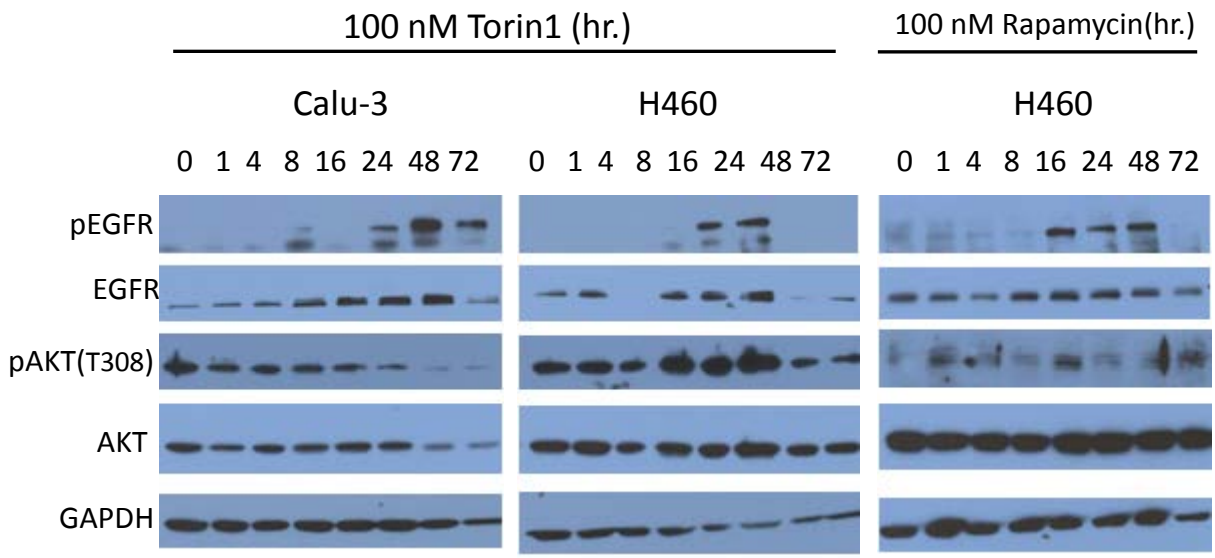


Figure 5.1: mTOR Inhibition Leads to Activation of Upstream Signaling Immunoblot of phospho- and total EGFR and AKT over the course of 72 hours of treatment with Torin1 (left 2 panels) or rapamycin, (right panel).

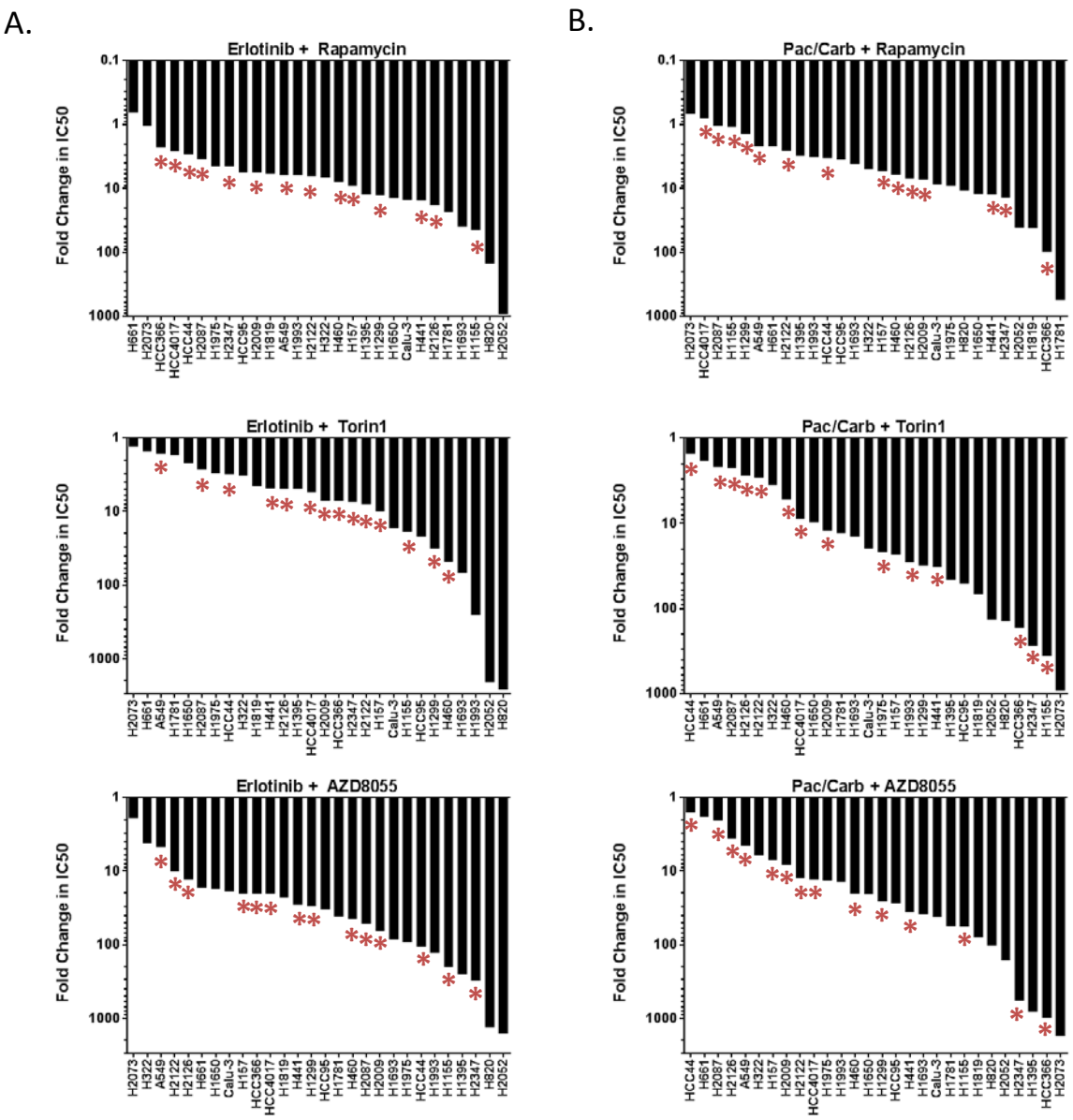


Figure 5.2: mTOR Inhibitors Sensitize NSCLC Cell Lines to Erlotinib or Paclitaxel/Carboplatin. Fold changes in IC50s between erlotinib (A) or paclitaxel/carboplatin(B) alone or in combination with Torin1 (upper), rapamycin (middle), or AZD8055 (lower). * indicates a cell line with a RAS mutation

Cell Line	Fold Sensitization ($IC50_{Erlotinib}/IC50_{Combination}$)			Combination Index (at IC50 Erlotinib)		
	Erlotinib			Erlotinib		
	+ 20nM Torin1	+ 20nM Rapamycin	+ 75 nM AZD8055	+ 20nM Torin1	+ 20nM Rapamycin	+ 75 nM AZD8055
A549	1.58	6.00	4.57	0.74	0.31	0.60
Calu-3	**16.14**	**14.49**	**18.13**	2.49	147.52	3.04
H1155	**18.29**	**41.90**	**192.86**	0.66	81.93	1.20
H1299	**30.65**	**12.16**	**28.78**	0.38	0.26	0.44
H1395	4.83	**11.93**	**242.72**	1.30	0.22	1.82
H157	9.60	8.66	**19.28**	1.04	0.24	0.56
H1650	2.13	**13.77**	**16.74**	0.66	0.32	0.45
H1693	**66.13**	**38.32**	**81.67**	1.03	1.83	2.72
H1781	1.65	**22.14**	**40.00**	1.58	0.19	1.53
H1819	4.31	5.68	**21.83**	0.97	281.85	4.53
H1975	2.91	4.24	**89.15**	0.87	0.44	1.88
H1993	**247.42**	6.00	**125.00**	0.79	0.38	1.69
H2009	6.93	5.42	**62.07**	0.43	0.43	0.58
H2052	**2000.00**	**888.89**	**1576.27**	2.40	26.00	2.78
H2073	1.25	1.00	1.85	2.55	6.88	44.95
H2087	2.57	3.27	**50.00**	0.77	0.42	0.28
H2122	7.69	6.12	9.85	0.21	0.31	0.51
H2126	4.79	**17.40**	**12.58**	0.51	0.79	0.92
H2347	7.16	4.24	**297.87**	1.00	0.36	5.38
H322	3.13	6.56	4.00	0.41	0.28	0.56
H441	4.66	**15.04**	**27.16**	0.71	0.16	0.61
H460	**45.65**	7.59	**43.27**	0.19	0.33	0.65
H661	1.47	0.63	**15.91**	1.03	1.75	1.07
H820	**2583.33**	**144.07**	**1295.77**	1.82	2.42	2.83
HCC366	6.96	2.18	**19.63**	0.56	0.68	0.31
HCC4017	5.31	2.50	**19.66**	0.59	0.58	0.80
HCC44	2.98	2.80	**102.00**	0.47	0.59	0.75
HCC95	**20.78**	5.33	**31.67**	0.47	0.41	0.65

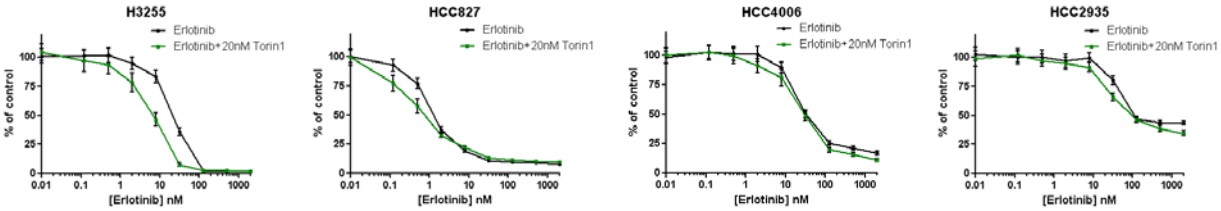
Table 5.1: Fold Sensitization and Combination Indices for Erlotinib + mTOR Inhibition in NSCLC Cell Lines
Left panel contains the fold difference in IC50 between Erlotinib alone and in combination with the three mTOR inhibitors. Green indicates a 4-fold or greater sensitization. Bolded and underlined text indicates a greater than 10-fold sensitization. Right panel contains the combination indices (CI) of the combinations with erlotinib and the mTOR inhibitors. Green indicates synergy, gray indicates additivity, and red indicates antagonism.

Cell Line	Fold Sensitization ($IC50_{Pac/Carb}/IC50_{Combination}$)			Combination Index (at IC50 Pac/Carb)		
	Paclitaxel/Carboplatin			Paclitaxel/Carboplatin		
	+ 20nM Torin1	+ 20nM Rapamycin	+ 75 nM AZD8055	+ 20nM Torin1	+ 20nM Rapamycin	+ 75 nM AZD8055
A549	2.14	2.10	4.30	1.11	0.52	0.63
Calu-3	**18.95**	8.10	**40.49**	4.18	84.12	3.68
H1155	**350.65**	1.03	**54.41**	1.91	1.02	1.27
H1299	**30.26**	1.35	**25.00**	0.54	0.79	0.92
H1395	**44.64**	2.97	**776.60**	1.04	0.43	2.15
H157	**22.36**	5.13	6.67	1.17	0.23	0.87
H1650	9.38	**11.49**	**19.85**	1.37	0.12	0.87
H1693	**13.80**	3.96	**37.36**	1.72	11.70	2.23
H1781	**12.58**	**526.32**	**54.00**	0.32	0.06	0.51
H1819	**65.43**	**40.43**	**78.08**	1.71	630.63	1.99
H1975	**20.95**	8.75	**12.75**	1.95	0.14	1.29
H1993	**27.85**	3.09	**13.60**	2.11	0.40	1.52
H2009	**11.94**	6.89	7.86	0.79	0.23	0.80
H2052	**131.40**	**38.86**	**158.14**	2.73	7.84	3.36
H2073	**901.64**	0.65	**1661.97**	2.95	4.80	5.36
H2087	2.17	1.01	1.96	0.56	1.05	0.89
H2122	2.83	2.44	**11.80**	0.64	0.45	0.36
H2126	2.71	6.67	3.46	0.87	0.30	0.61
H2347	**266.67**	**13.24**	**558.14**	2.87	0.12	5.69
H322	3.49	4.77	5.80	0.97	0.24	0.47
H441	**31.76**	**11.72**	**35.23**	1.09	0.11	1.07
H460	5.06	5.82	**19.58**	0.80	0.20	0.84
H661	1.78	2.11	1.75	0.88	0.51	0.96
H820	**135.62**	**10.31**	**100.00**	1.84	1.15	2.82
HCC366	**162.00**	**92.59**	**961.54**	0.36	0.04	0.25
HCC4017	8.57	0.76	**12.59**	0.94	1.38	0.70
HCC44	1.49	3.24	1.53	1.13	0.34	1.07
HCC95	**49.52**	3.39	**26.39**	1.65	0.34	1.04

Table 5.2: Fold Sensitization and Combination Indices for Paclitaxel/Carboplatin + mTOR Inhibition in NSCLC Cell Lines Left panel contains the fold difference in IC50 between paclitaxel/carboplatin alone and in combination with the three mTOR inhibitors. Green indicates a 4-fold or greater sensitization. Bolded and underlined text indicates a greater than 10-fold sensitization. Right panel contains the combination indices (CI) of the combinations with paclitaxel/carboplatin and the mTOR inhibitors. Green indicates synergy, gray indicates additivity, and red indicates antagonism.

A.

Erlotinib Sensitive



B.

Erlotinib Resistant

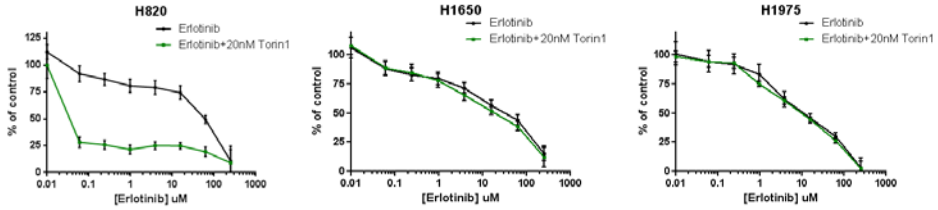


Figure 5.3: EGFR Mutant Cell Lines are not Further Sensitized to Erlotinib by Adding Torin1 Dose response curves of erlotinib sensitive lines (A) or erlotinib resistant lines (B) to erlotinib alone (black) and erlotinib+20nM Torin1 (green).

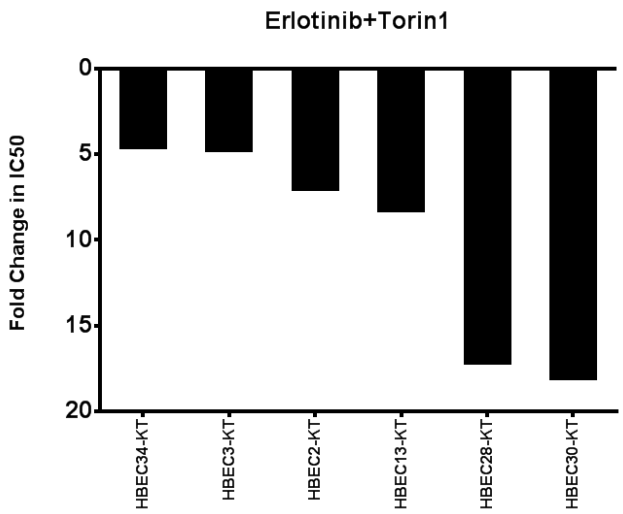


Figure 5.4: HBECS are Moderately Sensitized to Erlotinib with the Addition of Torin1 Fold changes in IC50s between erlotinib alone or in combination with Torin1.

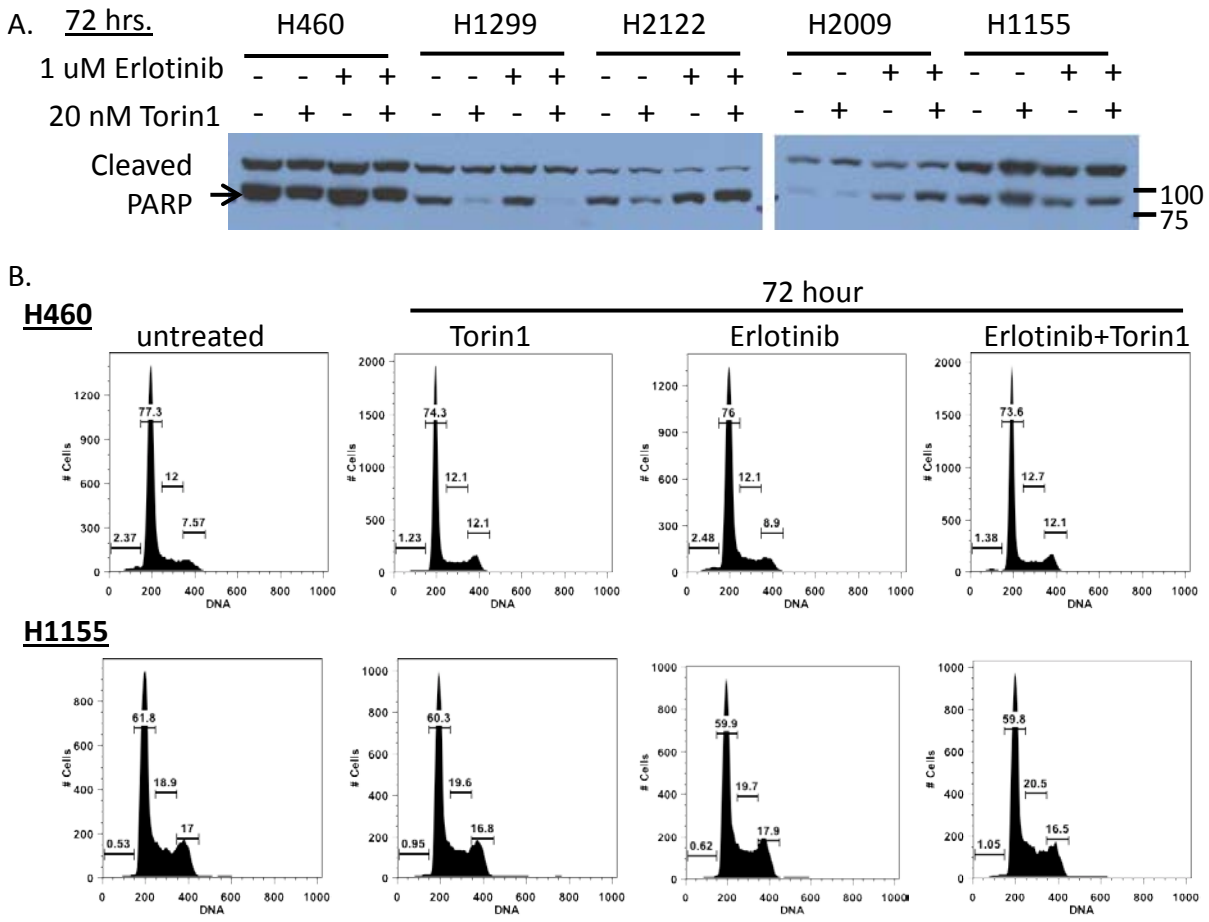


Figure 5.5: Erlotinib+Torin1 Does Not Lead to Increased Apoptosis Nor Changes in the Cell Cycle A. Immunoblot for PARP cleavage as an indictor for apoptosis induction in response to treatment with erlotinib and Torin1, alone or in combination. B. Cell cycle profiles determined by measuring DNA content by flow cytometry of cell treated with erlotinib and Torin1 alone or in combination.

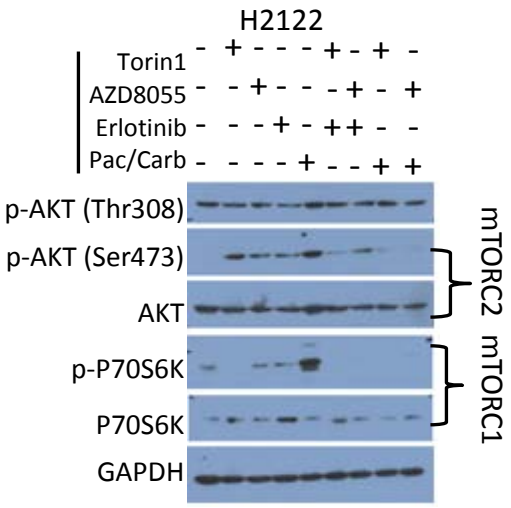


Figure 5.6: Drug Combinations Lead to Improved Inhibition of mTOR Activity Immunoblot of phospho- and total AKT and P70S6K following treatments with Torin1, AZD8055, erlotinib, paclitaxel/carboplatin alone or in combinations.

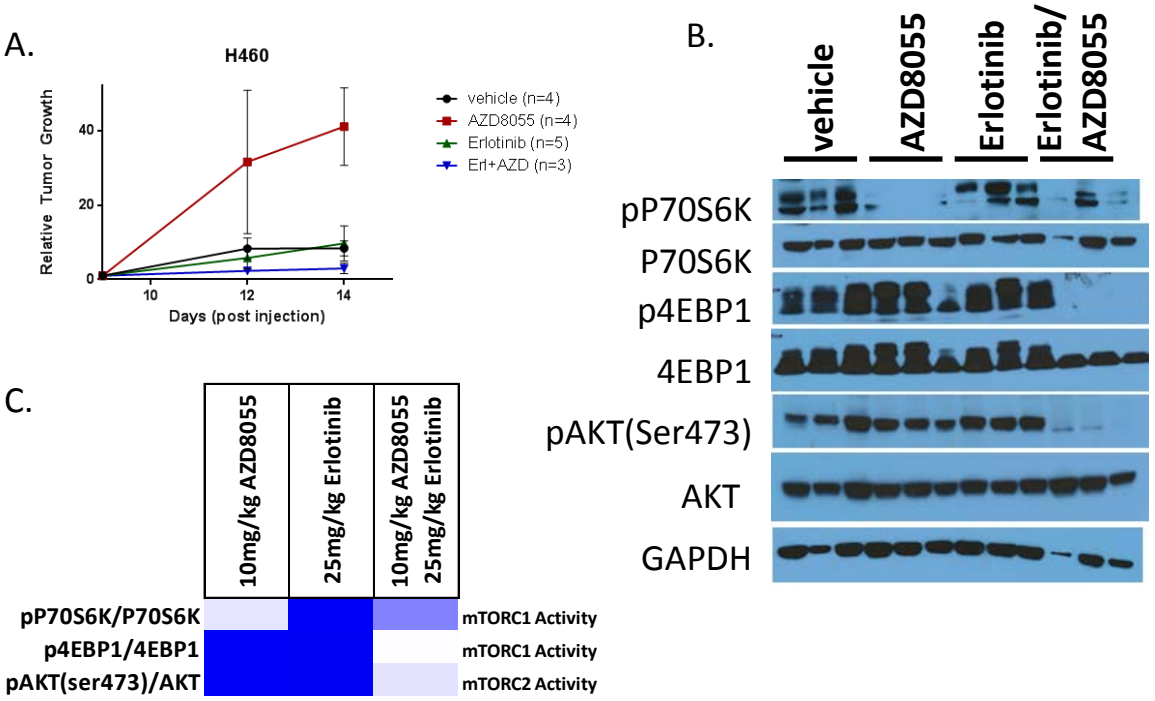


Figure 5.7 Combinations of AZD8055 and Erlotinib Reduce Relative Tumor Growth and Improve Inhibition of mTOR Signaling in a mTOR Inhibitor Resistant Xenograft

A. While H460 sub-cutaneous tumors are resistant to AZD8055 alone, combined treatments with erlotinib reduced the relative tumor growth. B. AZD8055 alone reduced mTORC1 activity on P70S6K in H460 tumors, however, AZD8055 combined with erlotinib significantly reduced mTORC1 activity on 4EBP1 and mTORC2 activity on AKT. C. Heat map representing the relative levels of mTORC1/2 activity in H460 tumors following daily treatment for five days.

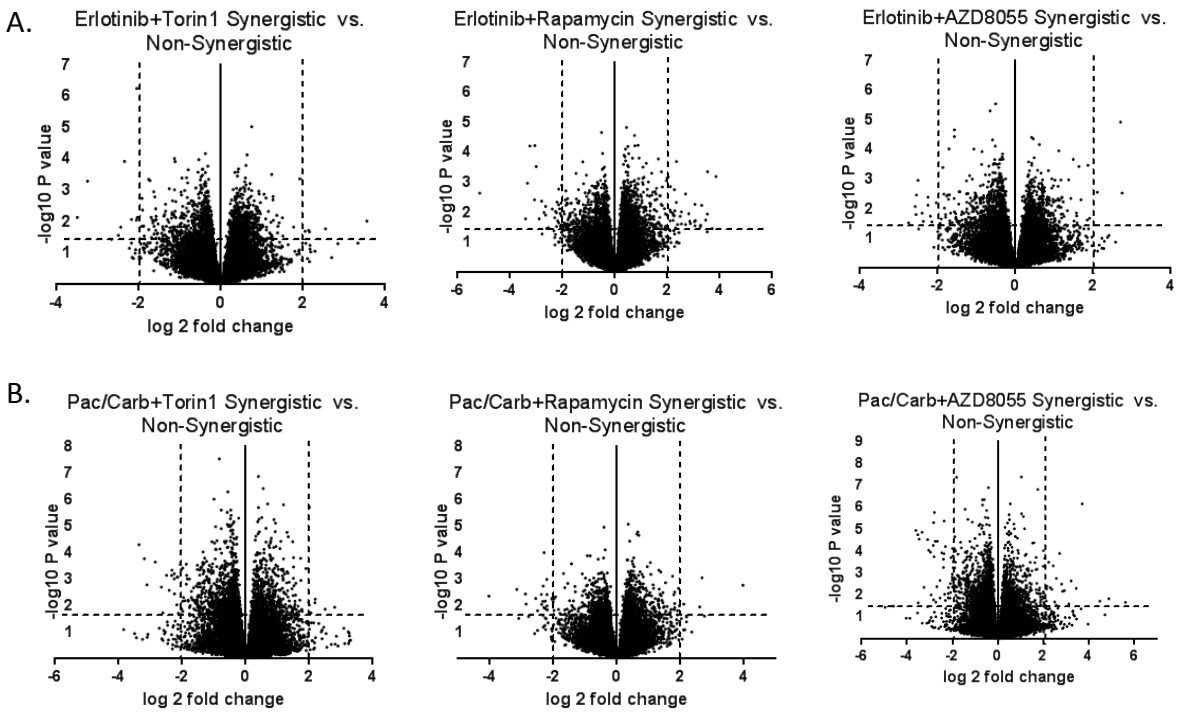


Figure 5.8: Differences in Gene Expression Between Synergistic and Non-Synergistic NSCLC Cell Lines
Volcano plots of log2 ratio of expression differences between erlotinib+ mTOR inhibitor (A) or Pac/Carb+ mTOR inhibitor (B) synergistic versus non-synergistic lines and –log10 of the p-values. Dots in the upper left-most and right-most represent genes which are significantly down- and up-regulated in sensitive vs. resistant lines, respectively. Left panels are combinations with Torin1, middle are combinations with rapamycin, and right panels are combinations with AZD8055.

Table 5.3: Differentially Expressed Genes in Erlotinib + mTOR Inhibitor Synergistic vs. Non-Synergistic NSCLC Cell Lines

Erl+Rap Synergy vs. Non-Synergy				Erl+Tor Synergy vs. Non-Synergy				Erl+AZD Synergy vs. Non-Synergy			
Gene ID	Symbol	log ratio	T-test P value	Gene ID	Symbol	log ratio	T-test P value	Gene ID	Symbol	log ratio	T-test P value
42359	KIF1A	-5.16	0.002	19226	QPRT	-3.50	0.007	10776	ID2	-2.58	0.008
25003	ARMCX2	-3.34	0.001	41772	CADM1	-3.25	0.001	14325	IGFBP5	-2.55	0.003
8180	ID2	-3.24	0.000	47816	DNAJC22	-2.35	0.000	22049	CLDN11	-2.51	0.001
11345	TMSB15A	-3.07	0.004	42851	UCP2	-2.18	0.008	3364	ALDH1A2	-2.25	0.004
10776	ID2	-3.06	0.000	15654		-2.06	0.009	13915	IGFBP5	-2.25	0.007
36748	MATN2	-3.00	0.000	5434	ProSAPiP1	-2.05	0.000	46584	ARHGAP4	-2.04	0.006
4643	CLIP3	-2.80	0.004	45337	APLP1	-2.04	0.007	21894	AHNAK2	2.11	0.003
42396	SLC2A10	-2.70	0.010	17608	CES1	3.56	0.009	40448		2.71	0.000
45792	NMU	-2.64	0.006					31728	PCDHB5	2.75	0.003
26113	CHST13	-2.39	0.008								
32513	HOXC6	-2.27	0.009								
38712	EFEMP2	-2.23	0.010								
30625	LRCH2	-2.21	0.004								
15946	HOXB7	-2.15	0.005								
44578	HOXC6	-2.14	0.002								
26383	GYG2	-2.11	0.007								
31947	SALL2	-2.06	0.005								
27486	PCSK1N	-2.03	0.001								
21425	CFH	2.04	0.001								
34711	DUSP5	2.10	0.008								
14441	NAMPT	2.12	0.001								
37849	STEAP1	2.13	0.009								
34509	IFITM1	2.21	0.010								
2579	CFH	2.27	0.002								
8515	FHOD3	2.29	0.009								
24645	IFI44L	2.34	0.005								
44700	PTHLH	2.35	0.000								
20793	XAGE1D	2.54	0.009								
24566		2.70	0.002								
27252	FAM113B	3.06	0.005								
19879	AKR1C4	3.31	0.006								
45581	KYNU	3.54	0.000								
22928	KYNU	3.86	0.001								

Table 5.4: Differentially Expressed Genes in Pac/Carb + mTOR Inhibitor Synergistic vs. Non-Synergistic NSCLC Cell Lines

P/C + Rap Synergy vs. Non-Synergy				P/C + Tor Synergy vs. Non-Synergy				P/C + AZD Synergy vs. Non-Synergy			
Gene ID	Symbol	PC+rap synergy vs non-synergy	T-test P value	Gene ID	Symbol	PC+tor synergy vs non-synergy	T-test P value	Gene ID	Symbol	PC+azd synergy vs non-synergy	T-test P value
629	NDN	-4.03	0.004	17614	OLFM1	-3.36	0.000	34785	DKK3	-3.61	0.000
4643	CLIP3	-3.15	0.002	2937	CXCR7	-3.19	0.000	26351	DKK3	-3.53	0.000
616	CPVL	-2.88	0.004	12260	SIRPA	-3.11	0.002	17614	OLFM1	-3.47	0.000
34785	DKK3	-2.86	0.009	32472	COL5A1	-2.85	0.000	19312	KCTD14	-3.34	0.000
12848	TMSB15B	-2.56	0.005	44782	GJA1	-2.65	0.002	38296	CMTM3	-3.25	0.004
8917	CPVL	-2.38	0.004	31640	CXCR7	-2.49	0.001	32472	COL5A1	-3.09	0.000
29051	BEX4	-2.37	0.009	40310	BAIAP2L2	-2.41	0.003	17576	CDH11	-3.05	0.000
857	TMSB15B	-2.33	0.007	32667	SPARC	-2.33	0.001	9325	ASPHD1	-2.97	0.000
20346	FSD1	-2.32	0.002	23966	CA12	-2.32	0.000	41022	CD14	-2.94	0.010
30216	CD302	-2.29	0.000	42626	FBLN2	-2.25	0.001	41524	NPTX2	-2.85	0.000
34846	SCARF2	-2.26	0.007	22049	CLDN11	-2.25	0.001	47187	FBLN2	-2.83	0.000
40774	DTNA	-2.21	0.001	47187	FBLN2	-2.24	0.004	10239	EPB41L3	-2.81	0.000
48088	NUDT11	-2.20	0.004	17045	CCDC28B	-2.17	0.009	42626	FBLN2	-2.80	0.000
33631	TRO	-2.20	0.010	45067	SFRP1	-2.15	0.001	47816	DNAJC22	-2.76	0.000
12710	NUDT11	-2.05	0.006	14859	DNM1	-2.14	0.001	5079	TSPYL5	-2.69	0.004
45751	ODZ3	-2.04	0.005	28752	NTNG1	-2.06	0.000	33574	SCG5	-2.67	0.000
11672	NKX3-2	-2.01	0.007	44794	NTNG1	-2.03	0.000	26048	TCEA3	-2.55	0.002
17335	ASS1	2.11	0.009	43218	CDKN2A	2.00	0.009	23103	GPR162	-2.47	0.000
16383	COL7A1	2.68	0.001	13393	ZNF667	2.01	0.000	45067	SFRP1	-2.40	0.000
10153	PTGES	3.97	0.002	21661		2.18	0.008	32667	SPARC	-2.40	0.001
				42310	ZSCAN18	2.19	0.006	39626		-2.38	0.000
								45435	CD14	-2.37	0.000
								17030	NRIP3	-2.35	0.009
								20346	FSD1	-2.31	0.000
								34115	METTL7B	-2.19	0.008
								41990	BEX1	-2.18	0.004
								32801	TM4SF18	-2.10	0.003
								27526	CD70	-2.09	0.007
								14859	DNM1	-2.07	0.000
								43961	LEPREL2	-2.02	0.001
								4424	CX3CL1	-2.01	0.002
								47558	TM4SF18	-2.01	0.001
								27818	ARMCX4	-2.00	0.000
								32806	PRSS3	-2.00	0.004
								46102	MST1R	2.06	0.000
								21804	EFNA1	2.14	0.005
								40280	BIK	2.15	0.004
								40255	CXCL16	2.23	0.000
								10461	CYP2J2	2.30	0.004
								25183	ITGB4	2.37	0.005
								11704	C2orf55	2.43	0.000
								10449	ITGB4	2.57	0.002
								18957	H2AFY2	2.71	0.000
								48632	MUC1	2.80	0.003
								40019	TACSTD2	2.84	0.006
								10183	ELF3	2.92	0.006
								21754	DLX5	3.22	0.002
								46678	MUC1	3.44	0.005
								14142	SLPI	3.70	0.000
								16256	MUC16	4.63	0.000

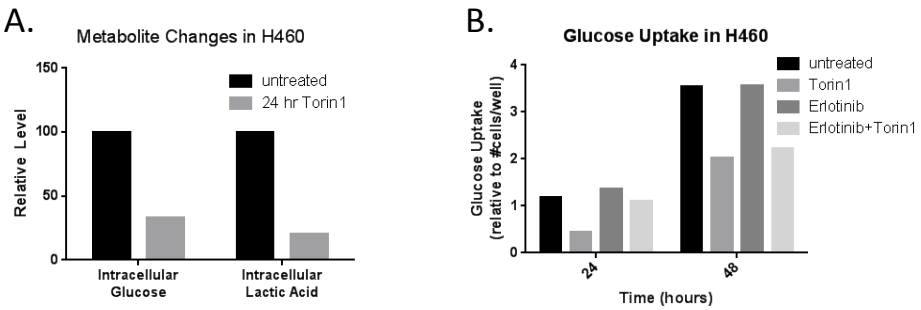


Figure 5.9: mTOR Inhibition Reduces Glucose Uptake in H460 Cells
A. GC-MS analysis indicated that glucose and lactic acid were reduced in Torin1 treated cells. B. Torin1 alone or in combination with erlotinib reduced the amount of glucose taken up by cells.

Table 5.5: Synergistic Lines Are Uniquely Vulnerable to Knockdown of 13 Metabolic Genes
Green boxes with Y indicate the cell line had a Z-score of less than -1 suggesting growth inhibition from siRNA targeting the indicated genes.

	Erlotinib+Torin1 Synergistic			Erlotinib+Torin1 **NON**-Synergistic								
Gene Names	**H460**	**H2009**	**H2122**	**HCC44**	**H1155**	**H1819**	**HCC4017**	**H1993**	**H2073**	**H1395**	**HCC95**	**HCC366**
TYR	**Y**	**Y**	**Y**	**N**	N	**N**	**N**	**N**	**N**	**N**	**N**	**N**
GLA	**Y**	**Y**	**Y**	**Y**	N	**N**	**N**	**N**	**N**	**N**	**N**	**N**
GAPDH	**Y**	**Y**	**Y**	**Y**	N	**N**	**N**	**N**	**N**	**N**	**N**	**N**
SGK3	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	N	**N**
AKT3	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	Y	**N**
MAPKAPK3	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	N	**N**
SNF1LK2	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	N	**N**
CDC42BPB	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	N	**N**
UGT2B15	Y	Y	Y	N	Y	**N**	**N**	**N**	**N**	**N**	N	**N**
MUSK	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	N	**N**
CDY1B	Y	Y	Y	N	N	**N**	**N**	**N**	**N**	**N**	Y	**N**
ATP4A	Y	Y	Y	Y	N	**N**	**N**	**N**	**N**	**N**	N	**N**
IL18BP	Y	Y	Y	Y	N	**N**	**N**	**N**	**N**	**N**	N	**N**

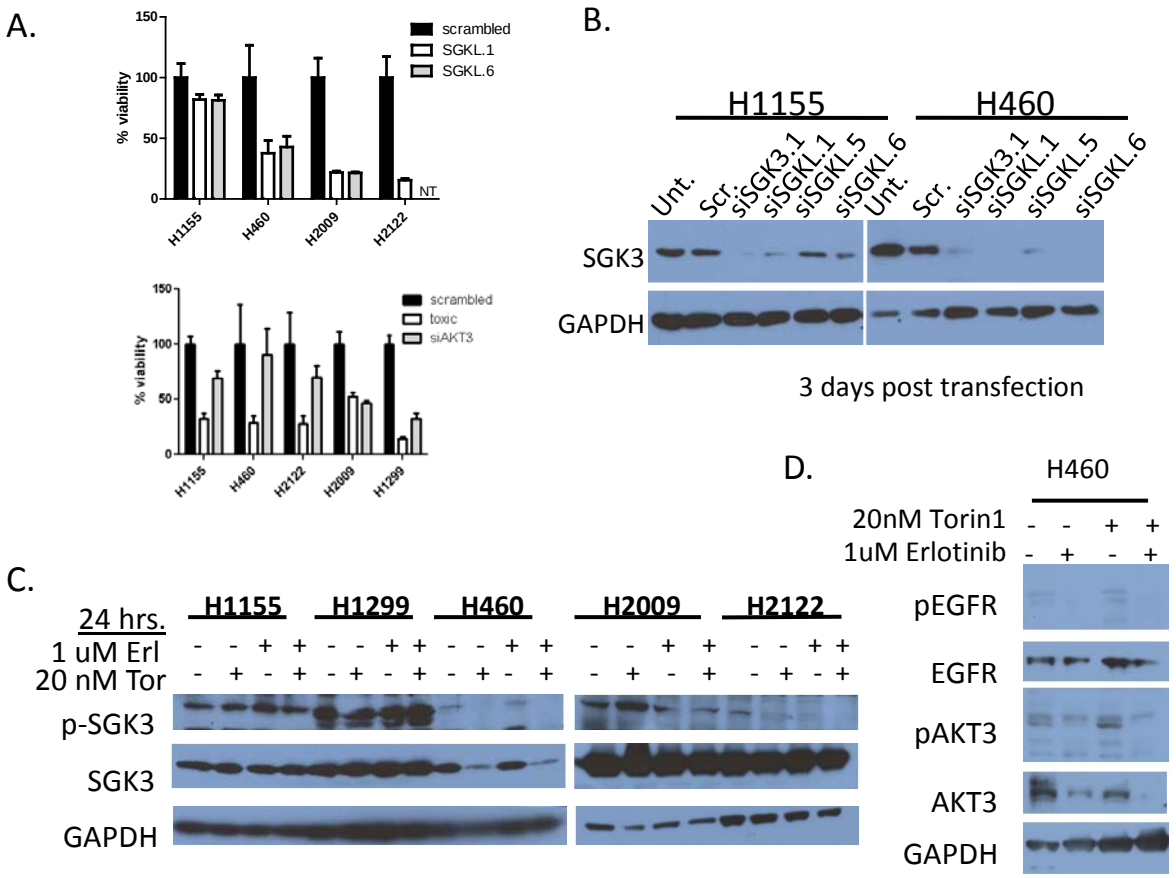


Figure 5.10: Association of SGK3, AKT1 and Synergistic Response to Erlotinib+Torin1 A. Viability of 4 NSCLC cell lines in response to siRNA mediated knockdown of SGK3 (upper) or AKT3 (lower). B. Validation of loss of SGK3 protein expression following siRNA mediated knockdown. C. Immunoblot of phospho- and total-SGK3 in response to erlotinib or Torin1 alone or in combination. D. Effect of erlotinib and Torin1 alone and in combination on phospho- and total-EGFR and AKT3.

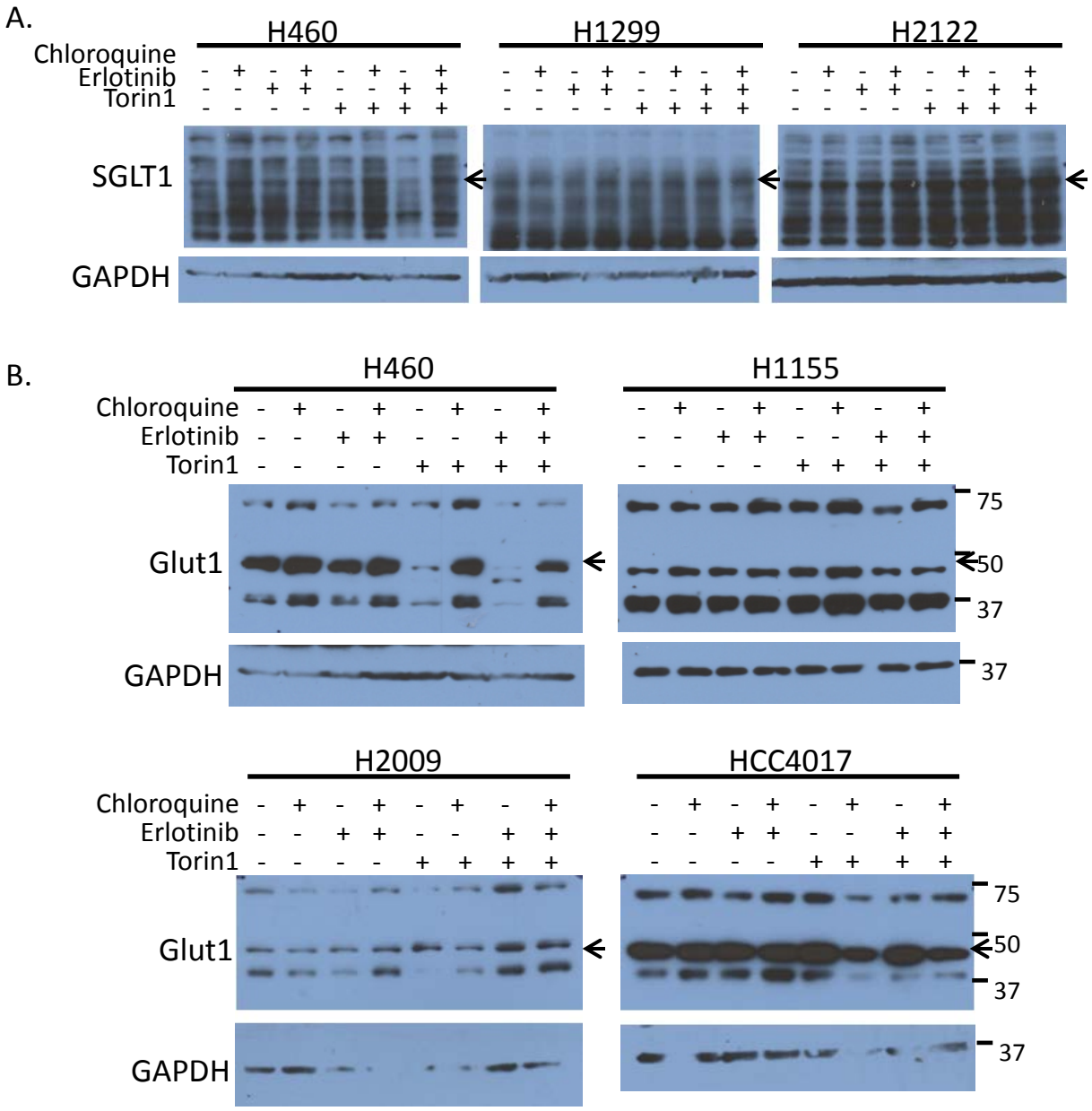


Figure 5.11: Effect of Erlotinib and Torin1 Drug Treatments on Expression of Glucose Transporters SGLT1 and Glut1 Immunoblot for the expression of SGLT1 (A) or Glut1 (B) in response to treatments with erlotinib, Torin1, or chloroquine alone or in combinations.

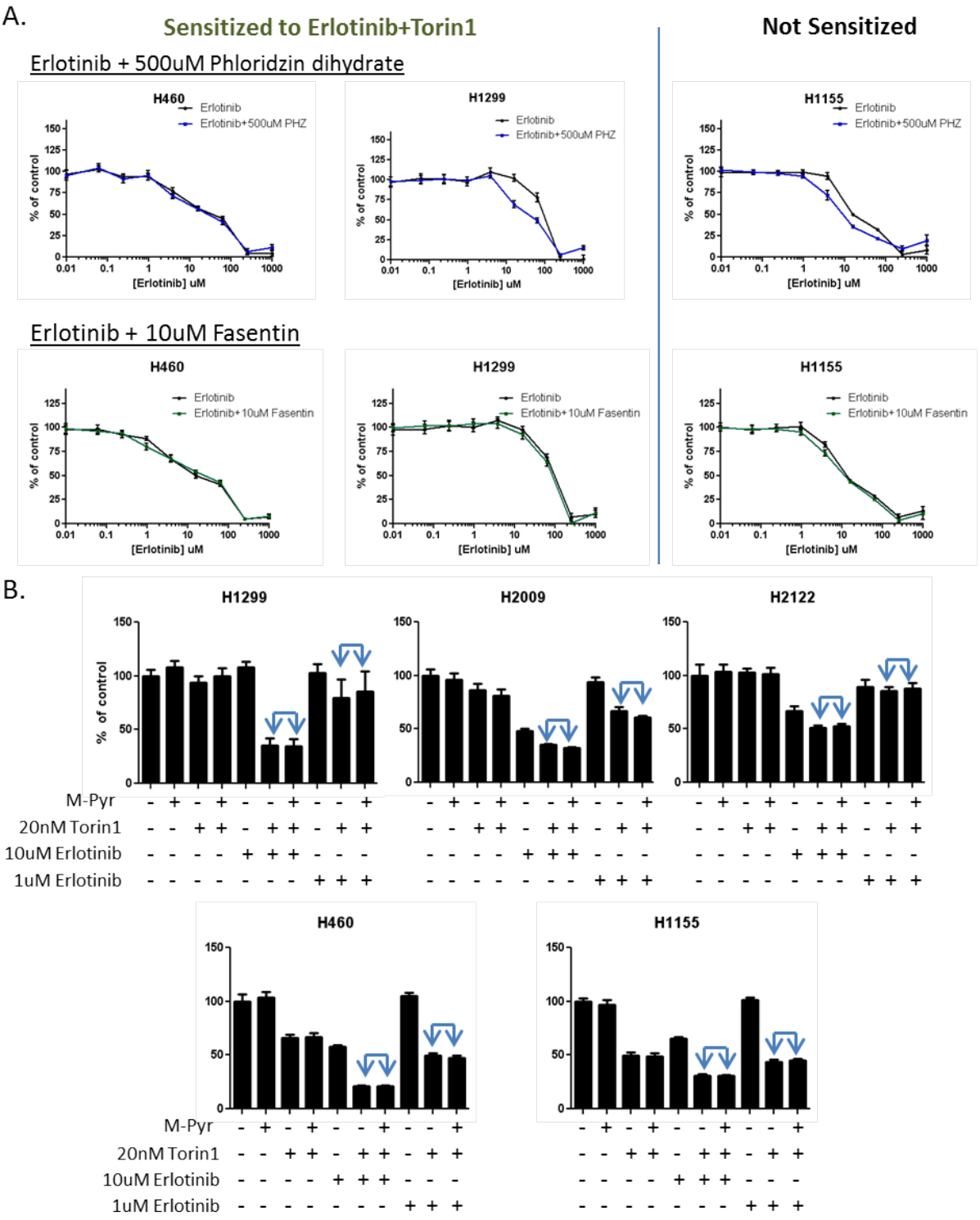


Figure 5.12: Inhibition of Glucose Transport Does Not Mimic the Synergistic Effects Seen with Erlotinib+Torin1, Nor Can This Effect Be Rescued by Membrane Permeable Pyruvate A. Dose Response curves for combinations with erlotinib and Phloridzen dihydrate (upper) or Fasentin (lower) in erlotinib+Torin1 synergistic (left panels) or non-synergistic (right panel) cell lines B. Viability of cell lines to Torin1 and erlotinib alone or in combination with and without the addition of methyl-pyruvate (m-Pyr).

Chapter 6: Autophagy Inhibition as a Lung Cancer Therapy

I. Abstract

Cells use the process of autophagy to rid themselves of damaged organelles and to break down proteins in order to recycle amino acids in conditions of low nutrient availability. The role of autophagy in cancer initiation and progression has confounded researchers due to the potential of the process to both prevent and promote tumorigenesis. As a result the most effective strategy to alter autophagy for the treatment of cancer has also been difficult to pin down. Previous chapters explored the use of mTOR inhibitors in NSCLC, in which the induction of autophagy is one downstream effect. Autophagy induction is thought to be an effective strategy if it can be activated to the extent that autophagic cell death occurs in the cancer cells. Alternately, autophagy can allow cancer cells to survive when nutrients are scarce, such as in the interior of a tumor, and also to overcome chemotherapy induced damage. As a result the inhibition of autophagy is also being explored as the therapeutic strategy. This chapter discusses the characterization of the response of NSCLC cells to a drug commonly used as an autophagy inhibitor, chloroquine, both as a single agent, and in combination with chemotherapy agents. We find that NSCLC cells display a very narrow range of response to chloroquine. In general, the dose of chloroquine required to achieve an IC50 is higher than the maximally achievable concentration (1 μM) in a patient, which is likely to limit its utility as anti-cancer agent. Furthermore, 1 μM chloroquine was incapable of sensitizing cancer cells to chemotherapy. We conclude that despite some reported success of chloroquine as a therapy in other cancer types, NSCLC cells show limited response.

II. Introduction

Autophagy

Autophagy is a process cells use to degrade damaged or unneeded organelles and proteins in order to recycle them to generate new proteins more efficiently (Choi, Ryter et al. 2013). Autophagy

becomes activated during times of cell starvation or stress and contributes to the maintenance of cellular integrity (Choi, Ryter et al. 2013). The process of autophagy involves the sequestration of cytoplasmic components into double-membraned compartments termed autophagosomes. Autophagosomes then fuse with lysosomes, delivering their content for degradation by lysosomal proteases (Choi, Ryter et al. 2013). Autophagy substrates were previously considered to be isolated through non-specific sequestration of cytosol. More recently it has been realized that several mechanisms of selectivity in the recognition of autophagy substrates exist; yet they still remain to be fully elucidated (Johansen and Lamark 2011, Choi, Ryter et al. 2013). Autophagy helps to turnover organelles, particularly mitochondria, clears poly-ubiquitinated protein aggregates, participates in the regulation of lipid metabolism, assists in immune responses, and protects from cell death by apoptosis. A greater understanding of the functions and regulation of autophagy has led to increased understanding of its important role in a variety of disease states, including metabolic and neurodegenerative disorders, cardiovascular and pulmonary diseases and cancer (Choi, Ryter et al. 2013).

Role of Autophagy in Cancer

The role of autophagy in cancer has confounded researchers due its multifaceted and seemingly opposing functions that could be in play. Autophagy has been reported to have both tumor-suppressive and tumorigenic properties (Kondo, Kanzawa et al. 2005, Amaravadi and Thompson 2007). In normal cells, autophagy is activated under stress in order to diminish damage and promote cellular senescence, a property that suppresses tumorigenesis (Altman and Rathmell 2009). However, autophagy activation under stress also promotes survival, dormancy, and regeneration, properties that potentially promote tumorigenesis (Gewirtz 2009).

It had been observed that early in tumorigenesis cancer cells have impaired autophagy, which leads an increase of genome damage and can stimulate inflammation and tumorigenesis (Kondo, Kanzawa et al. 2005, Mah and Ryan 2012). However, in an established tumor, autophagy enables tumor cells superior stress tolerance, and promotes cancer cell survival. Tumor cells must often survive in hypoxic environments with low access to nutrients. As a result these cancer cells may become dependent on autophagy for survival (Levine 2007). Furthermore, several chemotherapies and targeted agents have been demonstrated to lead to the activation of autophagy (Kondo, Kanzawa et al. 2005). Autophagy activation in response to drug treatments is thought to be a protective mechanism the cancer cells use in order to survive the stresses induced by these drugs. However, it is also possible for activation of autophagy to contribute to tumor cell death through cross-talk with apoptotic signaling or autophagic cell death (White and DiPaola 2009).

The exact role of autophagy in the tumor of a patient may vary depending on the particular alterations present in the tumor, which may influence the level of metabolic stress, the level of impairment, up-regulation of autophagy in the cancer cells, the stage of tumor progression, or other factors. Modulation of autophagy for the treatment of cancer is being investigated as a possible method to improve cancer therapies (Amaravadi and Thompson 2007). In this study, we investigated the potential utility of autophagy inhibition as a treatment for NSCLC. We aimed to identify subsets of autophagy inhibitor sensitive and resistant NSCLCs, and identify potential biomarkers to predict response, including oncogenotypes, gene expression differences, or presence of alterations in autophagy genes through mutation/methylation, etc. Furthermore, we tested the utility of autophagy inhibition to sensitize lung cancer cells to standard chemo- and targeted therapy agents as a method to prevent autophagic protection from cell death.

Chloroquine

In this study, the anti-malarial drug, chloroquine, was tested for treatment of NSCLC cells. Chloroquine inhibits autophagy due to its function as lysosomotropic agent. As such, it accumulates in lysosomes and raises their pH, which results in increased lysosomal permeability and prevents the fusion of the lysosome to the autophagosome, thereby preventing autophagic flux. Chloroquine and the related compound hydroxychloroquine have had promising results in preclinical models and are also being tested in combination with standard cancer therapeutics in clinical trials (White and DiPaola 2009).

To investigate the role of autophagy in lung cancer, NSCLC cells were screened for their response to autophagy inhibition using chloroquine. The goal of this screen is to identify subsets of NSCLCs that are sensitive or resistant to autophagy inhibition and to characterize oncogenotype and gene expression differences between these groups to identify predictive biomarkers.

III. Results

Cell Lines Display a Narrow Range of Response to Chloroquine

In order to determine the response of NSCLC cell lines to autophagy inhibition we first screened a panel of 63 cell lines for their response to chloroquine. Drug response phenotypes for the cancer cell lines were determined by generating dose response curves and using MTS reagent as a readout for cell viability. Cells were treated for 96 hours with 8 different concentrations of drug ranging from 0.008 μM to 125 μM. The IC50 was determined as a measure of comparing sensitivity or resistance to the drugs. Figure 6.1 summarizes the response phenotypes for the cell line panel to chloroquine.

The range of NSCLC cell line responses to chloroquine was very narrow. There was only a 10-fold difference in IC50 of the most sensitive and the most resistant cell line ranging from about 10 μM to about 100 μM. Such a narrow range of responses makes it difficult to identify functional biomarkers that will enable sensitive subsets to be predicted. Although there is a significant difference in the

response of the most sensitive and the most resistant cell lines, the narrow range of IC50s limits the statistical power when the goal is to correlate the drug response with other characteristics such as gene expression.

Furthermore, previous pharmacokinetic studies have indicated that the maximum level of chloroquine that is achievable in a human patient is about 1 μM (Augustijns, Geusens et al. 1992). This concentration is much lower than the level required to reduce the viability of a tumor cell. Therefore, the utility of chloroquine as a single agent for the treatment of cancer cells is likely to be inadequate due to the inability to get higher concentrations of drug delivered to the tumor.

Chloroquine Inhibits Autophagic Flux in NSCLC Cell Lines

Changes in the levels of autophagy are determined by analyzing the levels of the autophagosomal membrane bound form of essential autophagy protein LC3 (LC3-II). When autophagy becomes activated, LC3 is conjugated with a phosphatidylethanolamine (PE), which targets the protein to the membrane of the autophagosome. During autophagic flux, the autophagosomes fuse with lysosomes resulting in the degradation of proteins, including LC3. Inhibitors of autophagic flux, such as chloroquine, prevent the degradation of proteins through the autophagy pathway and lead to an observable increase in the levels of LC3-II (Klionsky, Abeliovich et al. 2008). Consistent with previously reports of chloroquine's function as an inhibitor of autophagy, treatment with this drug lead to a decrease in autophagic flux and an accumulation of LC3-II in NSCLC cells (Figure 6.2).

NSCLC Oncogenotype and Expression of Autophagy Related Genes Do Not Correlate with Response to Autophagy Inhibition

Previous reports have indicated that tumors that are driven by the Ras oncogene require autophagy for survival (Guo, Chen et al. 2011). Similarly, malignant progression of an oncogenic KRAS-driven mouse model of pancreatic cancer was reduced when autophagy was inhibited; however,

additional loss of p53 resulted in accelerated tumor onset with autophagy inhibition (Rosenfeldt, O'Prey et al. 2013). Additionally, autophagy inhibition has been shown to be an effective strategy in a MYC-driven model of lymphoma (Amaravadi, Yu et al. 2007). Therefore, we sought to determine whether lung cancers that are driven by these oncogenes are more susceptible to inhibition of autophagy by treatment with chloroquine. Of the ten most sensitive cell lines to chloroquine, 6 contain mutations in the Ras oncogene. However, there is no statistical difference in chloroquine IC50s of cell lines with KRAS mutations versus those with wild-type KRAS. Cell lines with mutations in LKB1 alone or in combination with KRAS also did not respond any differently to chloroquine than cell lines with wild-type versions of these genes. Similarly, there was no difference in response to chloroquine in EGFR mutant and wild-type lines (Figure 6.3).

In normal cells, the level of autophagy in cells is not regulated at the level of transcription. In cancer cells, however, it is unknown whether mRNA expression of autophagy genes correlates with levels of autophagy or response to autophagy manipulating drugs. Microarrays to determine mRNA expression of genes related to autophagy were analyzed in order to determine whether differential expression correlated with response to autophagy inhibition. Cell lines were clustered according the pattern of expression of 38 genes known to play a role in autophagy or it regulation (Figure 6.4A). HBEC cell lines demonstrated similar patterns of expression of these genes compared with one another and clustered separately from the cancer lines. Differences in culture medium of these cells potentially contribute to the separation of the HBEC lines. Cell lines generated from tumors from various histotypes did not cluster together based on their expression of autophagy genes. As further validation of the expression of genes related to autophagy in NSCLC cancer cell lines, RT-PCR was used to measure mRNA expression of three genes that function to negatively regulate autophagy (*AKT1S1, BCL2 and FRAP1*) and 5 genes that positively regulate autophagy *(ATG10, ATG16L2, ATG4C, ATG7, and ULK1*) (Figure 6.4B). Two cell lines, HCC2429 and HCC2450 expressed high levels of AKT1S1, and HCC1833 and HCC2429

express high levels of BCL2 relative to the other cell lines tested. HCC2450 also expresses high levels of ATG10 and ATG4C, relative to the other cell lines. However, there is no apparent correlation with expression of these genes and either the levels of autophagy or the response to autophagy inhibition.

Genome-wide analysis of microarray expression data was performed in order to determine whether there were any significantly differentially expressed genes between chloroquine sensitive and resistant cell lines. The top 15 most sensitive and 15 most resistant NSCLC cell lines based on the IC50s for chloroquine were used to calculate the log ratios of expression differences on a genome-wide scale using MATRIX software. Figure 6.5 portrays a summary of the results of the analysis. Genes that had a log2 of greater than 2 or lower than -2 (4 fold difference in expression) and a p-value of less than 0.05 were considered significantly differentially expressed between sensitive and resistant lines. The most significant down-regulated genes in sensitive cell lines are represented in the section of the graph above the horizontal dotted line and to the left of the left-most dotted line. The most significant up-regulated genes in sensitive cell lines are represented in the section of the graph above the horizontal dotted line and to the right of the right-most dotted line.

In chloroquine sensitive lines, there were 39 genes that were significantly under-expressed and 85 genes that were significantly over-expressed, compared to resistant lines. The largest difference in expression is a 10-fold difference for genes under-expressed in sensitive lines, and an almost 20-fold difference in expression for genes over-expressed in sensitive lines. The top underexpressed genes in chlroquine sensitive lines included a calcium binding protein, *S100P*, a ribosomal protein, *RPS4Y1*, a ligand for ephrin receptors, *EFNA1*, an enzyme in the arginine biosynthesis pathway, *ASS1,* and a membrane protein component of tight junctions, *CLDN7*. The top overexpressed genes in chlroquine sensitive lines included an actin binding protein in the filamin family, *FLNC*, a receptor tyrosine kinase, *Axl*, a serine-proteinase inhibitor, *SERPINE1*, and a G-protein regulator, *RGS4* (Table 6.1). Currently,

explanations for these expression differences in chloroquine sensitive and resistant lines are unknown as these genes have not previously been identified with autophagy or the response to autophagy manipulating drugs. Beclin-1, ATG genes, ULK1 and other autophagy related genes are not among those that are most differentially expressed between the 2 groups.

Autophagy Genes are Rarely Mutated in NSCLC Cell Lines

In some tumors autophagy is impaired, which can lead to accumulation of damaged organelles, improperly folded proteins, and genomic damage. Various alterations in essential autophagy genes and their regulation can result in impaired autophagy. Point mutations in *BECN1* are rare in human cancer, and unlikely to play a role in cancer pathogenesis (Lee, Jeong et al. 2007). In concordance with this fact, there are no mutations of the *BECN1* gene found in the panel of human NSCLC cell lines. Similarly, mutations in other autophagy genes including *ULK1, ATG4, ATG5, ATG7, ATG12*, and *MAP1LC3B* are rarely or never mutated in the cell lines.

In human breast, ovarian and prostate tumors, monoallelic disruption of the essential autophagy gene *BECN1* is found 40-75% of the time. In mouse models with this monoallelic loss (*BECN1+/-)* spontaneous tumorigenesis can occur, indicating Beclin-1 is a haplo-insufficient tumor-suppressor (reviewed in (Choi, Ryter et al. 2013). However, loss of *BECN1* also seems to be rare in the NSCLC cell lines. Copy number variations have been determined for 55 of the cell lines used for this study. Of these cell lines, only 3 demonstrated loss of 1 allele of the *BECN1* gene. Meanwhile, 15 had significant amplifications (CN>3) of the gene. There is no correlation between *BECN1* copy number and response to chloroquine. There is also no difference in Beclin-1 mRNA expression as measured by microarray in cancer cell lines compared to normal HBEC cells. Several (17/55) of the cell lines have loss of ATG5, with copy numbers less than 1.5. Monoallelic loss of ATG7, ATG4C, ULK1, and MAP1LC3B is

found in 5, 5, 6, and 9 cell lines, respectively. These copy number changes also do not correlate with drug response.

Chloroquine Does Not Sensitize Cancer Cell Lines to Starvation Conditions

Functional autophagy becomes more important for cell survival when access to nutrients is diminished. In order to determine whether autophagy inhibition reduced lung cancer cell survival under starvation conditions we compared cell viability of cells grown in HBSS in the absence and presence of chloroquine. The addition of chloroquine had no effect on viability of cells grown under starvation conditions at any time point between 2 and 48 hours in any of the cell lines tested (Figure 6.6A, B). When the cells were pretreated with chloroquine for 24 hours before switching to starvation conditions viability was only minimally reduced by 10-20% in 3 out of 4 cell lines tested (Figure 6.6C).

Inhibition of Autophagy Does Not Sensitize NSCLC Cell Lines to Standard Chemo- and Targeted-Therapy Agents

The utility of chloroquine as a single agent to treat lung cancer will likely be limited due to the requirement of higher than achievable concentrations of the drug to affect the viability of cancer cells. Even in the most sensitive cell lines, 10 μM chloroquine was needed in order to reduce growth by 50%, but the maximum drug concentration achievable in patients is 1 μM. Alternatively to finding modifications or mechanisms that improve drug delivery to the tumor, drug combination strategies may be useful. Autophagy has been demonstrated as a mechanism cancer cells use to overcome the stresses induced by chemotherapy drugs. Chloroquine is already widely used in patients for treatment of malaria and therefore drug tolerability, dosing schedules, and toxicity profiles are already well-known. As such, it would not be a great leap to begin testing this drug in patients as an autophagy inhibitor in combination with other cancer therapies. Nevertheless, pre-clinical studies of such drug combinations are needed in order to select any patients that may be predicted to respond synergistically to the

combination, and eliminate any that might be predicted to respond antagonistically. In order to determine whether such subsets exist, we combined 1 μM or 10 μM chloroquine in combination with varying doses of cisplatin (Figure 6.7A), paclitaxel (Figure 6.8A), paclitaxel/carboplatin doublet (Figure 6.9) or erlotinib (Figure 6.10) in a panel of NSCLC cell lines and assessed viability in response to the single agents compared to the combinations.

Overall, drug combinations with chloroquine were ineffective at sensitizing lung cancer cells to standard chemotherapy agents. Combinations with any of the standard chemo- and targeted agents with 1 μM chloroquine had no improvement over the single agents. Increasing the chloroquine concentration (10 μM) sensitized one cell line, HCC193, about 7-fold over cisplatin alone, but even this higher concentration was ineffective at sensitizing NSCLC cells to paclitaxel. Colony formation assays (Figures 6.7B, 6.8B, 6.10B) confirmed that despite inhibition of autophagy, physiologically achievable concentrations of chloroquine do not improve the responses of NSCLC cell lines to a variety of chemo- and targeted therapy drugs.

IV. Discussion

Research investigating the role of autophagy in the progression of cancer and the response to therapy has led to the idea that it is a double-edged sword. At early stages of cancer initiation, autophagy is frequently but reversibly turned off because of its potential be tumor suppressive (Kondo, Kanzawa et al. 2005, Amaravadi and Thompson 2007, Choi, Ryter et al. 2013). However, in later stages of cancer progression, active autophagy is beneficial to tumor cells and enables them to survive in low nutrient and hypoxic conditions and to overcome stresses induced by chemotherapy (Kondo, Kanzawa et al. 2005, Choi, Ryter et al. 2013). The aim of this study was to determine whether there is a subset of NSCLCs that are dependent on autophagy innately or to survive drug induced stresses by screening the cell lines' responses to autophagy inhibition with chloroquine treatment alone, or in combination with

chemo- and targeted therapy. We find that chloroquine does not effectively inhibit cell growth of NSCLC cells at physiologically relevant concentrations, nor does it sensitize cancer cell lines to other therapeutics.

Several previous studies investigating the utility of autophagy inhibition using chloroquine or other agents as anti-cancer agents have found a potential benefit in preclinical cell culture and animal models. The effects of various concentrations of chloroquine on the NSCLC cell line A549 were experimentally tested, and it was found that lower concentrations slowed cell growth and induced vacuolation and an increase in the volume of acidic compartments. At higher concentrations, chloroquine was found to induce apoptosis in this cell line (Fan, Wang et al. 2006). Autophagy inhibition using chloroquine or an shRNA against the autophagy gene ATG5 enhanced the level of cell death induced by tamoxifen in a Myc-induced model of lymphoma (Amaravadi, Yu et al. 2007). While we don't find any significant subsets of lung cancers that are dependent on autophagy, this result may be cell type specific and autophagy inhibition may be useful as a therapy in other cell types. More effective autophagy inhibitors may also expand the effectiveness of this class of drugs as an anti-cancer strategy.

Inhibition of autophagy is considered potentially useful as a strategy to combat protective autophagy initiated by treatment with other chemo- and targeted therapy drugs, or radiation (Kondo, Kanzawa et al. 2005, Amaravadi and Thompson 2007) Autophagy knockdown sensitized tamoxifen-resistant breast cancer cells to therapy by enhancing mitochondrial-mediated apoptosis (Qadir, Kwok et al. 2008). Similarly, bafilomycin A1, another late stage autophagy inhibitor, sensitized malignant glioma cells to temozolomide by enhancing cytotoxicity (Kanzawa, Germano et al. 2004). EGFR TKIs, gefitinib and erlotinib, have been shown to induce autophagy in resistant NSCLC cell lines A549 and H1299, and blockage of autophagy using chloroquine or siRNA knockdown of *ATG5* or *ATG7* attenuated erlotinib induced growth inhibition, suggesting that in these cell lines, autophagy activation resulted in drug

resistance (Han, Pan et al. 2011). Similarly, SKBR3 cells with acquired resistance to the anti-ERBB2 monoclonal antibody, trastuzumab, had higher levels of autophagy, which enabled them to survive and were exquisitely sensitive to inhibitors of autophagosome formation and function (3MA, LY294002, and bafilomycin A1) (Vazquez-Martin, Oliveras-Ferraros et al. 2009). Additionally, when treatment naïve cells overexpressing ERRB2 were treated with trastuzumab, surviving cells had increased levels of autophagy which facilitated their survival (Vazquez-Martin, Oliveras-Ferraros et al. 2009). Knockdown of autophagy genes using siRNAs enhanced the cytoxocity of radiotherapy in a variety of cancer cell lines (Apel, Herr et al. 2008).

One caveat that must be considered in studies using chloroquine *in vitro* is the concentration of drug used. The dose responses measured in this study used concentrations of chloroquine ranging from less than 10 nM up to over 100 μM. The median IC50 for the NSCLC cell lines was approximately 30 μM, with the most sensitive lines having an IC50 in the 10 μM range. The dose of chloroquine tolerated by patients is limited due to retinal toxicity (Augustijns, Geusens et al. 1992). Steady-state blood concentrations vary considerably and were found to range from 36.6 ng/ml to 3.9 μg/ml (equivalent to ~0.1-10 μM) in patients given 250 mg chloroquine sulphate per day, with the median blood concentration 474 mg/ml (1.4 μM) (Augustijns, Geusens et al. 1992). Studies using chloroquine to treat rheumatoid arthritis found that the mean plasma concentrations in responders and non-responders were 1.04 and 1.6 μM, respectively (Wollheim, Hanson et al. 1978). Therefore, for the purposes of this study, 1 μM chloroquine was considered a physiologically attainable concentration, though the actual concentration that accumulates in human tumors is unknown. *In vitro*, low concentrations of chloroquine (1 μM or less) are effective at inhibiting autophagy, but higher concentrations are required to affect viability. Since the concentration of chloroquine required to achieve 50% inhibition of even the most sensitive NSCLC cell lines is 10-times higher than the achievable blood concentration, we conclude that the use of chloroquine to treat lung tumors is not very likely to be effective. Commonly, other

preclinical studies that conclude chloroquine may be an effective therapeutic for this indication use concentrations from 10-100 μM. While these studies may indicate autophagy inhibition is a promising strategy, additional refinement of autophagy inhibiting agents may be required to get a robust clinical result.

Results from preclinical studies with autophagy inhibition suggest that the addition of these agents to standard chemotherapy ought to improve tumor response. As a result a number of clinical trials have been developed to analyze the potential benefit of such a strategy. One such study compared the effects of adding chloroquine to conventional chemotherapy and radiotherapy in a small set of glioblastoma multiforme patients (Sotelo, Briceño et al. 2006). They found that the median survival for the chloroquine group was 24 months compared to 11 months with placebo; however, larger-scale studies are needed to verify this trend. Autophagy inhibition is also being tested in breast cancer (NCT01292408), small-cell lung cancer (NCT00969306), in combination with carboplatin, paclitaxel, and bevacizumab in NSCLC (NCT00933803 , NCT01649947), in combination with AKT inhibitor MK2206 in solid tumors, prostate, or kidney cancer (NCT01480154), in combination with gemcitabine in pancreatic cancer (NCT01506973), among others. In addition to the modest responses observed thus far using autophagy targeting drugs, there is also a possibility that use of chloroquine in combination with anti-cancer drugs may result in additional toxicities. Anti-cancer drugs frequently accumulate in the kidneys, which are highly vulnerable to chemotherapy. Autophagy is thought to protect against acute kidney damage, which will be exacerbated by the addition of chloroquine to cancer therapy (Kimura, Takabatake et al. 2013). In addition to determining the efficacy of chemotherapy combinations with chloroquine on inhibiting tumor growth, additional adverse effects that may result from such a combination strategy will need to be closely monitored.

Due to a narrow range of response of the NSCLC cell lines to autophagy inhibition by chloroquine, we were unable to find any significant correlations with drug response and other molecular classifications including oncogenotype. However, previous studies have found that Ras-driven cells are dependent on autophagy for survival. Guo JY, et al. demonstrated that expression of either the $Hras^{V12}$ or $Kras^{V12}$ oncogene in immortal, non-tumorigenic baby mouse kidney epithelial (iBMK) cells led to a 10-fold increase in basal autophagy, and a deficiency in autophagy resulted in loss of viability in starvation conditions and reduced tumorigenicity due to metabolic stress(Guo, Chen et al. 2011). In a small panel of human bladder, lung, pancreatic, colorectal or prostate cancer cell lines with mutations in Ras (Hras, Nras, and Kras mutations were represented), all but lung cancer cell line H460 displayed elevated levels of basal autophagy. They found that chloroquine suppressed or attenuated growth of Ras mutant cell lines with high levels of basal autophagy, and a subset of cell lines was sensitive to lentiviral knockdown of ATG5 or ATG7 (Guo, Chen et al. 2011). A follow-up study using a genetically engineered Ras-driven NSCLC or PDAC mouse models confirmed that autophagy is required for tumorigenesis (Guo, Karsli-Uzunbas et al. 2013, Guo and White 2013, Rosenfeldt, O'Prey et al. 2013). Authors of Guo et al. describe the dependency on autophagy in Ras-driven tumors by explaining that energy depletion is amplified in these cells, which increases the dependency on autophagy to buffer the demand for energy through the preservation of mitochondrial function (Guo, Chen et al. 2011). In the panel of cell lines used in this study, only 1 (H460) had a co-mutation in LKB1. Presence of this mutation likely explains the difference in autophagy levels and response to autophagy inhibition in this cell line as proper function of this gene is important for maintenance of energy sensing and induction of autophagy in response to low nutrients. Therefore, cells with co-mutations in KRAS and LKB1 have a unique metabolic profile and may have a program of metabolic flexibility that allows them to survive even when autophagy is inhibited. Other mutations in addition to Ras may also influence the metabolic profile and dependence on autophagy. Two NSCLC cell lines, H1299 and H460, used in Guo, et al. were also tested for sensitivity to

autophagy inhibition in the present study. Results from the two studies agree that H1299 was much more sensitive to chloroquine treatment than H460. However, Guo, et al. tested for the effect of 30 μM chloroquine on growth inhibition, which is higher than what is considered a physiologically achievable concentration, and the reduction in growth rate of H1299 was minimal. Nevertheless, the notion that a subset of Ras-driven tumors may be dependent on autophagy has important implications for therapy, and suggests a need for the development and inquiry of more specific and potent autophagy inhibitors.

V. Figures

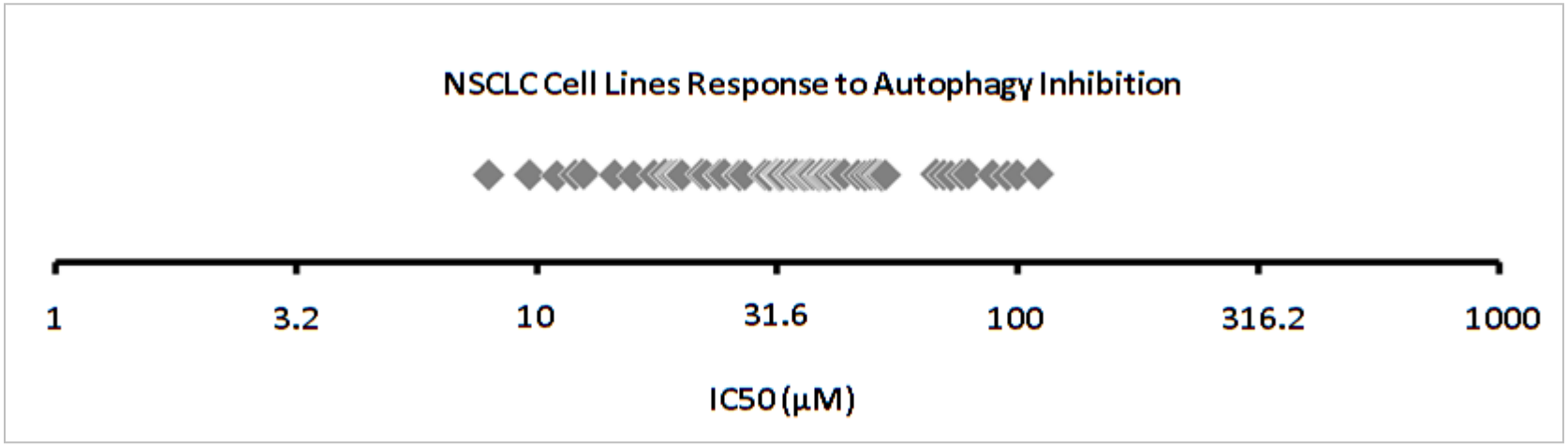


Figure 6.1: NSCLC Cell Lines Display a Narrow Range of Response to Autophagy Inhibition Each dot represents the IC50 of a particular cell line

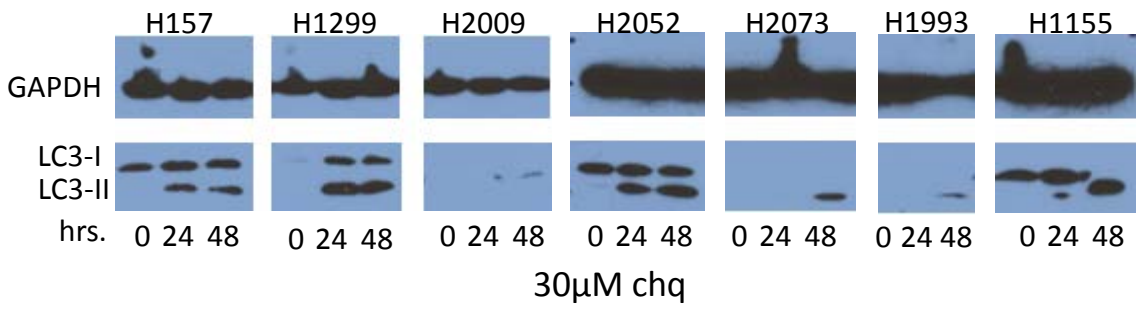


Figure 6.2: Chloroquine Leads to Autophagosome Accumulation in NSCLC Cells Immunoblot for LC3 following treatment with 30uM chloroquine in a panel of NSCLC cell lines.

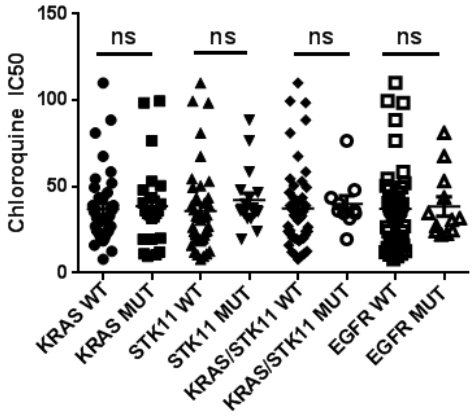


Figure 6.3: Common Oncogenotypes Do Not Predict Response to Chloroquine Comparison of IC50s of Kras, STK11, KRAS/STK11 co-mutants, or EGFR mutants vs. wild-type (WT).

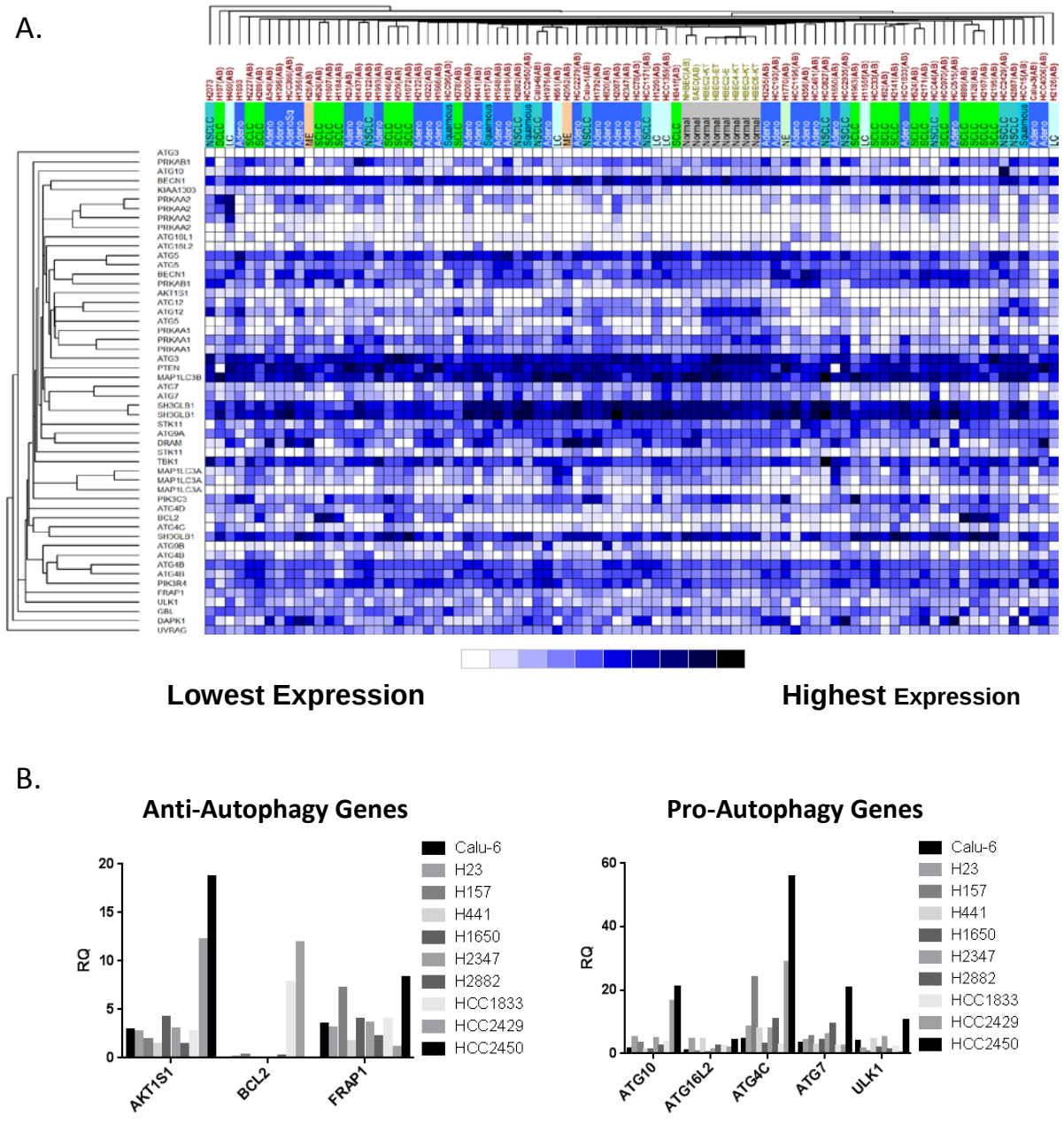


Figure 6.4: Variation in Expression of Autophagy Related Genes is Minimal and Does Not Correlate with Response to Chloroquine A. Cluster analysis of expression of genes related to autophagy across the panel of NSCLC cell lines and HBECs B. Quantitative PCR of three genes that inhibit autophagy and five genes that promote autophagy in a panel of NSCLC cell lines.

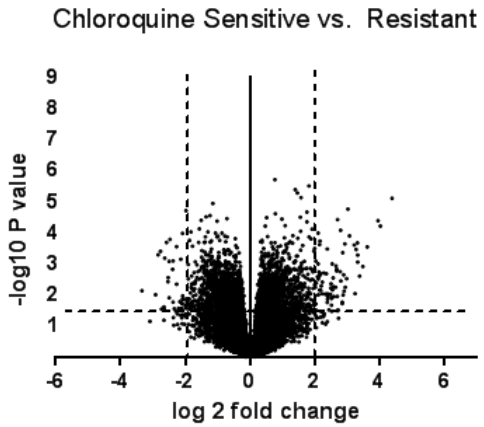


Figure 6.5: Differences in Gene Expression Between Chloroquine Sensitive and Resistant Cell Lines Volcano plots of log2 ratio of expression differences between chloroquine sensitive and resistant cell lines and –log10 of the p-values. Dots in the upper left-most and right-most represent genes which are significantly down- and up-regulated in sensitive vs. resistant lines, respectively.

Table 6.1: Differentially Expressed Genes in Chloroquine Sensitive and Resistant Cell Lines

Chloroquine Sensitive vs. Resistant			
Gene ID	Symbol	log ratio	T-test P value
10214	S100P	-3.34	0.007
38841	RPS4Y1	-2.92	0.009
23456	EFNA1	-2.84	0.000
1085	ASS1	-2.77	0.000
32866	CLDN7	-2.74	0.027
24423	HOPX	-2.66	0.027
40593	OVOL2	-2.66	0.023
604	NCALD	-2.64	0.001
3523	NCALD	-2.58	0.000
27285	IGFBP2	-2.54	0.004
7963	PARM1	-2.53	0.003
47621	GSTT1	-2.50	0.012
14111	LRRC26	-2.50	0.001
21804	EFNA1	-2.47	0.000
35660	GADD45G	-2.39	0.001
46200	LRRC26	-2.38	0.001
5452	FOXA1	-2.38	0.016
46678	MUC1	-2.34	0.026
7475	CRIP1	-2.29	0.032
17335	ASS1	-2.27	0.000
24557	SBK1	-2.27	0.004
4344	CKMT1B	-2.25	0.039
1141	SYK	-2.21	0.025
33178	SOX2	-2.18	0.042
22310	CKMT1B	-2.17	0.016
20501	MAL2	-2.16	0.042
12022	ZNF704	-2.14	0.001
30974	GABBR2	-2.14	0.027
20268	AGR2	-2.11	0.017
47985	CXCL17	-2.11	0.026
42275	FGFR3	-2.10	0.018
37643	MSMB	-2.09	0.013
17793	CPLX1	-2.09	0.000
14142	SLPI	-2.09	0.041
43558	ABCA4	-2.08	0.010
10985	CAPN8	-2.08	0.030
5568	HS6ST2	-2.07	0.013
22692	TJP3	-2.04	0.043
39685	CACNA1H	-2.00	0.022

Chloroquine Sensitive vs. Resistant			
Gene ID	Symbol	log ratio	T-test P value
6215	AP1S2	2.01	0.005
24891	IL7R	2.01	0.010
41041	ETS1	2.03	0.001
27139	TMSB15B	2.03	0.018
46828	GYPC	2.03	0.045
41430	RGS20	2.04	0.004
21873	COL6A2	2.06	0.030
45328	ABI3BP	2.08	0.002
45775	EVI2B	2.10	0.001
7060	AP1S2	2.10	0.003
22183	NLRP3	2.10	0.000
27782	CALD1	2.11	0.003
21290	MOXD1	2.11	0.009
41939		2.12	0.002
47341	NR2F2	2.14	0.000
47176	BDNF	2.14	0.007
2179	PROCR	2.16	0.002
46767	DSE	2.16	0.002
37615		2.16	0.000
1074	RAC2	2.17	0.049
23	F3	2.18	0.007
39784	MSRA	2.19	0.005
1194	APCDD1L	2.20	0.033
23403	KRT34	2.22	0.009
7065	CAV1	2.23	0.004
37004	CD44	2.26	0.047
31491	PTRF	2.27	0.002
32472	COL5A1	2.27	0.043
28458	GAS6	2.29	0.038
48098	PMP22	2.32	0.009
25099	CAP2	2.35	0.002
12586	IL1A	2.37	0.010
44615	ADAMTS1	2.38	0.000
8283	RFTN1	2.38	0.017
46929	VEGFC	2.39	0.009
17582	STC1	2.39	0.010
41934	THY1	2.39	0.013
45751	ODZ3	2.40	0.011
966	GAS6	2.43	0.029
36144	LAYN	2.48	0.009
4293	CALD1	2.49	0.006
23572	AOX1	2.50	0.004
36733	THBS1	2.51	0.020
25155	MARCH4	2.51	0.012
31640	CXCR7	2.52	0.012
2426	SRGN	2.52	0.032
35330	CAV1	2.53	0.001
35657	DKK1	2.54	0.006
44652	ADAM19	2.56	0.003
12848	TMSB15B	2.58	0.005
45067	SFRP1	2.59	0.011
22049	CLDN11	2.64	0.005
4995	MECOM	2.65	0.001
19768	F3	2.67	0.001
22401	FBN2	2.67	0.002
7593	CDH2	2.69	0.011
22080	IGFBP7	2.70	0.031
45054	FOSL1	2.70	0.000
3553	CPA4	2.71	0.029
48749	BCAT1	2.78	0.002
29608	F2R	2.79	0.000
16868	THBS2	2.80	0.003
45736	VIM	2.84	0.016
5590	VIM	2.84	0.018
27818	ARMCX4	2.86	0.000
2937	CXCR7	2.87	0.009
2126		2.89	0.002
9946	HS3ST3A1	2.90	0.007
48335	ADRB2	2.92	0.000
5816	IL1B	2.94	0.005
25146	LOX	2.98	0.003
12259	NRG1	3.02	0.000
47470	IGFBP6	3.07	0.000
3959	DAB2	3.21	0.000
32667	SPARC	3.24	0.009
25040	DAB2	3.27	0.000
39790	GLIPR1	3.31	0.000
3842	DFNA5	3.32	0.000
36747	C12orf75	3.32	0.001
19992	TMEM158	3.38	0.002
11120	RGS4	3.48	0.001
5149	SERPINE1	3.62	0.000
42041	AXL	3.95	0.000
30170	AXL	4.02	0.000
3864	FLNC	4.38	0.000

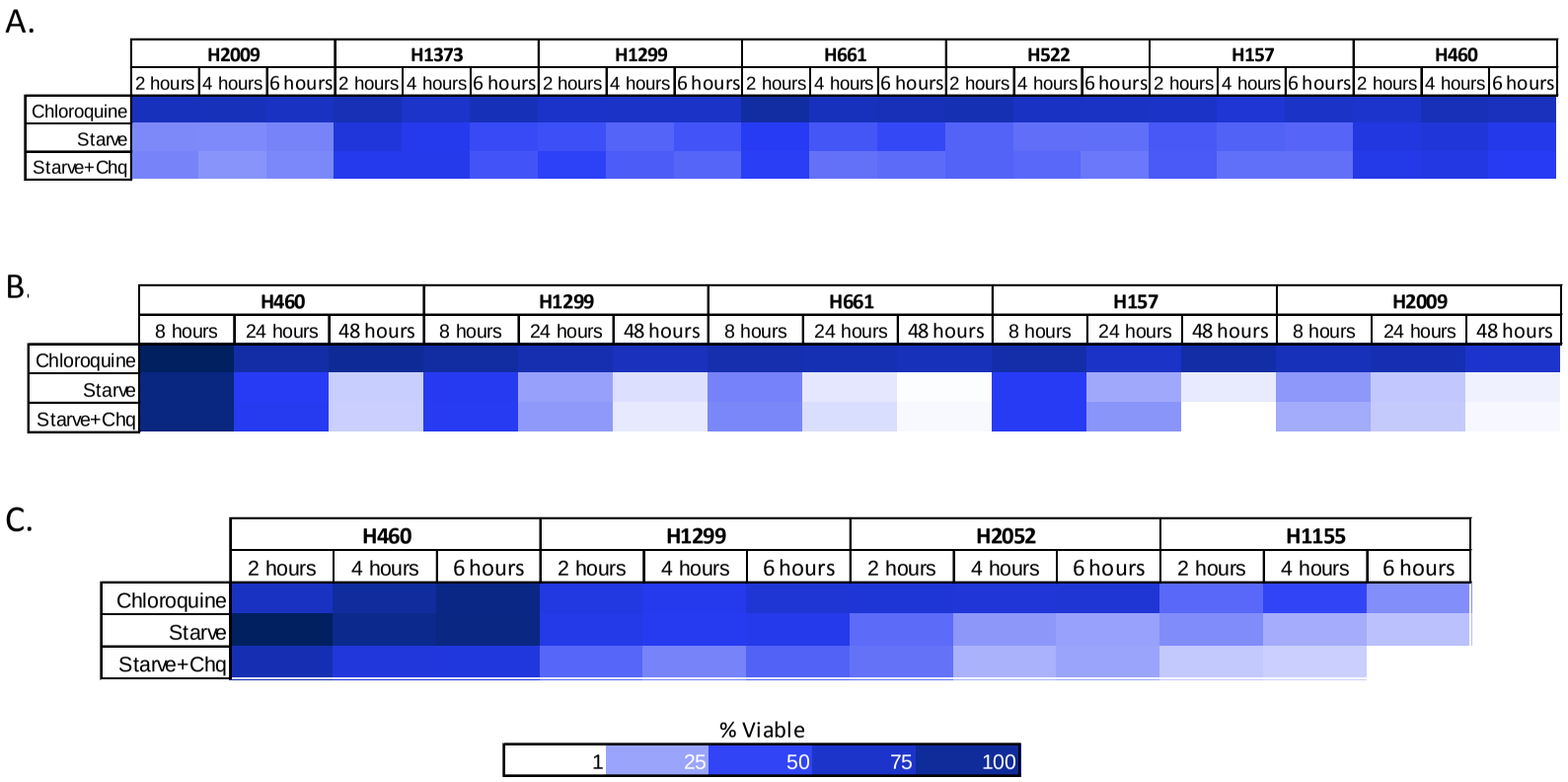
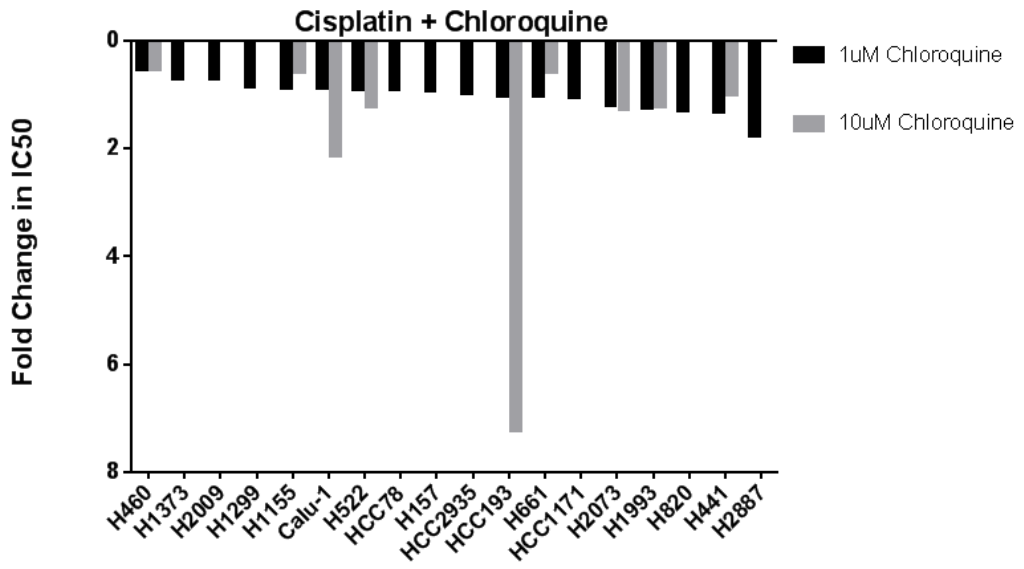


Figure 6.6: Chloroquine Does Not Sensitize NSCLC Cell Lines to Starvation Conditions Cells were treated with chloroquine, Hank's Buffered Salt Solution (HBSS), or the combination for short time points (A), or long time points (B). C. Cells were pre-treated with chloroquine for 24 hours and then exposed to starvation conditions for the indicated time points.

A.



B.

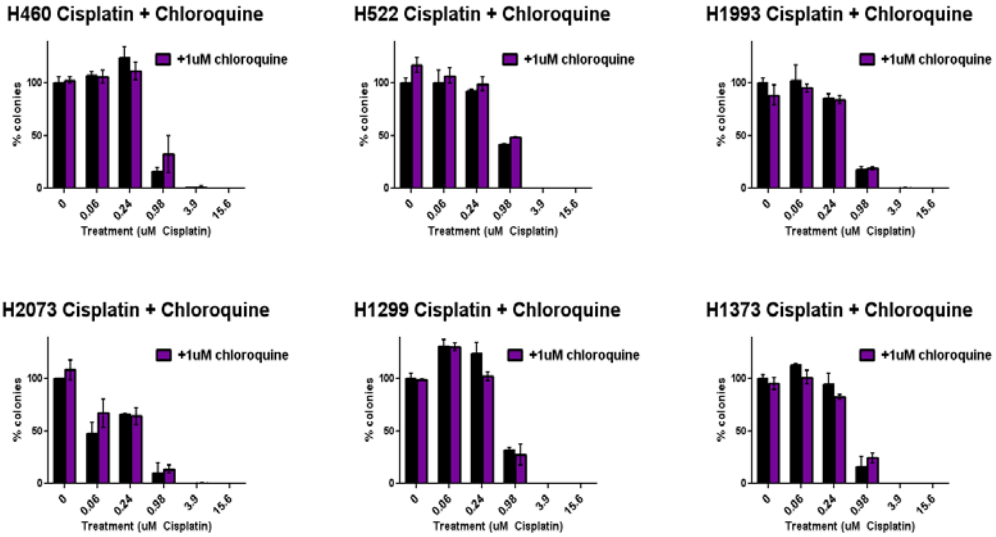
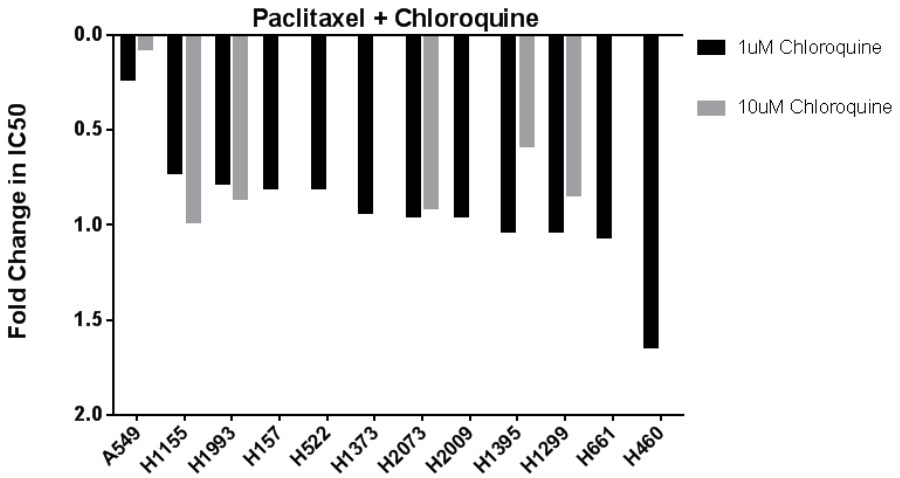


Figure 6.7: Chloroquine Does Not Sensitize NSCLC Cell Lines to Cisplatin A. Fold changes in IC50s between cisplatin alone or in combination with 1uM or 10uM chloroquine. B. Colony formation of NSCLC cells treated with cisplatin alone or in combination with 1uM chloroquine.

A.



B.

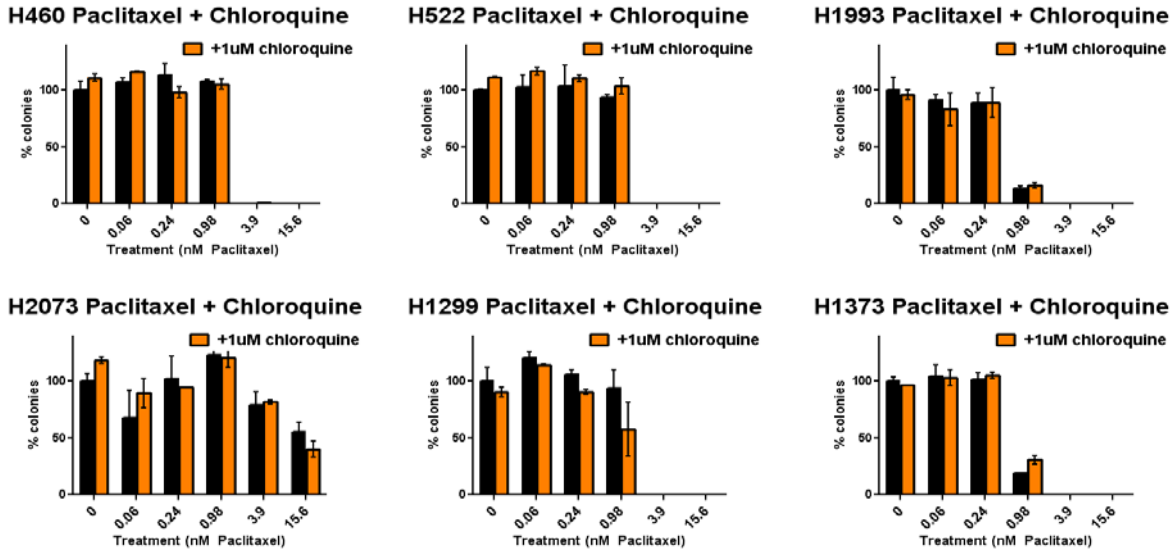


Figure 6.8: Chloroquine Does Not Sensitize NSCLC Cell Lines to Paclitaxel A. Fold changes in IC50s between paclitaxel alone or in combination with 1uM or 10uM chloroquine. B. Colony formation of NSCLC cells treated with paclitaxel alone or in combination with 1uM chloroquine.

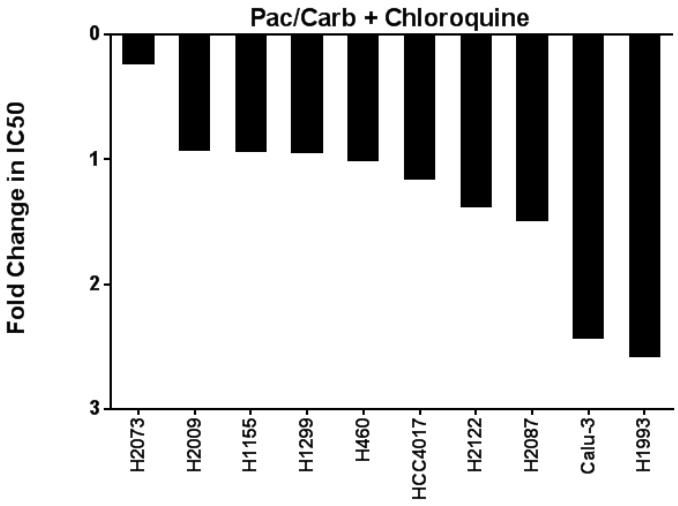
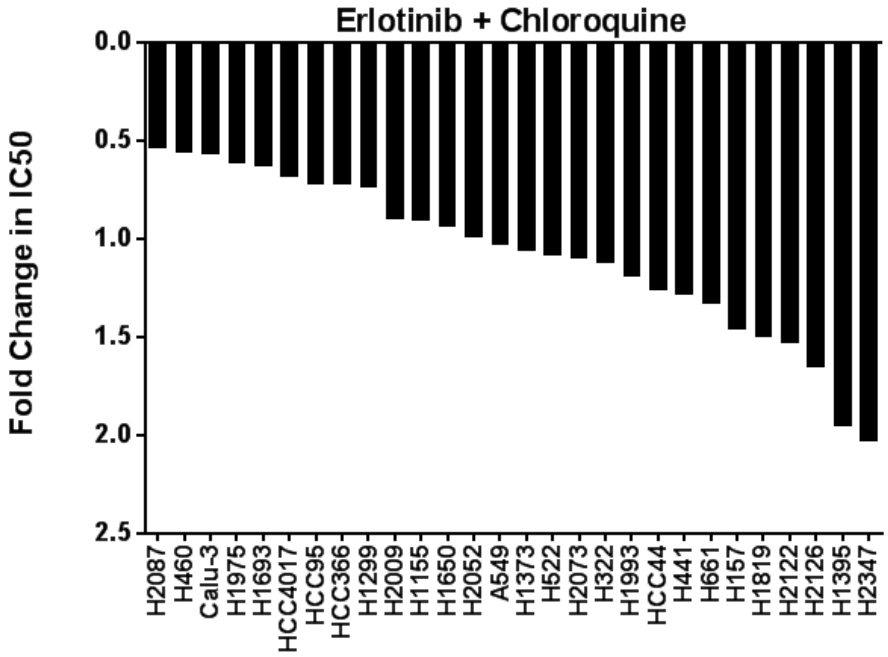


Figure 6.9: Chloroquine Does Not Sensitize NSCLC Cell Lines to Paclitaxel/Carboplatin A. Fold changes in IC50s between pac/carb alone or in combination with 1uM.

A.



B.

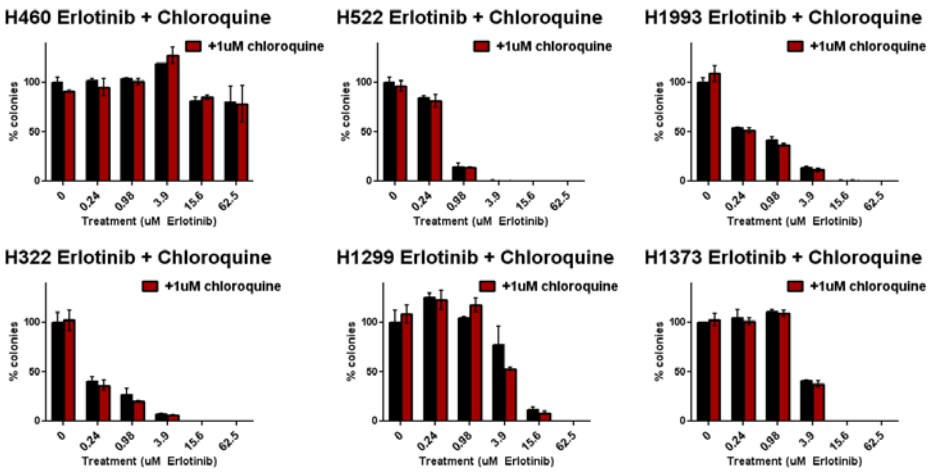


Figure 6.10: Chloroquine Does Not Sensitize NSCLC Cell Lines to Erlotinib A. Fold changes in IC50s between erlotinib alone or in combination with 1uM. B. Colony formation of NSCLC cells treated with erlotinib alone or in combination with 1uM chloroquine.

Chapter 7: Conclusions, Future Directions, and Perspectives

I. Conclusions

Our knowledge about the role of mTOR in the regulation of cell growth, proliferation, and the protein synthesis is continuing to increase. It is clear that deregulation of mTOR has broad implications in human disease including diabetes, obesity, autoimmune disorders, neurological disease, aging, and cancer (Laplante and Sabatini 2012, Santulli and Totary-Jain 2013). Although mTOR itself is not an oncogene, and it is not mutated in cancer, several other oncogenic alterations lead to aberrant regulation of mTOR. Most notably, activation of RTKs including EGFR and ERBB2, oncogenic KRAS, and loss of tumor suppressors PTEN and STK11/LKB1 occur frequently in lung cancer. As a central regulator in several pro-oncogenic pathways, mTOR is being explored as an important target for cancer therapy. Initial clinical trials with mTOR inhibitors have had only minimal positive results, but two mTOR inhibiting agents, everolimus and temsirolimus, have been approved for use in advanced renal cell carcinoma. In other cancer types, including lung cancer, early mTOR inhibiting agents have not been effective.

The aim of the present study was to explore the utility of novel mTOR inhibitors in NSCLC to compare with the classical rapamycin. We also explored the role of autophagy in cancer cell survival by characterizing the effects of the autophagy inhibitor chloroquine as a therapeutic agent in NSCLC. By screening a panel of NSCLC cell lines we aimed to identify subsets of sensitive and resistant NSCLC cell lines to each of these therapeutics and to identify potential biomarkers that could be used to predict response. Furthermore, we aimed to test the efficacy of these new targeted agents in combination with standard chemo- and targeted-therapies as a possible strategy to improve the current state of cancer care. The most important findings from the present study include:

1. A subset of NSCLCs are more responsive to rapamycin than to mTORC1/2 inhibition by Torin1, or AZD8055, and sensitivity to mTOR inhibition is associated with RTK activation such as ERBB2 amplification or EGFR mutation or amplification, while KRAS mutations were associated with resistance (Chapter 3).
2. RNAi knockdown of various components related to mTOR signaling and autophagy produce a heterogeneous growth effect response in NSCLCs cells, and potentially define subset-specific vulnerabilities (Chapter 4).
3. mTOR inhibitors sensitize NSCLC cells to standard targeted- and chemotherapy agents, erlotinib and paclitaxel/carboplatin doublet, in an additive or synergistic manner, with the greatest level of synergy occurring in cell lines that are resistant to single agent therapies, including those with KRAS mutations. (Chapter 5).
4. Inhibition of autophagy using chloroquine is not likely to be a successful therapeutic approach in lung cancer as no significant growth effect was seen at physiologically relevant concentrations, and no sensitization to standard chemo- or targeted-therapies were observed (Chapter 6).

II. Future Directions

Additional Mechanistic and Biomarker Discovery Research

Novel mTOR interacting partners and signaling effects are constantly being discovered. The role of mTOR signaling in cell biology and as a pro-tumorigenic player is continuing to be defined. As the knowledge of mTOR's functions increases, the opportunity to further describe mechanisms of inhibitor response and biomarkers to predict these responses will be further advanced.

The role of mTORC2 in cancer signaling has only recently been discovered and is still very poorly understood. This complex is now known to be responsible for the phosphorylation of AKT at Ser473, which greatly increases its activity and promotes tumorigenesis. Therefore inhibition of mTORC2 signaling is likely to be a valuable target for cancer therapy, at least in subsets of tumors. Indeed, targeting of mTORC2, but not mTORC1, was found to increase levels of serum starvation- or cisplatin induced apoptosis in breast cancer (Li, Lin et al. 2012). Clarifying the role of mTORC1 versus mTORC2 in lung cancer will require further studies that target each of these complexes separately. In the present study, experiments using RNAi knockdown of either raptor, to abolish mTORC1 activity, rictor, to abolish mTORC2 activity, or mTOR, which should inhibit the activity of both complexes were inconclusive. In theory using an siRNA against mTOR should yield similar growth effect phenotypes as a dual mTORC1/2 pharmacologic inhibitor, barring any off-target effects. Similarly, siRNA knockdown of raptor should yield similar growth phenotypes as an mTORC1 inhibitor. Because siRNA effects and drug responses compared in NSCLC cell lines did not coincide, despite validation of each hitting the expected target, the vulnerabilities of lung cancer cells to mTORC1 versus mTORC2 remain a mystery. The potential for pharmacological agents to have multiple targets may also contribute to the discrepancies between the drug and siRNA responses. Additional validation of RNAi knockdowns, perhaps using stable knockdowns and longer term assays, are required in order to further characterize the assortment of vulnerabilities to these complexes in NSCLC.

Additionally, the vulnerabilities of cancer cells to various components of mTOR signaling are not yet clear and require further study and validation. Addiction to a protein may not be easily recognizable through examination of expression differences or presence of a mutation. In this study, an siRNA screen that individually knocked down 55 components related to mTOR signaling and autophagy was performed on 26 NSLC cell lines. Preliminary results from the screen identified a heterogeneous response to the siRNAs, and suggest there are subsets of NSCLC that are more vulnerable to loss of

certain mTOR signaling constituents. In order to determine potential cancer-specific vulnerabilities, and to further characterize the effects of each knockdown and discover potential biomarkers to predict vulnerabilities, additional validation and characterization of the screen hits will be required.

Clinical Translation

Ultimately the goal of the present research is to translate the findings into the clinical setting. This study found that activation of RTKs including EGFR and ERBB2 are strong predictors of sensitivity to mTOR inhibition. These results suggest a need for trials that select patients with these alterations to examine the utility of mTOR inhibition in a relevant patient subset. These potential biomarkers will need to be validated as also being important predictors in patients. These receptors are already widely accepted as clinically relevant biomarkers for other targeted therapies and a number of tests already exist for their detection. Because tyrosine kinase inhibitors are commonly used as first line therapies for patients with mutations in these receptors, mTOR inhibitors are not likely to replace these agents in the clinic. However, because NSCLCs with EGFR TKI resistance mechanisms may also respond to mTOR inhibitors, these target agents could be employed to possibly delay the acquisition of resistance, or as second-line therapies in patients whose tumors no longer respond to EGFR inhibitors. Clinical trials that examine the utility of mTOR inhibitors in these contexts will be required.

The most likely benefit of mTOR targeted therapies will be with their use in drug combinations with standard of care agents. We found that combinations with mTOR inhibitors and erlotinib or chemotherapy doublet paclitaxel/carboplatin sensitized NSCLC cell lines to these standard of care agents. Clinical trials that aim to test mTOR inhibitors in drug combinations have already been proposed, and many are currently ongoing. Examples of such trials will be described below.

Results from the present study suggest that autophagy inhibition will not be a successful strategy for the treatment of lung cancer. Neither autophagy inhibition with chloroquine as a single

agent, or in combinations with standard chemo- and targeted-therapy agents significantly inhibited NSCLC cell line growth. Furthermore, chloroquine may exacerbate chemotherapy induced kidney damage as autophagy in the kidney is thought to be protective (Kimura, Takabatake et al. 2013). Chloroquine and Hydroxychloroquine are currently being investigated as autophagy inhibitors in combination with chemotherapy in clinical trials of a number of cancer types including lung, breast and colon (NCT01292408 , NCT01006369 , NCT01649947). It will be interesting to see whether any of these trials are successful in improving tumor responses using these combinations.

Current Clinical Trials

In May 2012 the results of a phase II "window of opportunity" trial undertaken by the North Central Cancer Treatment Group that tested mTOR inhibitor temsirolimus in treatment naïve NSCLC patients were published (Reungwetwattana, Molina et al. 2012). Use of this agent as a monotherapy failed to meet the required efficacy endpoint. They also did not find any association with levels of phospho- or total-p70S6K or AKT. They concluded that the use of a single targeted agent in an unselected population was an ineffective strategy for finding effective novel therapies. Newer trials to determine the safety and efficacy of dual mTORC1/2 inhibitors are underway. The mTOR kinase inhibitor screened in this study, AZD8055, has been tested for safety and tolerability in patients with advanced solid tumors (NCT00731263). The drug had manageable toxicity profiles, with fewer skin toxicities compared to rapalogues, but additional effects on liver function not previously seen with mTOR inhibitors. Several patients with a variety of primary tumors had stable disease with AZD8055 treatment, but did not fulfill the requirements of RECIST to indicate response (Naing, Aghajanian et al. 2012).

mTOR inhibitors are also being tested in combination therapies. Everolimus and erlotinib combinations are being explored for safety and efficacy in a number of solid tumors, including lung cancer (Papadimitrakopoulou, Soria et al. 2012). A combination of pan-ERBB TKI, neratinib, and

temsirolimus was recently reported in a Phase I trial to be safe and efficacious in both ERRB2 altered and uncharacterized lung and breast tumors (Gandhi, Bahleda et al. 2014). A Phase II study of docetaxel in combination with everolimus in NSCLC found the combination was well tolerated but had only modest efficacy in terms of tumor response in an unselected population (NCT00406276 , Ramalingam, Owonikoko et al. 2013). Two dual mTORC1/2 inhibitors are planned to be tested in combination therapies. A Phase I trial testing dosing of MLN0128 (INK128) in combination with paclitaxel with or without Trastuzumab is recruiting patients with advanced solid tumors (NCT01351350). Another investigational dual mTOR inhibitor CC-223 is recruiting NSCLC patients to establish a maximum tolerated dose for combinations with this drug with either erlotinib or azacitidine (NCT01545947). Results from these trials and follow-up Phase II trials to determine efficacy will determine the success or failure of mTOR inhibition as a targeted therapy for treating cancer.

III. Perspectives

Use of Cell Culture Models for Translational Research

The cancer cell line model has been both heralded and criticized as a tool for studying cancer biology phenomena and screening novel therapeutic agents. It has been speculated that cells grown *in vitro* culture for many years no longer represent the state of the original tumor. For example acquired mutations in p53 and silencing of the *MGMT* gene encoding a DNA repair protein have been reported as occurring in cultured cells (Harris, von Wronski et al. 1996, Taylor, Shu et al. 2000, Morton and Houghton 2007). Furthermore, only subsets of tumor biopsies are successfully used to generate cell lines, which may limit the representation of a number of patient tumors in cell line models. The representation of patient tumors in cell lines is further limited due to the fact that certain cell lines are more amenable to experimentation due to ease in management of growth conditions, and their ability to form liquid colonies or to grow as xenografts.

The most successful use of cancer cell lines in cancer research stems from the availability of large panels of cell lines that encompass a variety of molecular characteristics that closely represent human tumors. Lung cancer research is benefited by the availability of several hundred cell lines, most of which were established through efforts by Drs. John Minna and Adi Gazdar starting at the NIH and later in the Hamon Cancer Center at UT Southwestern. Common oncogenotypes including p53, KRAS, or EGFR mutation are well established molecular alternations known to be represented in cancer cell lines. Continuing efforts aim to molecularly classify the lung cancer cell lines to generate a multitude of potential biomarkers to be examined. New studies are underway to compare molecular characteristics such as mutations and gene expression of lung cancer cell lines with human tumor samples. Whole exome sequencing on 115 cell lines is complete and microarray expression has been performed. Similarly, nearly 1000 human lung tumor samples have been similarly profiled by The Cancer Genome Atlas (TCGA). Comparisons of mutation data and expression arrays between these cell lines and 302 TCGA tumors found that about 2/3 of the tumor samples have high concordance with established lung cancer cell lines (analysis by Dr. Luc Girard). While the spectrum of oncogenotypes found in human lung cancer are well represented in the cell lines, ongoing efforts to utilize novel methods to establish cancer cell lines will be important to further increase tumor representation in cell lines, particularly for squamous cell histology and matched tumor-normal pairs, which are currently underrepresented.

While there certainly are caveats to using cell lines as a human tumor model, there is overwhelming evidence asserting their importance for the progress of cancer research. Lung cancer cell lines have already proven their worth as models to test novel targeted therapies and to discover functional biomarkers that have relevance in patients. Initial clinical trials examining the utility of EGFR targeting agents erlotinib and gefitinib had disappointing response rates at only 10-20% in unselected populations (Jänne, Engelman et al. 2005, Zhang, Stiegler et al. 2010). Later it was discovered that activating mutations in *EGFR* such as deletions in exon 19 or point mutations in exon 21 are associated

with sensitivity to EGFR targeted therapies (Gazdar 2009, Gazdar 2010). NSCLC cell lines show similar response rates to EGFR targeting agents seen in patients, with approximately 10% of the cell lines having a sensitive phenotype. These cell lines contain the same mutations in *EGFR* that are found in lung tumors. Furthermore, cell lines also exist that represent patient tumors that have acquired resistance to EGFR targeted therapies including the T790M second-site mutation in *EGFR* and amplification of the *MET* oncogene (H1975, H820 for example). Lung cancer cell lines are also useful in predicting response to other targeted therapies such as ALK inhibitor, crizotinib, or ERBB2 inhibitor, trastuzumab, as EML4-ALK fusions and ERBB2 amplifications are also known lung cancer alterations that are represented in cell lines.

Targeted Therapy Paradigm

The use of chemotherapy agents did not appear until the mid-twentieth century when it was found that nitrogen mustard had an anticancer effect (Gilman 1963, Goldstein, Madar et al. 2012). Since then, chemotherapeutics have been discovered that affect cell division and cause DNA damage. These agents kill cancer cells, which are subject to aberrant cell division and often have deficits in DNA repair mechanisms; however non-cancerous cells are also affected in these patients, often at the cost of extreme adverse effects for the patients (Malhotra and Perry 2003). While novel agents that are better at killing cancer cells without affecting normal cells have always been sought after, it wasn't until significant advances in the molecular understanding of cancer cell functions toward the end of the 20th century occurred that targeted therapies really emerged (Goldstein, Madar et al. 2012). Knowledge of oncogenes lead to the theory of "oncogene addiction," which in turn spurred the development of compounds that directly targeted the alterations that cancer cells had come to depend on for survival (Weinstein and Joe 2008, Luo, Solimini et al. 2009). While initially the goal of cancer research was to find a magic bullet that eradicated tumors while leaving the patient unharmed, it soon became evident

that in most cancer types a variety of different oncogenes were capable of driving tumorigenesis in different patients (Strebhardt and Ullrich 2008, Luo, Solimini et al. 2009, Goldstein, Madar et al. 2012). Further molecular classification of cancer cells lead to the idea of "personalized medicine." The current goal of this approach is to perform analysis on a patient's tumor in order to identify the driving oncogene(s) and select a therapeutic agent that previous studies have demonstrated will be effective for that particular tumor (Goldstein, Madar et al. 2012).

While the clinical implementation of targeted agents and predictive biomarkers has improved initial response for patients with tumors in a variety of tissue types, significant progress must still be made in order to solidify the success of the targeted therapy paradigm. Overall survival for cancer patients has not had great improvement in the last few decades due to a variety of limitations in the current state of targeted therapy research. For lung cancer in particular, almost half of all tumors have unknown alterations that drive their growth (Pao and Girard 2011). Sorting out important driver mutations from passenger mutations is another challenge, especially in smoker lung cancer where the number of total mutations is magnified. Furthermore, mutant KRAS is a driving oncogene that is found in approximately 30% of lung adenocarcinomas (Kris MG, Johnsen BE et al. 2011, Pillai and Ramalingam 2014). To date, attempts to target Ras have been unsuccessful as this protein has not been amenable to inhibition using small molecules (Downward 2003). The inability to target Ras-driven tumors is a severe roadblock for cancer therapy, especially because these tumors are highly aggressive and often associated with resistance to standard chemotherapy agents as well, giving patients with these tumors with a poor prognosis. Finally, even when targeted therapy drugs are initially successful in treating a tumor, acquired resistance to the drugs is an almost universal phenomenon (Engelman and Settleman 2008). The acquisition of resistance mechanism is likely the result of tumor heterogeneity (Cirkel, Gadellaa-van Hooijdonk et al. 2014). Tumors consist of a collection of heterogeneous clones that are unequally represented in the initial tumor, many of which may have alterations that are not easily

detectable when the presence of driving oncogenes is first tested. While identification of novel cancer drivers in an important approach to improve targeted therapies by increasing our knowledge of targetable oncogenes, there will still be a need for cancer researchers to broaden their scope of inquiry to progress on the overall goal of reducing the cancer death toll.

In addition to molecular characterization and targeted therapy studies, greater understanding of other aspects of cancer progression may also lead to improved therapies. Research on tumor heterogeneity and the role of the tumor microenvironment and pro-inflammatory components in tumor initiation, progression and response to drugs are a few examples that have potential to expand the current scope of targeted therapy (Goldstein, Madar et al. 2012). Drug combinations and adjustments in drug dosing may further improve both new therapies and those already employed. A multi-faceted approach to cancer therapy that integrates biological knowledge and technological advances will ultimately advance or marginalize the current targeted therapy approach to cancer treatment.

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