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PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) AS A THERAPEUTIC TARGET IN NSCLC

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PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) AS A THERAPEUTIC TARGET IN NSCLC

DISSERTATION

A thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Christopher Wayne Stamatkin

Lexington, Kentucky

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Lexington, Kentucky

2014

To my family,

You have been, and always shall be my strength. We now can exhale, smile, and look forward to living. I love you. CS

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Chapter 1

1.1.1 Lung Cancer Overview

The genesis of the word cancer can be attributed to the Greek physician Hippocrates (460 - 370 B.C.), who is regarded as the “Father of Medicine.” His detailed study used the terms *karkinos* and *karkinoma* to distinguish non-ulcer forming and ulcer forming tumors [1]. The Greek definition of *karkinos* is crab, which among many other explanations is thought to describe the outward projections from a cancer akin to that resembling the shape of a crab. Centuries later, the Greek word was translated into Latin by the roman physician, Celsus (28 - 50 B.C.) giving us the modern word *cancer* [2].

Advancing twenty-two centuries, we now define cancer as the transformative biological response of a cell to both genetic and epigenetic changes shaped by environmental cues that together lead to uncontrolled cell proliferation and survival. This is a disease fundamentally occurring at the cellular level, and when compared to normal cells differ in a number of ways: control of growth, morphology, cell-cell interaction, membrane and cytoskeletal composition, protein synthesis, and gene expression [3].

At the causative core of these differences are two broad classes of genes that play an elementary role in the transformative progression to cancer called proto-oncogenes and tumor-suppressor genes. Oncogenes can be generally defined as any gene product capable of transforming cells. Proto-oncogenes play am evolutionarily conserved role in normal signaling networks controlling cellular differentiation, growth, survival and maintenance of tissues. Activation of

a proto-oncogene into an oncogene occurs through gain-of-function mutations that now express an oncoprotien capable of driving transformation process. Conversely, tumor-suppressor genes normally encode proteins that through various mechanisms act to limit cell proliferation [4]. These include proteins responsible for cell cycle control, and DNA repair, as well as critical mediators of apoptosis, among others. It is important to note that tumor-suppressor genes are recessive with regard to activity, meaning both copies of tumor-suppressor gene must be lost or inactivated to drive tumor progression. Familial inheritance of single copy mutant alleles for tumor suppressors, such as RB, APC, and BRCA1, dramatically increases the probability of a select tumor initiation [5]. Progression of cancers is highly variable and is dependent on a number of factors: namely the particular molecular drivers of disease, tissue of origin, immune system function, and micro-environmental cues [6].

As with other cancers, current treatment options for lung cancer are largely based upon stage of cancer upon diagnosis, however other factors such as overall health and lung function are important. For early stage (stage I/II) disease non-small cell lung cancer (NSCLC) patients are most likely to undergo surgery alone or in conjunction with adjuvant chemotherapy [7]. Patients with stage III diagnosis represent a heterogeneous group and treatment options are limited to the location of the tumor and whether surgery is possible. Patients may present disease ranging from resectable tumors with microscopic lymph node metastasis to large tumors involving multiple lymph nodes. Stage III NSCLC is addressed similar to early disease, albeit in a more aggressive manner with

increased use of radiotherapy or adjuvant chemotherapy. For newly diagnosed NSCLC, 40% of patients are found to have stage IV disease [8]. Treatment goals and options for stage IV NSCLC are no longer curative but rather to prolong survival and control cancer related symptoms. The first-line therapy for patients with advanced NSCLC are cytotoxic combination chemotherapy using a platinum based doublet (cisplatin or carboplatin) in tandem with a cytotoxic agents such as paclitaxel, gemcitabine, docetaxel, vinorelbine, irinotecan and pemetrexed [9, 10]. More recent clinical studies also indicate potential benefit of adding molecularly targeted inhibitors, bevacizumab or cetuximab, to the cytotoxic combination chemotherapy [11]. For patients having identified EGFR mutations, the use of EGFR tyrosine kinase inhibitors (TKIs) are indicated as a first-line treatment, in addition to second- or third-line treatment for advanced NSCLC [12, 13]. In this era of molecular therapeutics, the discovery of clinically responsive EGFR mutations found in a small subset of lung adenocaricinomas and the promise of EGFR targeted therapeutics stands as model for future personalized care for advanced stage cancers. We now realize the clinical practice is gradually shifting from therapy based on clinicopathologic features to implementation of biomarker driven treatments based on a molecular profile of a patient's tumor.

1.1.2 Lung cancer etiology and epidemiology

Exactly 50 years ago the landmark publication "Smoking and Health: Report of the Advisory Committee to the U.S. Surgeon General of the Public

Health Service" concluded that cigarette smoking is a cause of lung cancer in men and sufficient in scope to demand "remedial action" for the betterment of society [14]. The call to action outlined in the 1964 report was followed by increased public awareness and shift in attitudes to smoking related health risks. In subsequent years, further action to increase regulatory oversight of use, sales, and advertising of tobacco products. The current Surgeon General report now concludes that active cigarette smoking is a cause of 1.) nine types of cancer, 2.) cardiovascular disease, 3.) chronic obstructive pulmonary disease, 4.) acute respiratory illness, 5.) respiratory effects having adverse impact on lung growth and function, 6.) reproductive associated complications, as well as numerous other deleterious health effects [15, 16].

Lung cancer was once considered a rare disease, however the onset of mechanization and wide scale marketing at the end of the 19th century brought cigarette smoking to the mainstream leaving behind a lung cancer epidemic [17]. The worldwide burden of lung cancer attributable to smoking is 80% for males and greater than 50% for females [18]. Lung cancer is now the leading cause of cancer-related death both in the United States and globally, accounting for 228,190 new diagnosis and 150,480 deaths in the United States for 2013 alone. [19, 20]. According to a 2012 Centers for Disease Control (CDC) study the Commonwealth of Kentucky was ranked first among all states for having the most adult cigarette smokers [21]. As a matter of public health, it is incumbent on our federal, state, and local officials to engage in anti-smoking efforts at all levels, knowing the best way to prevent lung cancer is to not smoke.

Tobacco smoke is composed of greater than 60 known carcinogens, with nearly half of which having a strong association with the development of lung cancer [22]. Among these carcinogens the best studied are the damaging effects of polycyclic aromatic hydrocarbons and the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone, each of which introduce mutations via DNA adduct formation [23]. Chronic exposure to the carcinogens found in tobacco smoke eventuates mutations in critical genes such as p53 and K-RAS, which may serve as an initiating event or contribute to the progression of disease. Secondary causes of lung cancer are attributable to workplace agents such as asbestos, chromium, nickel, arsenic, and radon, as well as environmental factors such as second hand smoke and air pollution [24].

There are primarily two histologically distinct forms of lung cancer, non-small cell lung cancer (NSCLC) accounting for 85% and small cell lung cancer (SCLC) representing approximately 15% of all lung cancers [25]. The overwhelming majority of lung cancer patients with NSCLC (85%) or SCLC (98%) are current smokers [26]. NSCLCs are further divided into three major sub-types, squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinomas (LCC). Lung adenocarcinoma is most common histological subtype of lung cancer accounting for nearly 40% of all lung cancer [27].

1.1.3 Lung cancer genetics

Significant advances have been made in delineating the cellular and molecular mechanisms directing tumor initiation, maintenance, and progression

of lung cancer. Susceptibility and risk of lung cancer been shown to have strong correlation with hereditary genetic factors, this is underscored by the observation that only 10-20% of smokers develop lung cancer [28]. Epidemiological studies indicate a 2.5 fold increase in risk of developing lung cancer related to a family history of lung cancer [29]. These risks are associated with common inherited cancer syndromes such as germ-line inactivating mutations in tumor suppressor genes TP53 and RB among others [30, 31]. However, loss-of-function mutations in these genes are common to a variety of cancers, and to date no conclusive familial link specific to the development lung cancer has been identified.

Apart from the familial predisposition to the development of lung cancer, environmental factors such as tobacco smoke cause DNA lesions directly involved in tumor initiation and progression. Large scale genomic studies indicate a given lung tumor is comprised of a large number of somatic mutations that occur over time [32, 33]. Indeed, both histologically normal bronchial epithelial cells and pre-neoplastic cells express many of these genetic and epigenetic abnormalities, suggesting human lung cancers arise from normal epithelium through a multistep accumulation of successive genetic and epigenetic changes [34]. Among the most common, and best studied of these related to the pathogenesis of lung cancer are the tumor suppressor TP53, and the proto-oncogenes RAS and EGFR.

TP53 (encoding protein p53) is the most frequently mutated gene in human cancer. Wild-type p53 functions as a sensor that determines cell fate by orchestrating the cellular response (DNA repair or apoptosis) to cell stress and

DNA damage. As a transcription factor, activated p53 promotes the expression of cell cycle inhibitory proteins causing G_0/G_1 cell cycle arrest and the induction of apoptosis [35] . When p53 is compromised, no longer can cells detect DNA damage and appropriately dictate the cellular responses needed, thus increasing the likelihood for acquiring DNA lesions and increasing overall genomic instability. Mutations in TP53 are present in approximately 50% of NSCLC tumors and 90% of SCLC cancers [24]. Therefore, loss of p53 can be viewed as a critical step influencing the development of lung cancer.

Proto-oncogenes in lung cancer

The deregulated signaling pathways that result directly or indirectly from the variety genetic mutations involved in lung cancer are important targets for current and future cancer therapies. The oncogenic signaling molecules and related pathways involved are common among many human cancer types besides lung cancer such as KRAS, EGFR, PIK3CA, BRAF, HER2 and EML-4ALK [36].

As a cancer develops these deregulated growth signals often lead to "oncogenic addiction," whereby the tumor becomes completely reliant on these signals for survival. Consequently, if these oncogenic signals are blocked (e.g. by targeted drugs) those addicted cancers will die, whereas those cells not dependent remain largely insensitive to the particular drug activity [37]. The concept of targeted inhibition of tumor specific oncoproteins has led to tremendous genome wide sequencing and simultaneous drug development

efforts, to both identify, and develop rational therapeutics. The remainder of this section will focus those driver oncogenes most common to lung cancers, EGFR and K-RAS, and their interaction with PI3K signaling pathway.

Oncogenic EGFR signaling in lung cancer

The epidermal growth factor receptor (EGFR/ERBB1/HER1) is an important member of the cell surface receptor super-family of receptor tyrosine kinases (RTKs) that are critical mediators of cell signaling by extracellular growth factors. In addition to EGFR, the other family members include ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4, each of which have garnered much attention provided their association with the development and progression of many human tumors [38]. The EGFR proto-oncogene plays an important role in normal cell proliferation, apoptosis, and angiogenesis, both the receptor and its ligands are frequently overexpressed in the development and maintenance of non-small cell lung cancer (NSCLC) [39, 40].

Over the past decade, the development of small molecule inhibitors of EGFR activity were developed as anti-cancer agents, these being erlotinib (OSI Pharmaceuticals) and gefitinib (AstraZeneca) [41, 42] . Subsequent studies indicated a subset of patients with chemo-refractory NSCLC demonstrated dramatic, though often not sustained response to inhibitors of EGFR [43] . Among those patients exhibiting tumor regressions, response was found to correlate with mutations of EGFR found in the kinase domain (exons 18-24) of the molecule [44, 45]. EGFR kinase domain mutations are restricted to only a

subset of NSCLC, comprising approximately 10-20% of NSCLC cases [46, 47]. Deletions in exon 19 (most often del746-750) and point mutations in exons 18 (G718A/C), 20 (T790M) and 21 (L585R) lead to the increased and sustained activation of the receptor [48]. Downstream of mutant EGFR are the PI3K/AKT, RAS/MAPK, and STAT pathways, which are regulated and dependent on the deregulated EGFR signaling [49]. Treatment with EGFR-TKIs in these "EGFR addicted" NSCLC tumors shuts down the growth and survival signaling through these pathways and cells undergo considerable apoptosis. However, significant initial tumor regressions are only temporary in response to EGFR-TKI treatments; these cancers inevitably become resistant to therapy within 12 months [50-52]. However, complicating this effort are the approximately 10-20% of patients who do demonstrate a partial response to EGFR-TKI, but do not have identifiable EGFR mutations, suggesting that other mechanisms and pathway activity apart from mutant EGFR underlie responsiveness [53, 54].

Such insights into EGFR-dependent signaling highlight the ongoing difficulty in identifying clinically suitable patient populations for EGFR-TKI therapy and the potential for rational combination therapies targeting other compensatory drivers of disease.

Mutant K-RAS signaling in lung cancer

Approximately 30% of human tumors harbor RAS gene mutations [55], this includes a high prevalence in lung cancers at approximately 25-50% [56, 57]. The RAS family of proto-oncogenes (H-RAS, K-RAS, and N-RAS) are membrane

associated small G proteins that regulate critical signaling responsible for normal cell proliferation, differentiation, and survival [58-60] . The RAS proteins functionally serve as a link between the upstream receptor tyrosine kinases (RTKs), and the downstream serine/threonine kinases of MAPK pathway [61, 62]. RAS also plays an important role upstream of PI3K signaling, acting as a positive regulator of PI3K activation [63]. GDP-bound RAS is inactive, however conversion to active state, GTP-bound RAS, is carried out by guanine nucleotide exchange factors (GEFs). GTPase-activating proteins (GAPs) return RAS to inactive state by hydrolyzing GTP into GDP.

Mutations affecting KRAS represent greater than 90% of RAS mutations found in NSCLC, most having single base pair mutations in codons 12 and to a lesser extent codon 13 which makeup the guanine binding domain of RAS [64]. Furthermore, lung adenocarcinomas having activating mutations in codons 12 and 13 of KRAS are almost entirely found in patients with a history of cigarette smoking [65, 66], whereas EGFR mutations are more common in never smokers [28, 33]. Mechanistically, these mutations lock the RAS protein into the active GTP-bound conformation that leads to persistent signaling downstream via PI3K/AKT/mTOR and RAF/MEK/ERK pathway effectors leading to sustained proliferative and pro-survival signaling. Attempts to develop targeted inhibitors of RAS have thus far been unsuccessful which has made treatment of RAS mutant cancers difficult. Current drug discovery efforts are now aimed downstream of activated RAS, as indicated by the clinical emergence of RAF and MEK kinase inhibitors [67-69].

1.2 THE BIOLOGY OF PI3K

Phosphoiositide-3-kinases (PI3Ks) form a family of lipid kinases that functionally generate intracellular phospholipid species that affect a wide range of biological activities. Specifically, activated PI3Ks phosphorylate the 3-OH group of membrane lipid phosphatidylinositol (4,5) bisphosphate (PIP2) to form phosphatidylinositol (3,4,5) triphosphate (PIP3) that function as second messengers providing both specific spatial and temporal signals that modulate the activity of intracellular protein effectors responsible for a wide range of cellular functions [70-74] .

A large body of research has identified an evolutionarily conserved family of PI3K enzyme organized into 3 classes (Class I, Class II, and Class III) on the basis of structural characteristics, tissue distribution, regulation, and lipid substrate preference. The best characterized of these, Class I PI3Ks are composed of p110α (PIK3CA), p110β (PIK3CB), p110γ (PIK3CG), and p110δ (PIK3CD) [75-77]. Class II PI3Ks PI3K-C2α (PIK3C2A), PI3K-C2β (PIK3C2B), PI3K-C2γ (PIK3C2G) remain elusive, having are poorly understood roles in cell signaling [78] . Finally, Class III PI3K is only comprised of VPS34 (PIK3C3), which initial studies report having a role in endosomal sorting of proteins [79]. The isoform specific functional activities of the class I PI3Ks and involvement in human cancers will largely be the focus of further discussion below.

1.2.1 Class I PI3Ks

Structural organization of p85:p110 heterodimers

The catalytic p110 subunits are further sub-divided into class IA composed of (p110α, p110β, p110δ), each associating with the p85-type (p85α or p85β) of regulatory subunit, whereas class IB sub-group (p110γ) binds either p101 or p87 regulatory subunits [80-82].

The class IA regulatory p85 α/β and catalytic p110 α/β/δ are multi-domain proteins that functionally interact with upstream and downstream effectors (Figure 1.1.). The full length p85 isoforms, α or β, are composed of N-terminal Src homology 3 domain (SH3) then a breakpoint cluster region (BCR) homology domain flanked by proline rich domains, and three SH2 domains (nSH2, iSH2 and cSH2) [71]. The p110 catalytic subunit is composed of N-terminal adaptor binding domain (ABD), Ras binding domain (RBD), C2 core structure, helical domain and the kinase domain [83] (Figure 1.1.).

Activation of class IA PI3Ks is strictly regulated by the obligate regulatory subunits association with the catalytic subunit [84]. Importantly, the regulatory interactions of p85 that control enzymatic activity differ depending on the particular class IA p110 isotype to which it is associated. Regulation of p110α and δ catalytic activity is mediated by two groups of inhibitory contacts: (I) SH2 (N-terminal SH2) domains of 85α make contacts with the helical domain, C2, and the kinase domain; and (II) the iSH2 (inter- SH2) domain of p85 forming inhibitory contacts with the ABD and C2 domains of p110α (Figure 1.1.; solid lines 1 & 2) [83]. The c-SH2 (C-terminal SH2) of p85 does not provide any inhibitory

involvement in for the p110α isoform. However, for p110β this is not true, in addition to the regulation by the nSH2 and iSH2 contacts, p110β has an additional inhibitory contact between cSH2 domain of p85 and the kinase domain (Figure 1.1., dashed line 3). These differences in p85:p110 regulation unique to p110α/δ and p110β comprise functional molecules that have very different interaction and activation requirements among the PI3K isoforms which ultimately impart isoform specific function in cells [85-87].

Regulatory and catalytic mechanism of PI3Ks

A common feature of PI3K and other protein kinases are the shared bi-lobal structural organization of the kinase domains where ATP is bound between the lobes [88]. The N-terminal lobe of PI3Ks is composed of highly conserved five-stranded β-sheet and the regulatory element kα3 helix oriented tightly along the helical and ABD domains [89]. The C-terminal lobe is composed of mostly α-helices that are oriented to allow for membrane interaction, receptivity of lipid substrates, and the p85 regulatory subunit [87]. A distinct structural element of the C-lobe are the kα10, kα11, and kα12 helices which make up the regulatory arch that encloses the activation and catalytic loops required for catalysis [90]. Multiple confirmations of kα12 helices have been identified, which have been attributed to its central role in membrane binding and thus the overall activity of the kinase [91]. The helix is thought to maintain the closed-inactive conformation of the enzyme in absence of lipid membrane, thus limiting substrate access to the active site. The kα10, kα11, kα10 helices of the regulatory arch are also

important because they interact with nSH2 and cSH2 of p85 as well as other regulatory proteins that modify the active confirmation of catalytic loop [92]. The catalytic domains of PI3K are spatially oriented by the conserved hydrophobic domains called the regulatory (R-spine) and catalytic spine (C-spine). Together these build the scaffold for the catalytic loop and placement of ATP [90, 93]. Thus, in the presence of membrane lipid substrate, binding by the activation loop and helix kα12 causes the conformation change needed fully accommodate the lipid substrate, and simultaneously relieves inactive confirmation in the catalytic center to its fully active state [87] .

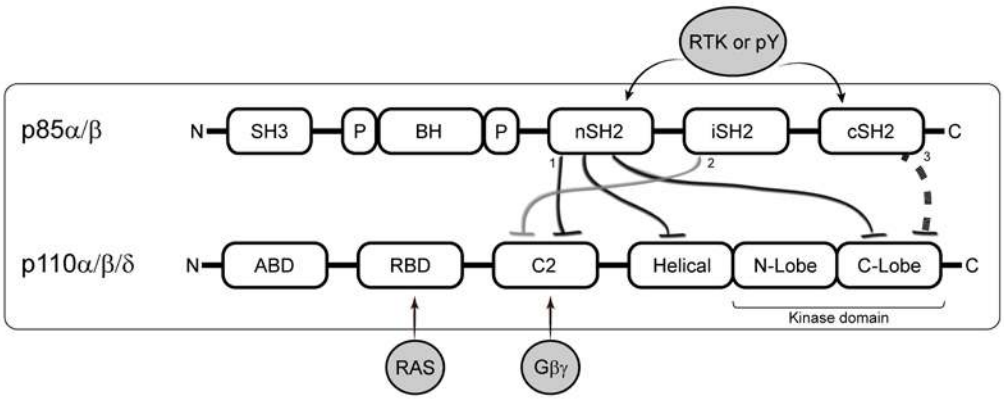


Figure 1.1. The domain structures and regulatory interactions of class IA PI3Ks. Illustration depicts the interaction of class IA PI3K heterodimers with various upstream signaling molecules known to activate PI3K. Solid Black and grey lines (1 & 2) represent common inhibitory contacts between p85 regulatory and p110 catalytic subunits. Dashed line (3) represents a unique third inhibitory contact found only in p85:p110β interaction.

1.2.2 Canonical Class I PI3K activation

The canonical PI3K signaling pathway can be activated by RTKs (with or without adaptor molecules), RAS, or GPCRs (Figure 1.2.).

Activation by receptor tyrosine kinases

In quiescent cells, cytosolic PI3K p110 catalytic subunits are stabilized and sustained in the inactive state by p85 regulatory subunit interaction. Upon growth factor stimulation, receptor tyrosine kinases (RTKs) can activate class I PI3K through the binding interaction between the SH2 domains (Rous-sarcoma-oncogene homology-2 domains) of the p85 regulatory subunit with phosphorylated-tyrosine (pYXXM) motifs of the receptor, or via receptor associated adaptor molecules [94, 95]. The phospho-tyrosine (pY) – SH2 engagement releases imposed inhibition of p110 catalytic site, and them into close proximity to membrane resident lipid substrate phosphatidylinositol 4, 5,-biphosphate (PIP2) to generate phosphatidylinositol 3, 4, 5,-triphosphate (PIP3) [96-98].

Activation by RAS super family of G proteins

RAS activation of class I PI3Ks occurs through a direct binding with the RAS binding domain (RBD) of the p110 catalytic subunit [99-101]. However, activation is not uniform for all PI3K isoforms. Differential activation of PI3Ks is thought to be dependent on the particular RAS family members involved, or in the degree of activation among the PI3K isoforms, due in large part to the

marked sequence variation among the isoforms in the RBD of p110 [90, 102, 103]. The canonical activation of PI3K through RAS occurs in conjunction with activated RTKs, the recruitment of adaptor molecule growth factor receptor-bound protein 2 (GRB2) and the guanine nucleotide exchange factor (GEF) protein son-of-sevenless (SOS). This signaling complex both activates and brings RAS into proximity with p110 PI3K [100, 104]. RAS mediated activation of p110α can be restricted by p85, however this inhibition can be released in response to p85 SH2 domain binding to (pY) motifs [105]. This suggests RAS activation of PI3K is a tightly orchestrated event, where p85 regulatory subunit can modulate responsiveness to RAS by sequential activation of catalytic subunits by RTKs, and that class IA PI3Ks may only be responsive to RAS after prior RTK activation.

G-protein-coupled receptor mediated activation of PI3K

The superfamily of heterotrimeric G-protein-coupled receptors (GPCRs) are cell surface signaling proteins involved in a wide range of physiological functions and diseases, including cancer development and metastasis [106]. Among the class IA PI3K isotypes, p110β is uniquely found to be activated by GPCRs [107, 108]. Binding and activation of p110β occurs through direct Gβγ heterodimer interaction with the catalytic p110 subunit occurring independently of p85 regulatory subunit [109, 110].

In addition to direct PI3K activation, the functional cross-talk between GPCRs and growth factor receptors is reported to contribute to the progression

of lung, breast, colon, prostate, and head and neck tumors by activation of PI3K [111]. Multiple GPCRs are found capable to transactivate EGFR signaling by promoting autocrine and paracrine release of EGF-like ligands [112, 113]. The GPCR-induced growth factor shedding is mediated by members of ADAM (a disintegrin and metalloproteinase) family of zinc-dependent proteases, most noteworthy being the tumor necrosis factor-α (TNFα) converting enzyme (TACE) [114]. Taken together, in solid tumors the GPCR driven PI3K signaling is mediated directly through the p110β isoform, and can drive particular growth factor signaling upstream of PI3K by transactivation of receptor tyrosine kinases such as EGFR.

Effectors of PI3K signaling

The oncogenic nature of PI3K pathway signaling is the result of interactions that deregulate transcription and translation controls responsible for cell mass and cell cycle progression, as well as the direct modulation of pro-survival signaling [115]. Aberrant activation of PI3K/AKT signaling is a common feature to NSCLC, playing a critical role in the initiation and progression of the disease. [116-118]. The deregulated hyperactivation of AKT is observed in most NSCLC cell lines and NSCLC tumors [35, 36], and found to promote resistance to chemotherapy and radiotherapy [119, 120]. Recent characterization of AKT in lung cancer cell lines identified persistent activation of AKT attributed to PTEN loss, activating mutations in EGFR or PIK3CA, or amplification of ERBB2 [118].

The Ser/Thr kinase AKT (the mammalian homologue of the retroviral transforming protein v-Akt, or also known as PKB, due its homology to PKA and PKC) is the best studied effector of PI3K, as it was among the first proteins found to contain the pleckstrin homology (PH) domain required for phosphoinositide binding [121, 122]. The PH domain was shown to bind to phosphatidylinositol 4,5 P2, and AKT is rapidly activated by growth factors and dependent on upstream PI3K lipid kinase activity [123]. The generation of phosphatidylinositol 3,4,5 P3 and phosphatidylinositol 3,4 P2 can directly bind to the PH domain of AKT allowing for activation phosphorylation at Thr308 by phosphoinositide-dependent kinase 1 (PDK1) [124-126]. However, initiating Thr308 phosphorylation is required for partial activation; an additional phosphorylation at Ser473 is needed for full AKT activation [127]. The phosphorylation at Ser473 is carried out by the mammalian target of rapamycin complex 2 (mTORC2) [128, 129].

Now 20 years since the discovery of AKT, we appreciate the central role as an effector of PI3K that is frequently activated in cancer leading to deregulated metabolism, proliferation and apoptosis (Figure 1.2.). The PI3K dependent activation of AKT occurs in 2 peaks affecting cell growth and G_1 cell cycle progression. The first occurs within minutes of growth factor stimulation (at the G_0/G_1 transition), and second occurring in advanced stage of G_1 phase [130-132]. Activated AKT has a number of direct substrates that have critical roles in transcriptional regulation of the cell cycle. Prominent examples are the cell cycle inhibitor protein p27 (also known as KIP1), the forkhead box transcription factors

(FOXO), glycogen synthase kinase 3 (GSK3), serum and glucocorticoid-induced kinase 1 (SGK1), tuberous sclerosis complex 2 (TSC2) and nuclear factor kB (NFκB) [133-135]. Upregulated PI3K/AKT signaling regulates cyclin-dependent kinase (Cdk) activity by inducing cyclin D synthesis and blocking its degradation, an effect directed by the inhibitory AKT phosphorylation of GSK3β [136]. Cell cycle entry is mediated by PI3K/AKT signaling through FOXO transcription factor controlled expression of cyclin G2 and p27 [137, 138]. Another important molecule inactivated by AKT phosphorylation is TSC2, which functionally connects the activation of PI3K with mTOR signaling. Active TSC2 is unphosphorylated (Ser939 and T1462), whereby it heterodimerizes with TSC1 to negatively regulate GTPase activity of the Ras-like small GTP-binding protein RHEB [139, 140]. The active GTP-bound RHEB can directly interact to positively regulate mTORC1 (mammalian target of rapamycin complex 1) [141] . In addition to TSC2, AKT activates mTOR by phosphorylating regulatory protein subunit PRAS40 (proline-rich AKT substrate of 40kDa) (Ser246) to alter the inhibitory raptor-PRAS40 interaction [142].

The protein kinase TORC1 functions as an evolutionarily conserved environmental sensor. This multifunctional protein integrates to cellular energy and nutrient status and diverse environmental cues such as growth factors, hormones and mitogens to coordinate the proper homeostatic cellular responses. The PI3K/AKT activation of TORC1 promotes POL I- and POL III- RNA polymerase dependent transcription, thus regulating the abundance of protein synthesis machinery in the cell [143, 144]. Furthermore, mTORC1 can directly

regulate protein synthesis by phosporylating effectors S6K (p70 S6 kinase) and 4EBP (translation initiation factor 4E-binding protein) proteins [145].

To date, TOR is known to exist as two multi-subunit complexes known as TORC1 (TOR complex 1) and TORC2 (TOR complex 2) [146] . These TORC complexes demonstrate distinct protein constituents, substrate specificity, regulation, and sensitivity to rapamycin [147]. The mammalian mTORC1 and mTORC2 assemblies both contain mTOR, mLST8 (mammalian lethal with SEC13protein)/Gbl (G-protein b-protein subunit-like) and Deptor (EDP domain-containing mTOR-interacting protein However, the interaction with Raptor and PRAS40 denotes mTORC1 complex, whereas the presence of Rictor, mSIn1 (mammalian stress-activated MAPK-interacting protein 1) and protor 1/2 (protein observed with Rictor 1/2) are representative of the TORC2 complex [146, 148]. Little is known about the functional roles of the mTOR-associated proteins, however Raptor and Rictor proteins are established to function as molecular scaffolds needed for assembly of complexes, and PRAS40 is understood to be a functional suppressor of mTORC1 signaling [149]. As compared to mTORC1 the regulation and function of mTORC2 remains unclear. mTORC2 is responsible for phosphorylating AKT (Ser473), SGK1 (Ser422), and PKCα (Ser657) and is insensitive to rapamycin [150].

Downstream of mTORC1, the ribosomal protein S6K phosphorylates its own group of target proteins most of which are responsible for protein synthesis and regulation of cell size [148, 151]. The best studied target of S6K being rpS6 (ribosomal protein S6), an integral constituent of the 40S ribosome [152].

Concurrently, TORC1 promotes protein synthesis by phosphorylation of 4EBP1 which is responsible for initiating cap-dependent translation by eiF4E (eukaryotic initiation factor 4E) [149].

1.3 Activation of PI3K/AKT pathway in Lung Cancer

The PI3K pathway is commonly deregulated in lung cancer as a result of genetic modifications affecting one or more pathway components leading to hyperactive PI3K signaling [153]. Activation of PI3K in cancer is generally brought about by oncogenic RTKs acting upstream, K-RAS, PTEN loss, or mutations in PI3K itself. In lung cancers, multiple mutations are reported that control PI3K pathway activation, most notable are EGFR and K-RAS (discussed previously) and to a lesser extent by mutations in ERBB2, MET, PIK3CA, and PTEN.

EGFR and cognate ligands are frequently highly expressed in NSCLC occurring in approximately 40-80% of tumors [154], whereas activating kinase domain mutations comprise approximately 10-20% of tumors [155]. Activating mutations present in codons 12 and 13 of GTPase domain of KRAS are observed in approximately 15-25% of NSCLCs [156] . Combined, activating mutations affecting either EGFR and KRAS account for approximately 40-50% of all NSCLC cancers [157]. Crystal structures of the mutant EGFR harboring the TKI-sensitive L858R and G719S mutations show constitutive kinase active through disruptive auto inhibitory interactions [51, 158]. The constitutive signaling through mutant EGFR primarily activates PI3K/AKT and RAS/MAPK pathway

[159]. Lung cancers with mutant EGFR respond to TKI treatment with simultaneous abrogation of both of these survival/proliferation pathways inducing apoptosis and significant tumor regression. Whereas, KRAS-active lung cancers depend on signaling occurring downstream of EGFR and are therefore largely de novo resistant to TKI therapy.

Mutations in PI3K contribute by activating PI3K enzymatic signaling and therefore play an important role in tumorigenesis. A number of somatic mutations have been identified in the p85 regulatory subunit that lead to activation of all class IA isoforms leading to unregulated cell survival and proliferation, however tumor specific mutations in p85 remain largely unexplored. With respect to the catalytic subunit of class I PI3Ks, gain-of-function oncogenic mutations have only been identified in the p110α (PIK3CA) isoform. Mutations in PIK3CA are common to a number of cancers in particular breast and colorectal cancers, and account for approximately 3% of NSCLCs [160-162]. Importantly, NSCLC tumors having PIK3CA mutations more frequently occur concurrent with EGFR or K-RAS (61.8%) mutations than alone (31.2%), and were mutually exclusive with loss of function mutations in PTEN [163, 164]. The co-existence of mutant EGFR and other molecular aberrations affecting IGF-1, PTEN, and PIK3CA were reported to decrease sensitivity to EGFR-TKIs [155, 165]. Likewise, the coexistence of these molecular alterations illustrates the difficulty with assigning individualized single-agent targeted treatments, and further highlights the potential benefit for combination therapies.

Although activating mutations of PIK3CA occur frequently in a number of cancers, the loss of PTEN phosphatase still remains the most common mechanism of activation of PI3K pathway found in human cancers [166, 167]. The genetic and/or epigenetic loss of PTEN phosphatase allows for uncontrolled PI3K kinase activity and activation of effector AKT signaling, resulting in unchecked proliferation and pro-survival signaling [168]. In both SCLC and NSCLC PTEN is rarely mutated, however considerable reduction in expression is common feature in lung cancers [169, 170]. Indeed, loss of PTEN expression and AKT overexpression confers poor prognosis in NSCLC [171]. Importantly, loss PTEN activity is found to contribute to erlotinib resistance in EGFR-mutant lung cancer by uncoupling AKT signaling from direct control of EGFR [172].

1.3.1 PI3K isoforms in cancer

Recent interest in the role of PI3K isoforms in cancer have revealed both unique and redundant functions related to tumorgenesis and signaling for the p110α and p110β isoforms, each of which represent the ubiquitous class IA catalytic subunits of PI3K residing in tissues of origin for most tumor types. p110δ and p110γ isoform expression is largely limited to cells of the immune system [75, 173]. The expression of p110α and p110β are essential for survival and each have non-redundant role with other class IA PI3Ks, as knockout mice demonstrate embryonic lethality at E9.5-10.5 and E3.5 for each respective isoform [174-176].

Both p110α and p110β isoforms are thought to play differential roles in transformation driven by alterations in particular components of the PI3K pathway and therefore have tumor specific activities. For example, tumors overwhelmingly receive PI3K activity through the p110α isoform when harboring "hotspot" mutations in PIK3CA or ERBB2 mutations such as those commonly found in breast and bladder cancers [177, 178]. Whereas, PTEN-null cancers which are common in tumors of the prostate and glioblastoma are thought to be mediated through p110β [179-183]. This divergence is further evident in that mutations affecting PIK3CA and PTEN rarely co-exist in the same cell, and thought to be mutually exclusive [184, 185]. Tumors with mutant PIK3CA often possess alterations in other components of the PI3K pathway such as RAS, and ERBB2/ERBB3 [178, 186]. Pharmacological and knockdown studies confirm signaling to AKT is directed by 110α in breast, colon, and endometrial cancers having co-existing mutations of PIK3CA with RAS or ERBB2/ERBB3 [187].

The hotspot mutations in p110α have been found to cluster in two locations, the helical domain (E542K and E545K) and the in the C-lobe of kinase domain (H1047R) [161, 188]. The helical domain mutations prevent the regulatory interaction of p85 nSH2 domain by disrupting electrostatic interactions between helical residues and nSH2 [189]. Activation of helical domain mutants is no longer responsive (pY) phosphopeptides, but are still dependent on RAS to induce oncogenic transformation [190, 191]. The most common PIK3CA mutation in lung cancer, H1047R, which affects the C-lobe kinase domain conformation of kα4-kα5 and kα11-kα12 loops responsible for membrane

interaction are likely oncogenic due to increased membrane association [192]. Consistent with this observation, the H1047R mutants do not require RAS activation for oncogenic transformation because the adoption of semi-activated confirmation of the kinase domain due to constitutive membrane localization [193].

Paradoxically, apart from p110α (PIK3CA), no other oncogenic mutations have been identified in any of the other class I PI3K catalytic isoforms. Furthermore, the transformative capacity of chicken embryo fibroblasts (CEFs) differs between the E545K and the H1047 PIK3CA mutants, and among the class IA isoforms. Transformation required overexpression of a mutant variant for p110α, as compared to isoforms p110β, δ, and γ which are inherently oncogenic when expressed in as wild-type proteins [194]. Elegant studies using p110α/β chimeras to analyze the C2-iSH2 brake demonstrated that disruption of this inhibitory interface is partially responsible for transforming potential of wild-type p110β and is the likely cause of high basal p110β signaling [110]. However, in the same study p110δ demonstrated differing characteristics in regards to the intact C2-iSH2 interaction, thus indicating a higher order factors are responsible for p110δ wild type transforming potential [110]. The disruption of this p85:p110 inhibitory contact may also explain the failure for p110β effectively signal downstream of activated RTKs relative to p110α as wild-type proteins [107].

The frequency to which PIK3CA is activated in human cancers, and the important role PTEN plays as the negative regulator of PI3K pathway activation, it could be assumed that p110α would function as the dominant isoform signaling

downstream of PTEN loss. Rather, a number of recent studies put that assumption into question by identifying the catalytic activity of p110β as the essential isoform for signaling and growth of PTEN-null cancers [181-183, 195]. This work has been validated using PTEN-null human cancer cell lines for prostate, brain and breast where researchers found that the both pharmacological inhibition using isoform-selective inhibitors of p110, and genetic knockdown of p110β, not p110α impaired downstream activation of AKT. Although controversial, a recent study has implicated p110β in specific growth of ERBB2-driven mammary tumors and K-RAS driven tumors [195]. Finally, p110β is the only class IA PI3K that signals downstream of GPCRs. This occurs via direct interaction with p110β catalytic subunit and Gβγ subunit, and activation occurs independent of the p85 regulatory subunit [196]. Although these studies demonstrated that p110β plays a dominant role in PI3K signaling in PTEN-deficient tumors, it is likely that this reliance is determined by the signaling inputs and the genetic context. Likewise, the contributions of GPCR-driven tumors and the relationship with p110β signaling remains elusive, but may hold promising opportunities. It is clear that p110α and p110β demonstrate distinct and overlapping functions in cell signaling and tumorigenesis, continued investigation is needed as both pan-isoform and isoform-selective PI3K inhibitors move into the clinic.

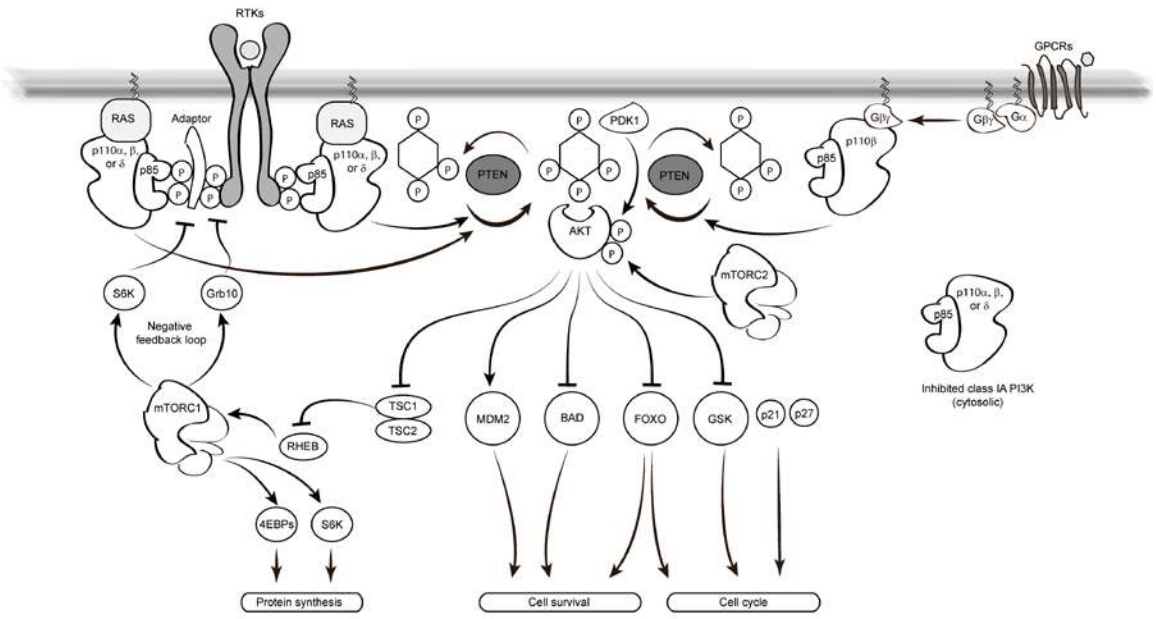


Figure 1.2. Canonical class IA PI3K activation. PI3K transduces signals from activated receptor tyrosine kinases, RAS, and GPCRs. RTKs and RAS can interact with multiple class IA isoforms, however each signal preferentially through p110α and p110δ isoforms, and to a lesser extent the p110β isoform. Conversely, p110β is the only isoform activated by GPCRs. Activation of the PI3K pathway leads promotes cell survival, cell cycle progression and protein synthesis.

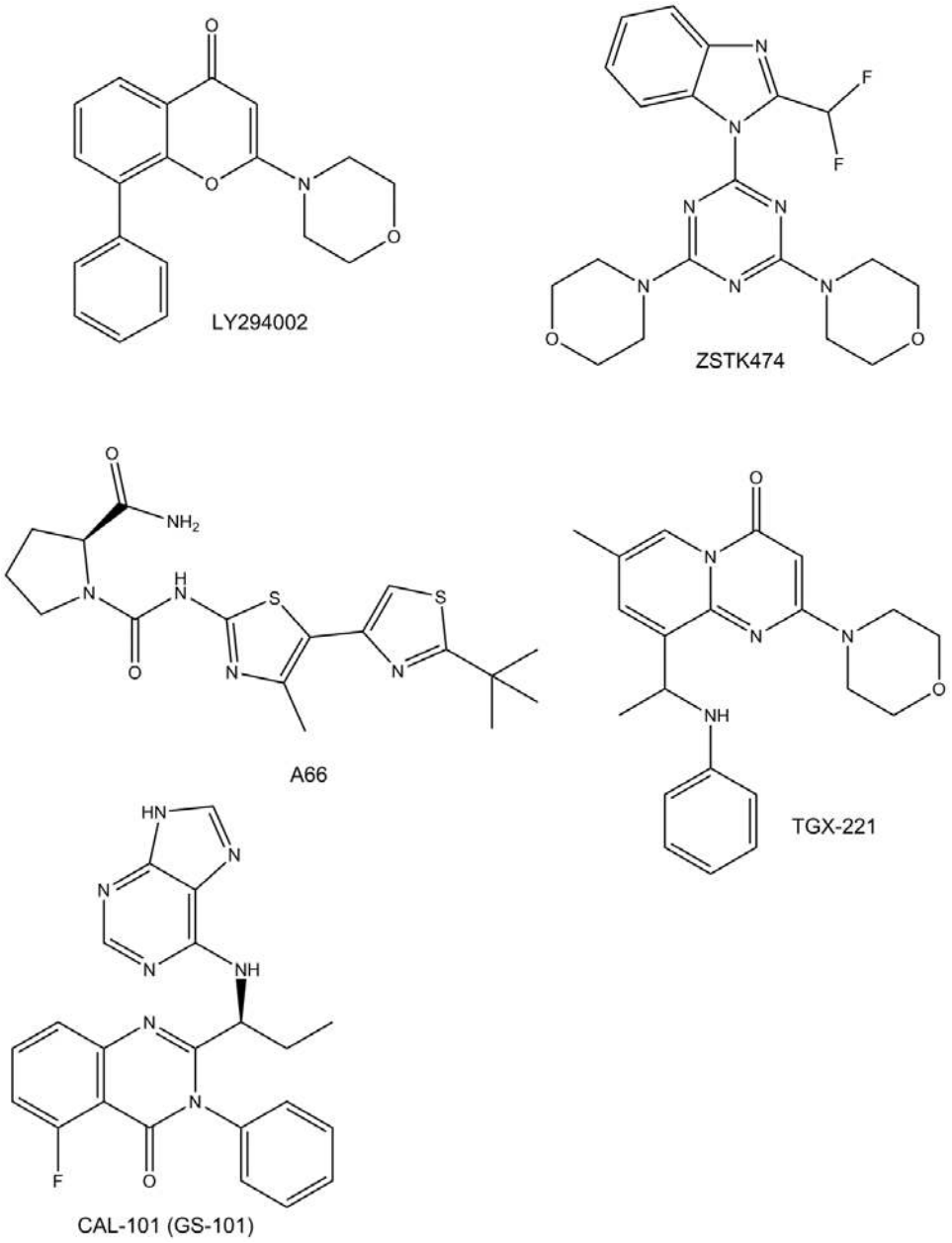


Figure 1.3. Chemical structures of pan-class I and isoform-selective PI3K inhibitors. LY294002 was the first synthesized PI3K inhibitor. ZSTK474 is a pan-class I PI3K inhibitor. A66, TGX-221 and CAL-101 are isoform-selective inhibitors of p110α, p110β, p110δ respectively.

Clinical development and therapeutic limitations of PI3K inhibitors

Unfortunately, the majority of PI3K pathway inhibitors are currently in early clinical development, thus a paucity of clinical data for efficacy and toxicity. PI3K inhibitors fall into 3 general categories: pan-class I PI3K, isoform-selective and dual PI3K/mTOR inhibitors. The first generation of PI3K inhibitors were pan-class I inhibitors, the natural compound wortmannin and the first synthesized drug LY294002 [197, 198]. These drugs demonstrated potent in vitro activities against PI3K signaling, however both showed considerable toxicities in animal studies and failed to reach clinical evaluation. Notwithstanding, more than 15 different agents are currently in various stages of clinical evaluation, a number of which utilize the functional aryl morpholine moiety present in LY294002 [199-201] (Figure 1.3.). Among those currently being investigated, early indications suggest that pan-PI3K/mTOR inhibitors may demonstrate the greatest clinical promise.

The advantage of pan-PI3K/mTOR inhibitors is a more complete shutdown of the PI3K/AKT/mTOR signaling axis, which may remain active in some cancers capable of bypassing single isoform or pan-PI3K, AKT, and mTOR inhibitors related feedback activation of the pathway [202-210]. Continued research is needed to define the innate and acquired mechanisms of resistance that reconstitute PI3K signaling or activate parallel pathways during inhibitor treatment. PI3K and mTOR are structurally similar, both belonging to the PI3K-related kinases (PIKK) superfamily, and therefore similarly targeted by certain inhibitory compounds [82]. Preclinical in vitro studies suggest pan-PI3K/mTOR inhibitors have broader efficacy across a variety of genotypes as compared to

pan-PI3K or mTOR targeting rapamycin analogs [202, 211, 212]. NVP-BEZ235 was the first member of the pan-PI3K/mTOR class of inhibitors to enter phase I clinical trials in patients with advanced solid tumors [213]. However, problems with increased toxicity may prove pan-PI3K/mTOR inhibitors are unsuitable for some genetic contexts or use in combination therapies. Currently, we are unable to make the side by side comparison of clinical efficacy and toxicity between dual pan-PI3K/mTOR and pan-PI3K inhibitors due to lack of clinical data. One may speculate that pan-PI3K inhibitors, having improved specificity relative to dual pan-PI3K/mTOR inhibitors, may be better suited for combination regimens with other targeted or chemotherapeutic agents. According to the ClinicalTrials.gov database, currently there a total of 19 combination trials using pan-PI3K inhibitors plus chemotherapy, and 9 reported for PI3K/mTOR inhibitors with chemotherapy.

Isoform-selective PI3K inhibitors are in clinical development with the goal of targeting specific alterations in PI3K while circumventing off target toxicity associated with inhibiting multiple isoforms. In particular are the development of p110α specific inhibitors to target tumors driven by activating PIK3CA mutations, and p110δ selective inhibitors for hematological malignancies. As compared to pan-PI3K isoform inhibitors, isoform-selective inhibitors of PI3K have a more narrow activity profile among differing tumor types and will require careful patient selection based upon biomarkers of sensitivity [214]. The emergence of isoform-selective inhibitors of PI3K, and the perceived decrease in toxicity raises the possibility of combining these agents with other targeted inhibitors. The use

of isoform-selective PI3K inhibitors with other targeted agents may offer more personalized therapeutic option as compared to a use with pan-PI3K inhibitors.

Recently development and preclinical characterization of p110α selective inhibitors have shown differential patterns of sensitivity and resistance related to the genetic context. The effects of p110α selective inhibitor BYL719 treatment in a large panel of cancer cell lines indicated sensitivity in tumors harboring PIK3CA activating mutations or amplifications or HER2 amplifications, but resistance in those with PTEN and BRAF alterations, and coexisting PIK3CA and KRAS mutations [215, 216]. Furthermore, results from phase I clinical trials with p110α selective inhibitors (BYL719 and GDC-0032) suggest potential benefit for these agents in selective tumors bearing PIK3CA mutations [217, 218]. In contrast with p110α selective inhibitors, recent studies suggest tumors having loss of function PTEN mutations are sensitive to p110β selective inhibitors. Currently there are three p110β selective inhibitors in early clinical trials, AZD8186, GSK2636771, and SAR260301 for treatment of advanced solid tumors with PTEN deficiency [219-221].

The p110δ inhibitor CAL-101 (GS-1101) is the best studied isoform-selective PI3K inhibitor to date. Currently in phase III trials, CAL-101 is being investigated for non-Hodgkin lymphoma (NHL) and chronic lymhocytic leukemia (CLL). The success of CAL-101 has provided the proof of concept that isoform-selective PI3K inhibition may be clinical relevant if acting in the appropriate cellular context. The p110δ isoform is primarily expressed in neutrophils, monocytes and lymphocytes, as well as lymphoid tissues such as the thymus,

lymph nodes and spleen, thus playing a central role in immune signaling through PI3K [222]. Targeting p110δ impairs signaling known to promote CLL migration and retention in bone marrow and/or lymphoid tissues, as well as directly block proliferation and survival of these cells. Collectively there are three p110δ selective inhibitors in development, CAL-101 (GS-1101), AMG 319, and IC488743 (GS-9820) for treatment of lymphoid malignancies.

1.4 PROJECT OVERVIEW

The PI3K pathway is firmly established a major conduit for tumor proliferation and survival and is actively involved in the development and progression of lung cancers. Thus, inhibitors of PI3K may be useful as a cytotoxic or sensitizing agents for treatment of NSCLC. Although inhibitors of the pathway are currently in clinical trials, rational and targeted use of these compounds, alone or in combination, requires an understanding of isoform specific activity in context. In this body of research, the ultimate goal is to evaluate the therapeutic potential for isoform-selective inhibitors through detailed study of PI3K pathway activation, proliferation, and survival in a lung cancer model system.

An overarching question to which this dissertation addresses is whether single-agent PI3K inhibitors can be clinically effective cancer therapeutics. To date, most all clinically successful targeted therapies have been targeted inhibitors against tyrosine kinases, such as BCR-ABL, KIT, EGFR and ERBB2. The common thread among these is that target inhibition leads to the

downregulation of multiple signaling pathways, not just one as is the case with PI3K. One major limitation of single-agent PI3K inhibitor effectiveness is the presence of negative feedback signaling and pathway crosstalk. Prominent examples being the feedback observed with rapamycin treatment where mTORC1 impairment leads to reactivation of PI3K signaling through a feedback inhibition of IGF-1 and the extensive cross pathway regulation between constituents of PI3K and ERK pathways [203]. Given this, there is compelling rational for combining PI3K pathway inhibitors with other targeted therapies to improve clinical efficacy.

To address these questions, the following testable hypothesis were proposed. **First, individual class IA PI3K isoforms p110α, p110β, and p110δ represent novel targets for therapeutic intervention in mutationally diverse NSCLC. Secondly, therapeutic sensitivity and resistance to PI3K inhibition are mediated by pathway compensation due to presence of coexisting mutations, which can be overcome by combination treatment of targeted inhibitors.** Together this work delineates the activity of PI3K in mutationally-defined NSCLC, discovered through the use of pan-class I and isoform-selective inhibitors of PI3K may direct the clinical use of PI3K inhibitors as a single-agent or in combination with EGFR-TKIs for select patient populations.

CHAPTER 2

2.1 OVERVIEW

Lung cancer leads to the greatest morbidity and mortality of all cancer deaths in the United States [19]. To combat these diseases, new therapeutic agents and therapeutic strategies are necessary, and these strategies should include patient-specific therapy to minimize toxicity and cost. Targeting therapies to actionable mutations found in non-small cell lung carcinomas (NSCLC), such as mutation/amplification of epidermal growth factor receptor (EGFR) gene [223], activating point mutations in p110α isoform of PI3K (PIK3CA) [224], and in the KRAS gene [225], and deletion of the PTEN gene [172], may help achieve personalized treatments. Importantly, each of these genetic alterations can lead to increased survival signals that converge on the PI3K/AKT/mTOR cascade. While the PI3K pathway is a central node for control of both cell growth and cell proliferation in normal epithelial cells, aberrant pathway activation in lung tumorigenesis dysregulates cell metabolism, proliferation, apoptosis, and angiogenesis [226-228].

Many cancers depend on a hyperactive PI3K pathway. Thus, increased attention has been placed on development of pharmacological inhibitors of PI3K enzymes that are often mutated or improperly expressed in tumors [229]. Specifically, activation of class IA PI3Ks generates plasma membrane lipid second messenger molecules by phosphorylating the 3'-OH position of phosphoatidylinositol 4,5 bisphosphate (PIP2) to produce phosphatidylinositiol-3,4,5-trisphosphate lipid (PIP3). While PIP3 levels are tightly regulated by the

cell, net pathway activation is determined by a delicate balance between the opposing activities of PI3K lipid kinases and 3- and 5- lipid phosphatases such as PTEN. Recent evidence suggests that there are specific cellular contexts and physiological roles for each class IA PI3K isoform: p110α (PIK3CA), p110β (PIK3CB), and p110δ (PIK3CD) [230, 231]. Cell-specific activities of PI3Ks include selective recruitment of p110 isoforms to activated receptors and second-messenger signal diversification by interaction with various PI3K p85 isoforms. Additionally, activated receptor tyrosine kinases, G-protein coupled receptors, RAS and other small GTPases can signal through cell type-specific PI3K isoforms [107, 181, 182, 232-234]. Application of these data to drug discovery efforts revealed a role for PIK3CD inhibition for treatment of B-cell malignancies and autoimmune diseases [235, 236]. Finally, class IA PI3Ks may also possess non-catalytic activity, serving as molecular scaffolds for other signaling proteins. Thus, therapeutic targeting of individual PI3K class IA proteins may have clinical promise in solid tumors by impeding a host of PI3K functions necessary for cancer cell growth.

We hypothesized that PI3K isoform-selective inhibitors would have clinical value for personalized treatment of lung cancers. Herein, we describe the effects isoform-selective inhibition of class IA PI3K enzymes on pathway activation, proliferation, and viability in genetically-diverse NSCLC cell lines. Our results show that differences in PI3K isoform mutation status, signaling pathway mutations and/or amplifications, and importantly, the apparent compensatory

activity of PI3K isoforms may be critical considerations for personalizing cancer therapy using PI3K inhibitors.

2.2 METHODS

Cell Lines and Culture Conditions

Human NSCLC cell lines obtained from American Type Culture Collection were A549, H460, H1650, H1975, and PC9. The lines were frozen at low passage for future use and subsequently were confirmed by STR testing (Bio-synthesis Inc, Lewisville, TX.). All cell lines were propagated as monolayer cultures at 37˚C in 5% CO_2 using growth media containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glucose, sodium pyruvate, HEPES buffer and penicillin-streptomycin (Life Technologies, Grand Island, NY). The mutational status of each cell line (http://www.sanger.ac.uk/genetics/CGP/cosmic/) is found in Figure 2.1. A**.**

Reagents

IC488743 and CAL-101 (GS-1101) were provided by Gilead Sciences (Foster City, CA). A66, TGX-221, and ZSTK474 was purchased from Selleckchem (Houston, TX). All inhibitors were dissolved in DMSO (Sigma Aldrich Corp, St. Louis, MO; BP231-100) to a stock concentration of 10mM, stored at -20C, and diluted to indicated final concentration in RPMI 1640 containing FBS at time of use.

PI3K activity ELISA

PI3K isoform inhibitor specificity was determined in vitro using an ELISA assay (Millipore, Billerica, MA; 17-493) according to manufacturer's instructions. The biotinylated-PIP3 well values are set to 100. Experimental signals are divided by biotinylated-PIP3 then multiplied by 100 to give percent positive signal. IC_{50} values and growth curves were calculated using Graphpad Prism software. Figures represent percent relative to control for each drug/isoform concentration as indicated. For detailed instructions, refer to the following document:

http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/ca49bfd0cd79e94b852577c3007bcebd/$FILE/PI3K.pdf

Cell Proliferation Assessment

The growth inhibitory activity of each compound was tested on cells using the Alamar Blue viability and Trypan Blue exclusion assays [237, 238]. In the Alamar Blue assay, cell lines were plated at 2 x 10^3 cells/well in 96-well cell culture plate (USA Scientific, Ocala, FL; CC7682-7596), then were treated 24 hr later with PI3K inhibitors) for 72 hr diluted in RPMI 1640 containing 1% serum. For the Trypan Blue exclusion assay, cells were plated at 1 x 10^4 cells/well in 24-well cell culture plate (USA Scientific, CC7682-7524). The cells were synchronized 24 hr later by changing media to RPMI containing 0.1% serum. After 24 hr serum deprivation, cells were released in RPMI containing 1% serum with PI3K inhibitors for 72 hr. Cells were trypsinized, and mixed 1:1 with trypan

blue for visual counting of both viable and dead cells. Experimental concentrations from (0.3 - 30μM) of A66, TGX-221, IC488743 and CAL-101 were tested. GI_{50} values were calculated using Graphpad Prism software.

Cell Cycle and Apoptosis Analysis

For the cell cycle study, NSCLC cell lines were plated in 60-mm dishes (Corning, Corning, NY; 25382-381). After 24 hr, the cells were treated with ZSTK474 and IC488743 at the indicated GI_{75} concentrations for 24 and 48 hr. Following incubation, cells were trypsinized, quenched with serum, washed, resuspended in phosphate buffered saline (PBS), and fixed in cold 70% ethanol. Cell cycle distribution was assessed by staining with propidium iodide (PI) and evaluated by BD FACS Calibur flow cytometery. Data were analyzed with CellQuest software (BD Biosciences, San Jose, CA). Analysis of apoptosis was performed using the same conditions as the cell cycle study, where whole-cell lysates were analyzed by western blot analysis for cleavage of PARP as described below (Cell Signaling Technology, Danvers, MA).

Analysis of Protein Expression

NSCLC cells were plated in 60-mm dishes. After 24 hr, plating media was removed, and cells were synchronized in starvation media RPMI 1640 containing 0.1% FBS for 24 hr. Concentration-dependent response experiments were carried out using indicated drug concentrations for 3 hr. Similarly, time-dependent response to drug exposure was undertaken at 1μM concentration of

each isoform-selective inhibitor at indicated time intervals. After drug treatment, cells were harvested, pelleted by centrifugation, and lysed with M-PER protein extraction reagent (Thermo Fisher Scientific, Waltham, MA; 78503). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for antibody exposure. Signal was elicited using chemiluminescent reagents (ThermoScientific/Pierce). Primary antibodies used in these experiments were phospho-AKT (Ser473), total AKT, phospho-ERK1/2 (Thr302/Tyr204), total ERK1/2, phospho-S6 ribosomal protein (pS6RP) (Ser235/236) phospho-GSK3B (Ser9), α-tubulin, PTEN, LC3β, PARP, p85, cyclin D1, HSP90, Histone H3, PI3K p110α, p110β (Cell Signaling Technology, Danvers, MA; 9271, 9272, 9101, 4695, 4858, 5558, 2125, 9559, 2775, 9532, 9661, 4255, 3011, respectively) and PI3K p110δ (Santa Cruz Biotechnology, Santa Cruz, CA; sc-7176).

Cytosolic and Nuclear Protein Extraction

A549, PC9 and H1650 NSCLC cell lines were treated with indicated concentrations of agents, representing the estimated GI_{75} concentrations for IC488743 and ZSTK474. After 48 hr exposure cells were trypsinized, quenched with serum, pelleted, and washed once with PBS. Nuclear and cytosolic compartments were separated according to NE-PER Nuclear and Cytoplasmic Extraction Kit protocol (ThermoScientific/Pierce, 78835). Western blot analysis for cyclin D1, and controls for cytosolic and nuclear fractionation, HSP90 and Histone H3, were performed as described above.

Short Interfering RNA Knockdown (siRNA)

Cells were plated in 6-well dishes in growth medium without antibiotics. After 24 hr and immediately prior to transfection, the media was changed to RPMI 1640 containing 1% FBS without antibiotics. Using isoform-specific PI3K p110β siRNA (Smartpool-Dharmacon, Thermo Fisher Scientific, Waltham, MA; L-003019-00) (25 nM) was pre-incubated with RNAiMAX transfection reagent (Life Technologies, 13778-150) then added to cells for 24 hr. siRNA-containing media was replaced with RPMI 1640 containing 1% serum with or without 1μM CAL-101 or IC488743 for 3 hr. Cells were then harvested and extracts prepared for western blot analysis, as previously described in analysis of protein expression.

Statistical analyses

Cell proliferation assays were completed three times with three replicates per experiment (Figure 2.2., Table 2.2.). The technical replicate values were averaged for the three experiments and plotted, mean ± standard deviation (SD). ELISA experiments were completed once with technical replicate measures. IC_{50} values were calculated using Graphpad Prism.

Pharmacodynamic analysis represents three independent experiments quantified by densitometric analysis, pAKT (S473) normalized to tAKT, and averaged to reflect changes in pAKT levels (Figure 2.3.). Image J software was used for densitometric analysis of pAKT and tAKT levels from films. The error bars for each agent and cell line combination represents the SEM, n=3.

Cell cycle and apoptosis data represent the mean ± SD of 3 separate experiments, (Figure 2.6.). Figures were generated using Graphpad Prism. Pearson correlation calculation was performed using GraphPad Prism software to compare paired GI_{50} values for each inhibitor quantified in serum-synchronized cells vs. asynchronous cells. The r-value represents the correlation coefficient.

2.3 RESULTS

PI3K isoform expression levels or mutation status alone do not dictate response to PI3K inhibitors.

We initiated the experimentation by determining the expression levels at the gene and protein level for class IA PI3K enzymes in a panel of NSCLC cell lines (Figure 2.1.). Gene expression levels of PI3K isoforms was measured in NSCLC cells using Affymetrix DNA microarray technology. Normalized Affymetrix signal intensity values revealed that the p110β isoform is the most highly expressed of the three isoforms assayed while the p110δ isoform was poorly expressed. Expression of important pathway members was also determined. PTEN demonstrated varying levels of expression among cell lines [239]. Even though gene expression levels vary among PI3K isoforms, levels of protein expression for PI3K isoforms change only modestly (Figure 2.1. B). PTEN expression is the most variable among pathway members assessed. All cell lines show activation of AKT (S473) and varying levels of activated ERK1/2. Finally, mutation status for important driver mutations (EGFR, KRAS, PIK3CA, or PTEN) is also indicated for the cell lines utilized.

To assess the contribution of each class IA PI3K enzyme in lung cancer cells to proliferation and AKT phosphorylation, we chose pharmacological inhibitors of PI3K isoform activity. Inhibitors of three chemotypes previously shown to possess isoform-selective activity were selected: A66 (aminothiazole) [240]; TGX-221 (morpholinochromone) [241]; CAL-101 (quinazolinone purine) [242], and IC488743 [243] (Table 2.1). Briefly, A66 preferentially inhibits p110α, TGX-221 preferentially inhibits p110β isoform, CAL-101 and IC488743 are potent p110δ inhibitors, and the latter also had significant inhibitory activity against p110β. We used the pan-PI3K inhibitor, ZSTK474, which has low nanomolar IC_{50} values for all three class IA isoforms as a control [244]. Although the IC_{50} values for these inhibitors have been independently reported [240-243], we quantified the kinase activity of A66, TGX-221, CAL-101 and IC488743 using a uniform ELISA assay (Figure 2.2.). The resulting IC_{50} values are consistent with those previously reported (Table 2.1.).

To determine whether isoform-selective PI3K inhibitors affect NSCLC cell proliferation and survival, Alamar Blue and Trypan Blue exclusion assays were used. We tested both cell lines in asynchronous and synchronized growth conditions to determine if cell cycle position affected activity of the agents. First, asynchronously growing cells were treated for 72 hr with the pan-PI3K inhibitor, ZSTK474, or each of four isoform-selective inhibitors to determine the half-maximal concentration for growth inhibition (GI_{50}) using the Alamar Blue assay (Table 2.2., Figure 2.3.). In cells synchronized by serum starvation, treatment with PI3K inhibitors was carried out for 72 hr and assayed by Trypan Blue

exclusion (Appendix I: Table 2.3.). There was significant agreement between inhibition of asynchronous and synchronous cells as determined by match-ranking of the inhibitors based on the calculated GI_{50} ($r= 0.651$, $p= 0.001$).

In summary, pan-PI3K isoform inhibition with ZSTK474 was effective at blocking proliferation in all NSCLC cell lines achieving GI_{50} values at nanomolar concentrations. Treatment with isoform-selective inhibitors for p110α, p110β, and p110δ enzymes was largely ineffective at impairing proliferation at concentrations conferring isoform selectivity. H460 and H1975 cells have the greatest sensitivity to the PIK3CA inhibitor, A66 (GI_{50} 8.1μM and 1.7μM) and demonstrated the greatest overall responsiveness PI3K inhibition compared to other cell lines tested. Conversely, PC9 and H1650 cell lines were found to be the least sensitive to all PI3K inhibitors tested. In particular, PC9 cells are most resistant to TGX-221 and CAL-101 (GI_{50} >100μM), and H1650 cells are most resistant to A66 (GI_{50} >200μM). Finally, cell cycle position does not appear to alter sensitivity in any cell line.

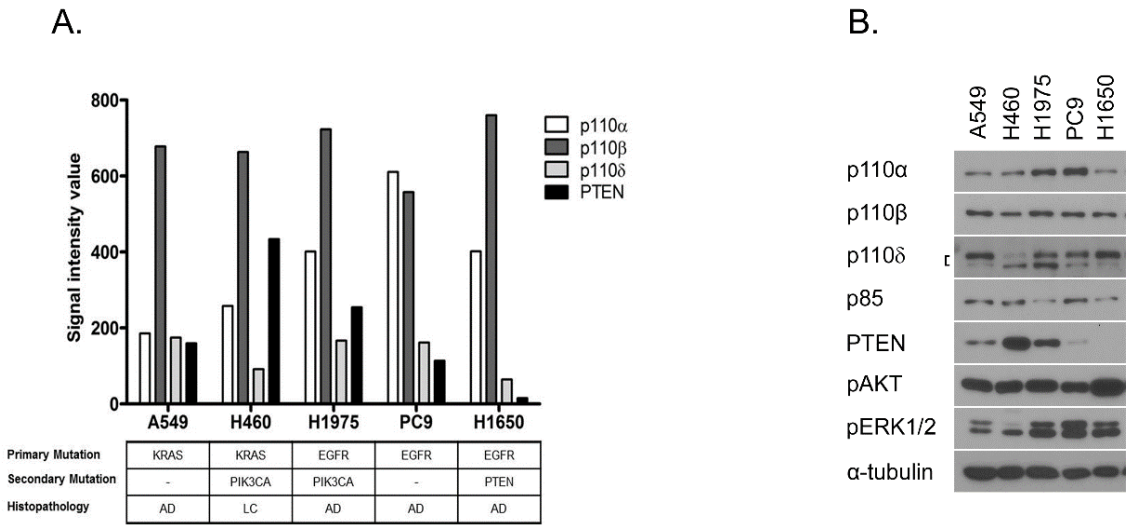


Figure 2.1. Expression of class IA PI3K p110 isoforms and PTEN among NSCLC cell lines. (A) Relative mRNA expression of p110 isoforms and PTEN were measured by Affymetrix microarray analysis. NSCLC cell line mutation status and histopathology are indicated below as Adenocarcinoma (AD) and Large cell carcinoma (LC). **(B)** PI3K p110/p85 isoform and pathway effector protein expression in NSCLC cell lines was determined by immunoblotting analysis. Experiments were performed using sub-confluent proliferating cells maintained in 10% serum containing media.

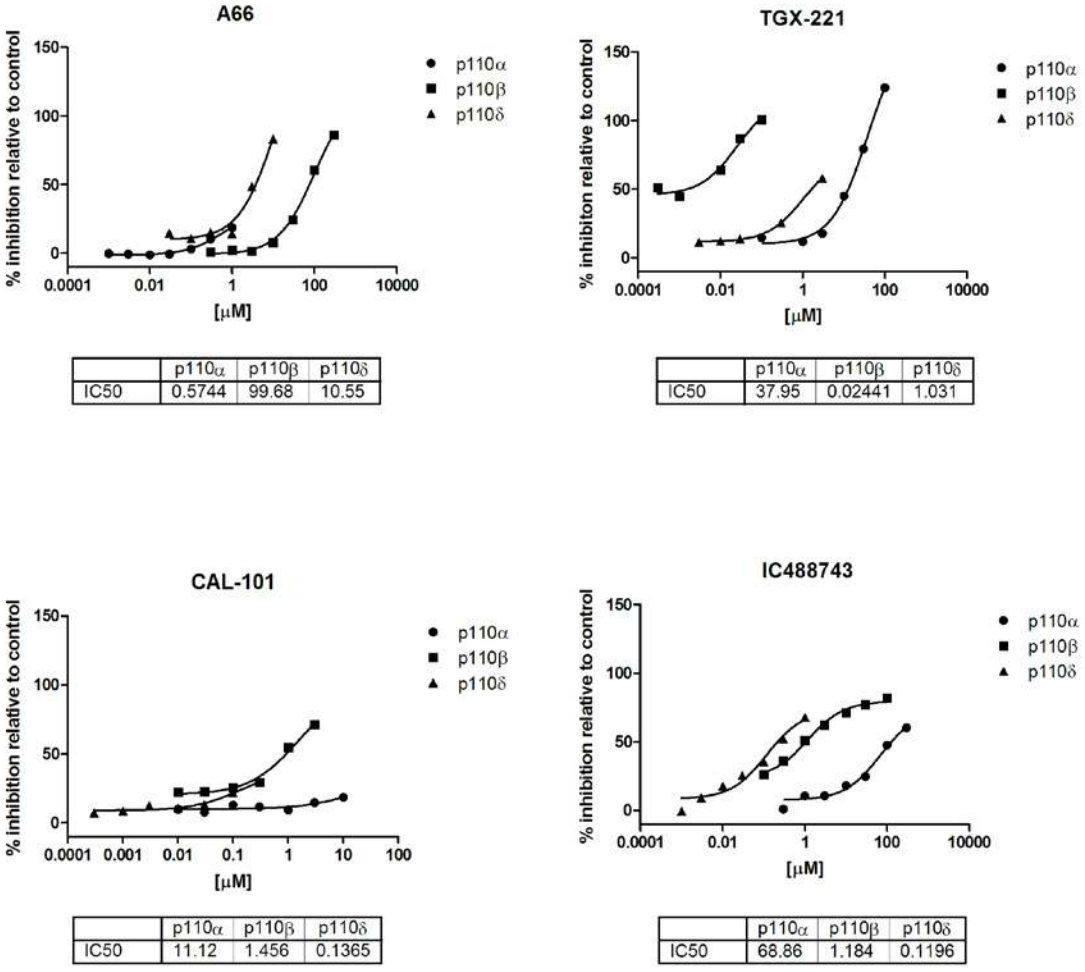


Figure 2.2. Inhibitor specificity measured by Class IA PI3K isoform activity ELISA. PI3K isoform inhibitor specificity was determined in vitro using an ELISA assay according to manufacturer's instructions. Points represent the average of replicate wells in single experiment. IC_{50} values indicating drug selectivity for each of the PI3K isoforms p110α, β and δ are found in table below each plot (μM).

IC_{50} (μM)	p110α	p110β	p110δ	p110γ	mTOR
Pan-PI3K inhibitor					
ZSTK474	0.017	0.053	0.046	0.049	>10
Isoform-selective PI3K inhibitors					
A66	0.032	>12.5	>1.25	3.48	>50
TGX-221	5	0.005	0.100	>35	>10
CAL-101	0.82	0.56	0.002	0.089	>1
IC488743	>20	1.2	0.005	-	-

Table 2.1. PI3K inhibitor IC_{50} selectivity profile. Table indicates previously published biochemical selectivity's for the class I PI3K isoforms and mTOR for each inhibitor tested in study (μM).

GI_{50} (μM)	A549	H460	H1975	PC9	H1650
Pan-PI3K inhibitor					
ZSTK474	0.33	0.15	0.21	0.40	0.69
Isoform-selective PI3K inhibitors					
A66	15.64	8.10	1.74	23.31	>200
TGX-221	24.19	26.78	21.88	120.5	50.14
CAL-101	22.09	10.66	10.85	131.6	62.10
IC488743	22.80	17.45	14.28	32.15	24.45

Table 2.2. GI_{50} values of PI3K inhibitors for NSCLC cell lines. A549, H460, H1975, PC9, and H1650 were treated with increasing concentrations of PI3K inhibitors: ZSTK474, A66, TGX-221, IC488743, and CAL-101 for 72 hr (0.03 - 100μM). Cell proliferation was determined by Alamar Blue viability assay. Non-linear curve fitting and GI_{50} values were generated using Graphpad Prism.

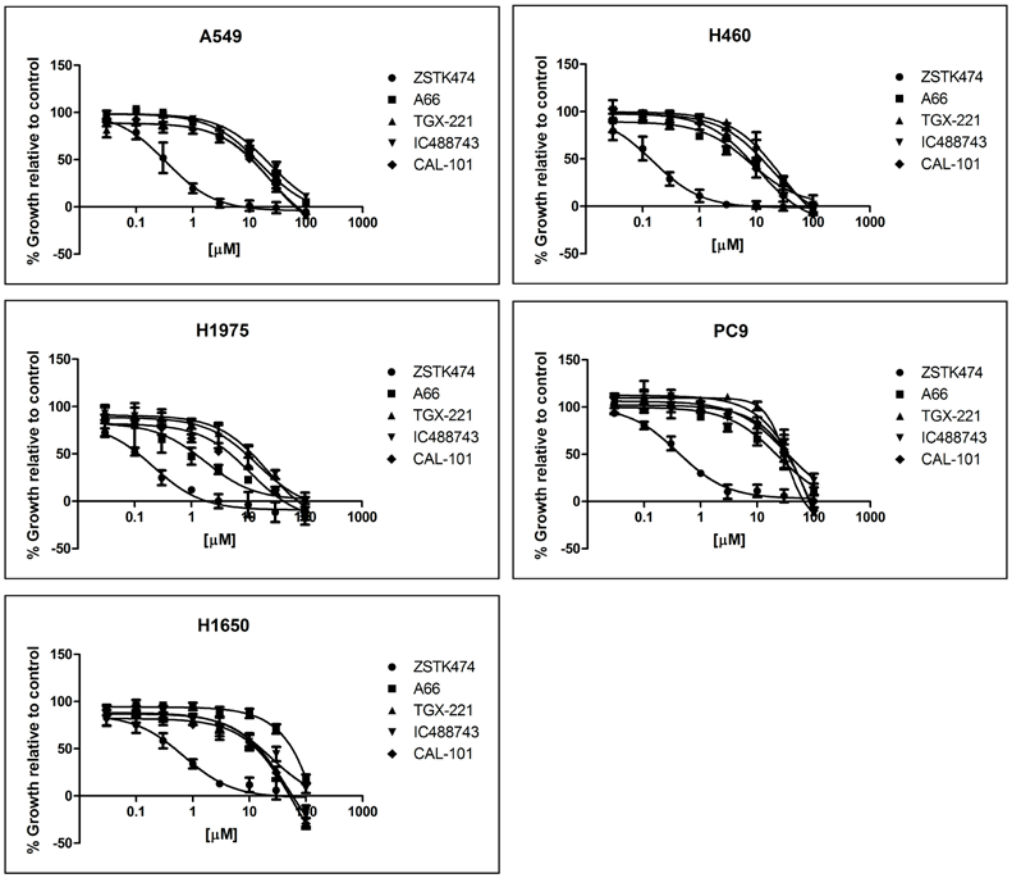


Figure 2.3. Growth inhibition activity PI3K inhibitors against a panel of 5 NSCLC cell lines. A549, H460, H1975, PC9, and H1650 were treated with increasing concentrations of PI3K inhibitors: ZSTK474, A66, TGX-221, IC488743, and CAL-101 for 72 hr (0.03 - 100μM). Cell proliferation was determined by Alamar Blue viability assay. Each experiment was performed with triplicate cultures, for 3 independent experiments (n=3). Error bars represent standard deviation (SD). Experimental results were normalized to 24 hr plated cells and divided by untreated control to determine (% growth relative to control). Non-linear curve fitting and GI_{50} values were generated using Graphpad Prism.

Inhibition of PI3K/AKT blocks survival signals necessary for proliferation

To assess whether the lack of proliferative response of the cell lines to isoform-selective inhibitors of the PI3K enzymes was due to inadequate target inhibition, we tested AKT activation over a range of inhibitor concentrations by western analysis of serine 473 phosphorylation after 3 hr of treatment. The ratio of pAKT/tAKT was determined by densitometric evaluation of the immunoblots and is plotted for each cell line and isoform-selective inhibitor at indicated concentrations (Figure 2.4.).

Consistent with the proliferation assays, H1975 and H460 cell lines demonstrate the greatest reduction of pAKT at nanomolar concentrations of A66 while H1650 demonstrated only minor reduction in pAKT even at high concentrations (Figure 2.4. A). Treatment with the p110β inhibitor, TGX-221, had little effect on AKT phosphorylation in all cell lines except for the H1650 cells (Figure 2.4. B). H1650 cells demonstrated nearly 75% reduction in pAKT at nanomolar levels of TGX-221. Treatment with the p110δ inhibitors (CAL-101 and IC488743) reduced AKT phosphorylation below 50% at the highest concentrations used for most cell lines (Figure 2.4. C and 2.4. D). As compared to the isoform-selective inhibition, treatment with the pan-class I PI3K ZSTK474 was followed by complete loss of pAKT at all concentrations except for at 0.1μM, whereby we achieve >50% decrease in all cell lines tested (Figure 2.5.). We did not observe any change in pERK1/2 phosphorylation in response to 3 hr ZSTK474 treatment.

Recent studies identify the catalytic activity of p110β as the essential isoform for signaling and growth of PTEN-null cancers, we sought to further validate these findings by testing the isoform-selective inhibitors utilized in this study in the PTEN-null prostate cancer cell line, PC3 (Figure 2.6.). We note activated PI3K/AKT signaling and basal expression levels of class IA PI3K isoforms at similar levels to those found in NSCLC cell lines. As indicated by the pharmacodynamic and proliferative responses to isoform-selective inhibitor treatment, PC3 cells demonstrated the greatest sensitivity to the p110β inhibitor, TGX-221, and were least responsive to the p110α inhibition. Treatment with the p110δ inhibitors were able to diminish pAKT signaling, but had limited effects on proliferation.

To determine the duration of inhibition of effector molecule (pAKT and pS6RP) phosphorylation, a time course was performed with 1μM of each inhibitor on three NSCLC lines (A549, PC9, H1650) (Figure 2.7.). At this concentration (1μM), each compound is predicted to be largely on-target, having a minor impact on other PI3K isoforms. The duration of activity was consistent with both the proliferation and concentration-dependent treatment assays. Specifically, the effect of each inhibitor against A549 cells was sustained over time, as measured by reduction in phosphorylation of AKT and S6. The PC9 cells displayed minimal response to each agent demonstrated by uninterrupted pAKT levels, except for A66. However, like the other cell lines, pS6 was reduced over time with each inhibitor relative to untreated cells. Like PC9 cells, H1650 cells also demonstrated sustained responses to each agent as measured by absence of S6

phosphorylation. However, A66 is ineffective at reducing pAKT levels, unlike other agents relative to the untreated control. The accumulated data suggest that loss of a single PI3K class I isoform might be compensated by remaining isoforms. To test this hypothesis, we undertook an experiment to couple gene depletion with pharmacological treatment.

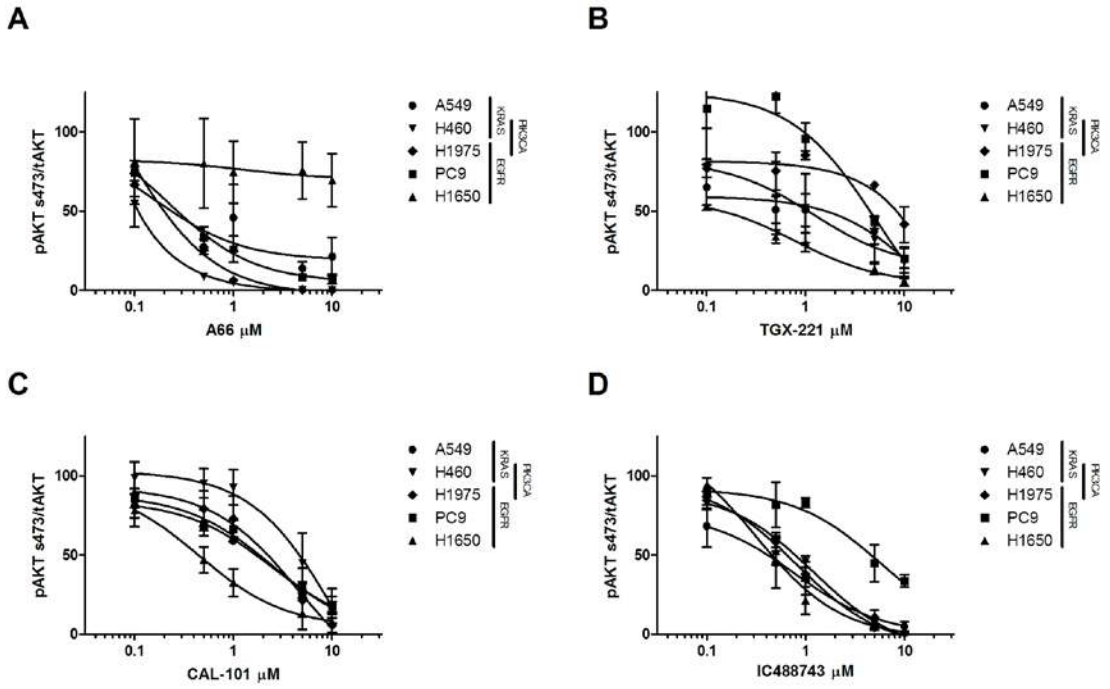


Figure 2.4. Pharmacodynamic responses to PI3K isoform-selective inhibition. The relative levels of phosphorylated AKT in NSCLC cell lines (A549, H460, H1975, PC9, H1650) treated with increasing concentrations (0.1-10μM) of PI3K inhibitors **(A)** A66 **(B)** TGX-221 **(C)** CAL-101 and **(D)** IC488743. Cells were synchronized by serum-starvation for 24 hr prior to release in RPMI 1640 media containing 1% serum with or without drug for 3 hr. Cell lysates were collected for immunoblotting analysis with pAKT (S473) and tAKT antibodies. α-tubulin serves as the normalization control. ImageJ software was used for densitometric analysis of pAKT and tAKT levels. Three independent experiments were quantified, pAKT (S473) was normalized to tAKT, and data were averaged to reflect relative changes in pAKT (S473) activation. Error bars represent mean ± SEM. PIK3CA mutant cell lines are indicated.

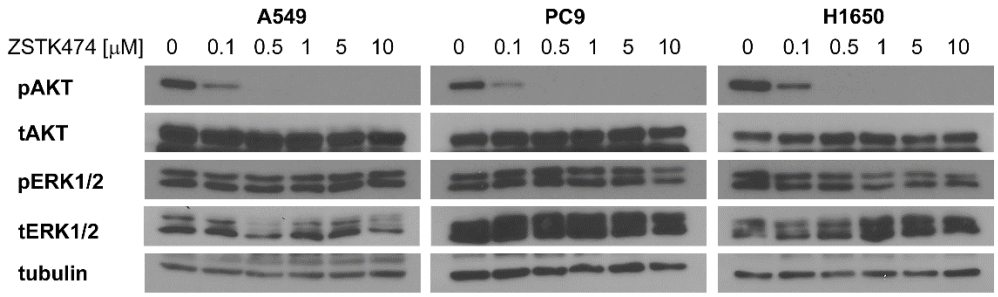


Figure 2.5. Pharmacodynamic responses to pan-class I PI3K inhibitor ZSTK474. The relative levels of phosphorylated AKT in NSCLC cell lines (A549, PC9, and H1650) treated with increasing concentrations of ZSTK474 (0.1-10μM). Cells were synchronized by serum-starvation for 24 hr prior to release in RPMI 1640 media containing 1% serum with or without drug for 3 hr. Cell lysates were collected for immunoblotting analysis with pAKT (S473), tAKT, pERK1/2, tERK and α-tubulin antibodies.

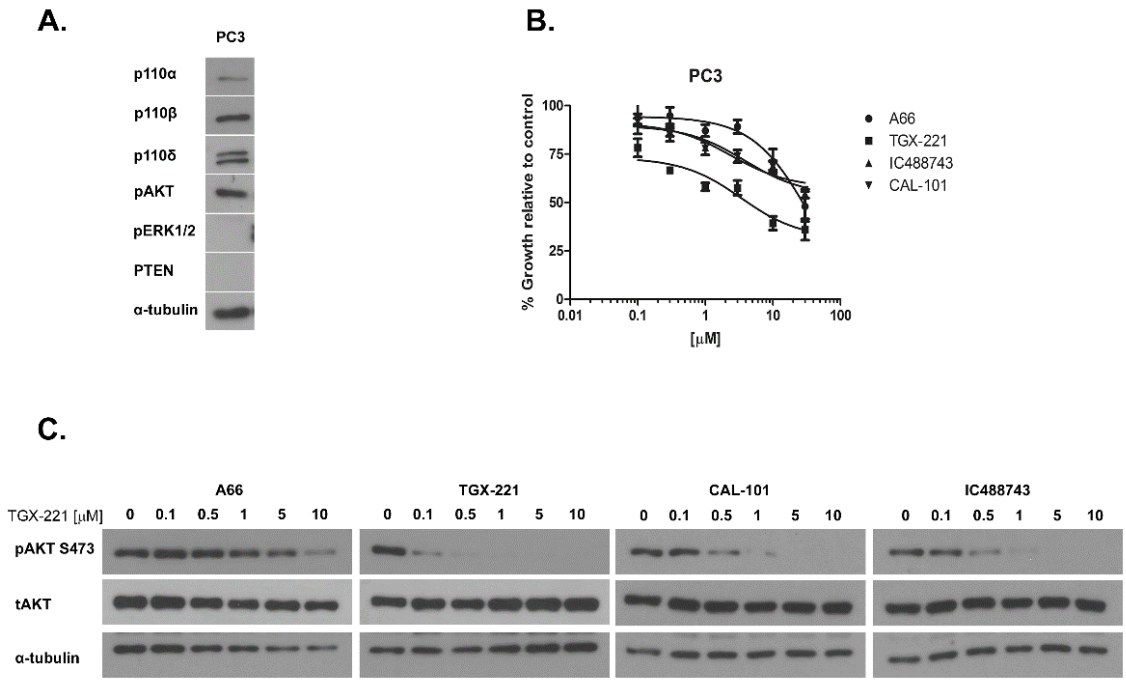


Figure 2.6. Basal class IA PI3K expression, proliferation and pharmacodynamic responses to isoform-selective inhibitors in PTEN-null prostate cancer cell line PC3. (A) Class IA p110 isoform and pathway effector protein expression as determined by immunoblotting analysis. Proliferating cells in 10% serum containing RPMI were harvested 24 hr after plating. **(B)** PC3 cells were treated with increasing concentrations of PI3K inhibitors; A66, TGX-221, IC488743, and CAL-101 for 72 hr (0.03 - 30μM) and proliferation was determined by Alamar Blue viability assay as described previously. **(C)** The relative levels of phosphorylated AKT after treatment with increasing concentrations of A66, TGX-221, CAL-101 and IC488743. Cells were synchronized by serum-starvation for 24 hr prior to release in RPMI 1640 media containing 1% serum with or without drug for 3 hr. Cell lysates were collected for immunoblotting analysis with pAKT (S473), tAKT, and α-tubulin antibodies.

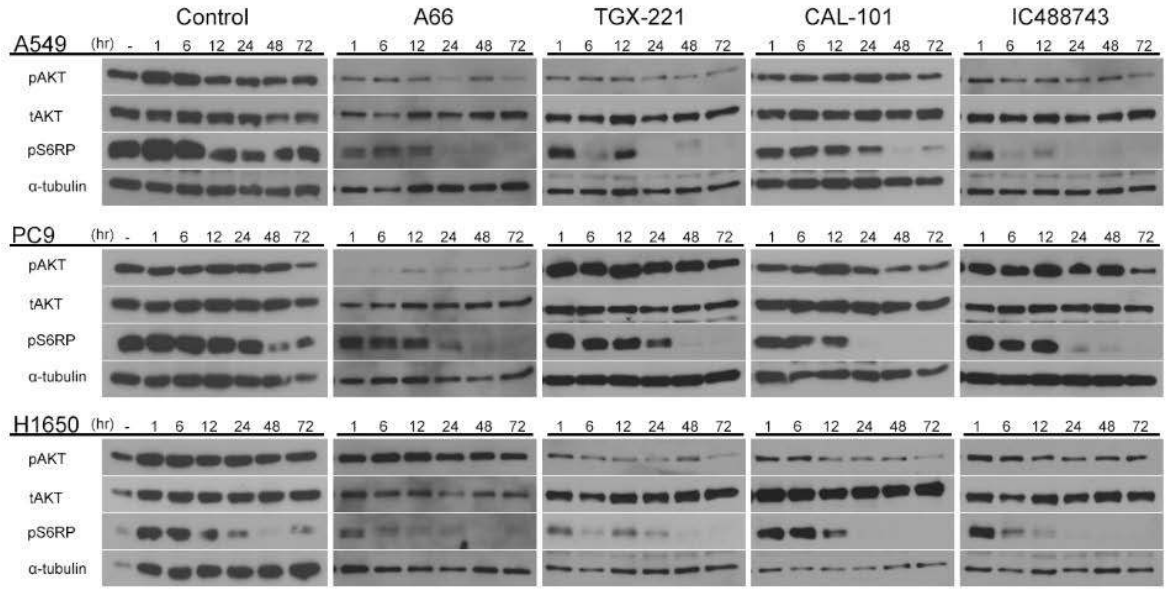


Figure 2.7. Isoform-selective PI3K inhibitor treatment demonstrates sustained time-dependent inhibition of PI3K signaling. A549, PC9 and H1650 cells were treated with 1μM isoform-selective inhibitors in RPMI 1640 containing 1% serum for regular time intervals of 1, 6, 12, 24, 48, 72 hr after drug addition. Untreated controls were tested using identical conditions but without drug addition. Samples were collected at each time point for immunoblotting with indicated antibodies as described above. α-tubulin serves as the normalization control.

siRNA knockdown of p110β does not phenocopy effects observed with PIK3CB and D inhibition.

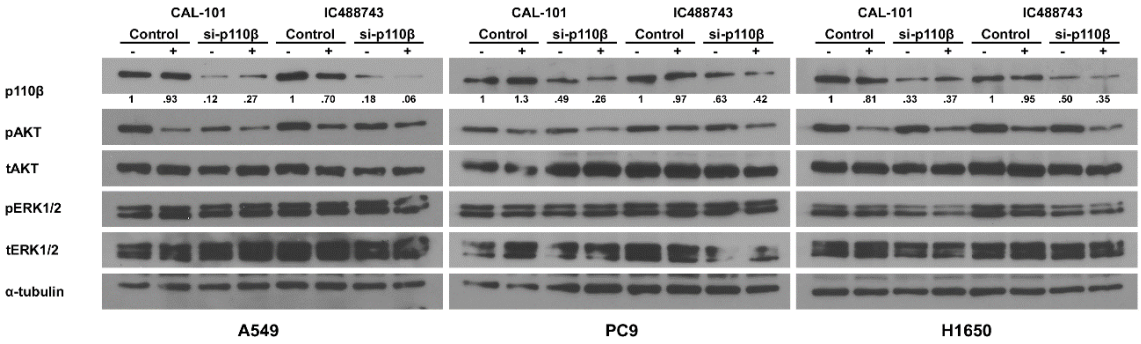
The previous experiments provided evidence that inhibition of proliferation and loss of AKT signaling manifested at concentrations of each inhibitor that exceeded selectivity for a single isoform. Only the pan-inhibitor, ZSTK474, was effective at inhibiting proliferation at sub-micromolar concentrations, suggesting that in most cells PI3K activity was necessary. Further, the cell lines with activating PIK3CA mutations (H460 and H1975) were the most sensitive to A66, as previously described in the breast cancer literature [218, 245, 246]. We hypothesized that PI3K isoforms may be able to compensate for one another. We tested this hypothesis on the cells least sensitive to PIK3CA pharmacological inhibition (A549, PC9, H1650). Expression of p110β was ablated using short-interfering RNA (siRNA) then cells were treated with either CAL-101 or IC488743 for 3 hr at 1μM concentration.

Silencing of p110β isoform reduced expression of p110β in A549 by 80%, in PC9 by 55%, and in H1650 by 61%, as measured by densitometry (Figure 2.8. A). However, partial ablation of the p110β isoform alone did not significantly reduce phosphorylation of AKT at S473 compared with IC488743 or CAL-101 treatment alone in all cells, suggesting that p110α and p110δ isoforms, or residual p110β, can compensate for the genetic loss of p110β. Importantly, the observed decrease in pAKT after p110β ablation with CAL-101 treatment was not superior to treatment with CAL-101 alone. Similarly, treatment of p110β-depleted cells with IC488743 prompted a minor decrease pAKT levels compared with the

either p110β-depletion or IC488743 treatment, suggesting that PIK3CA is responsible for the remaining activity (Figure 2.8. B).

Interestingly, pERK1/2 signaling was reduced in H1650 cells in the p110β knockdown cells for all conditions (plus or minus CAL-101 or IC488743), but not the other lines (Figure 2.8. A). Densitometric analysis of pERK1/2 showed reduction of signal of nearly 50%, suggesting that one or both isoforms play a role in RAS/MAPK signaling in H1650 cells which may be independent of the phosphorylation of AKT. These data suggest that both the AKT and RAS/MAPK signaling cascades may be linked by multiple independent PI3K isoforms, and functionally these may have kinase independent roles such as a molecular scaffold or adaptor protein.

A.



B.

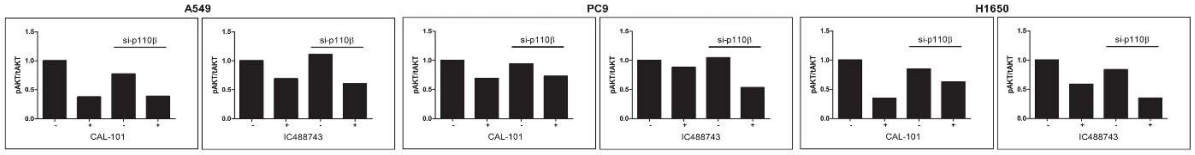


Figure 2.8. Functional redundancies among class IA isoforms maintain PI3K pathway activation after siRNA-mediated ablation PIK3CB and pharmacological inhibition PIK3CD. (A) A549, PC9, and H1650 cells were transiently-transfected with siRNA specific for p110β for 24 hr (25nM), then treated with p110δ inhibitors CAL-101 or IC488743 (1μM) for 3 hours before harvesting. Cell lysates were prepared for immunoblotting with indicated antibodies. **(B)** Densitometric analysis of pAKT (S473) levels normalized to tAKT expression levels.

Treatment with IC488743 or ZSTK474 promotes cell cycle arrest in G_1 but not apoptosis NSCLC cells

Inhibition of p110β and p110δ isoforms by genetic and/or pharmacological treatment reduced cell proliferation and inhibited PI3K signaling in A549, PC9, and H1650 NSCLC lines better than isoform-specific inhibitors alone. We sought to understand if reduced proliferation was due to cell cycle arrest or induction of apoptosis, given that the potential consequences on normal and tumor cells.

To identify the cause of reduction in proliferation, we first investigated the cell cycle profile of cells treated with the IC488743 as compared to the pan-isoform inhibitor ZSTK474 at the individual GI_{75} concentrations for cell lines A549, PC9, and H1650. We chose to assess the compounds at the GI_{75} because this is the relative concentration where a proliferative phenotype was observed with IC488743. Importantly, when evaluating differences in sensitivity between the two inhibitors, ZSTK474 was on average >50-fold more potent than IC488743 (1.3μM vs 73.3μM) among the three cell lines. After 24 hr of treatment, both compounds led to significant accumulation in G_1 phase for each cell line with concomitant loss in the S phase compartment (Figure 2.9. A). At 48 hr, we observed a sustained arrest in G_1 in A549 and H1650, however PC9 cells appear to exit G_1 and re-enter into cell cycle. Additionally, at both 24 and 48 hr we detected no change in the % sub-G_1 population suggesting apoptosis was not being induced by inhibitor treatments for all cell lines tested (Figure 2.6. B).

Using identical conditions as outlined above, we also assessed pathway and apoptotic signaling at 24 and 48 hr after treatment with IC488743 and

ZSTK474. As expected, both drugs inhibited phosphorylation of AKT S473 at each time interval. We also observed re-activation of pERK1/2 signaling in the PC9 and H1650 cells (Figure 2.9. C). The increase in pERK signaling in response to treatment was most pronounced in response to ZSTK474 in the EGFR mutant cell lines H1650 and PC9, with only a minor increase observed in KRAS-active A549 cells. We also evaluated cleaved PARP as an apoptotic signaling molecule. We observed no change in cleaved PARP levels with treatment which is consistent with our inability to detect significant changes in sub-G_1 fraction after treatment (Figures 2.9. B and 2.9. C).

To characterize treatment effects on cell cycle progression, we evaluated nuclear and cytosolic levels of cyclin D1 after 48 hr treatments (Figure 2.9. D). Cyclin D1 should be extruded from the nucleus of cycling cells. After 48 hr, cyclin D1 accumulated in the cytosol with treatment of IC488743 or ZSTK474 relative to the controls in A549 and H1650 cells. However, in PC9 cells we observed the presence of nuclear cyclin D1 in drug treated samples, suggesting progression into the cell cycle. Fractionation of the nuclear and cytosolic compartments was confirmed by histone H3 and HSP90 localization.

From these data, only pan-PI3K inhibition or an isoform-selective inhibitor utilized at non-selective concentrations can diminish PI3K/AKT signaling sufficient block proliferation while not inducing apoptosis. However, we note G_1 blockade may be temporary, and taken together with apoptotic failure are likely the result of compensatory mechanisms that increase pERK signaling to escape PI3K/AKT inhibition.

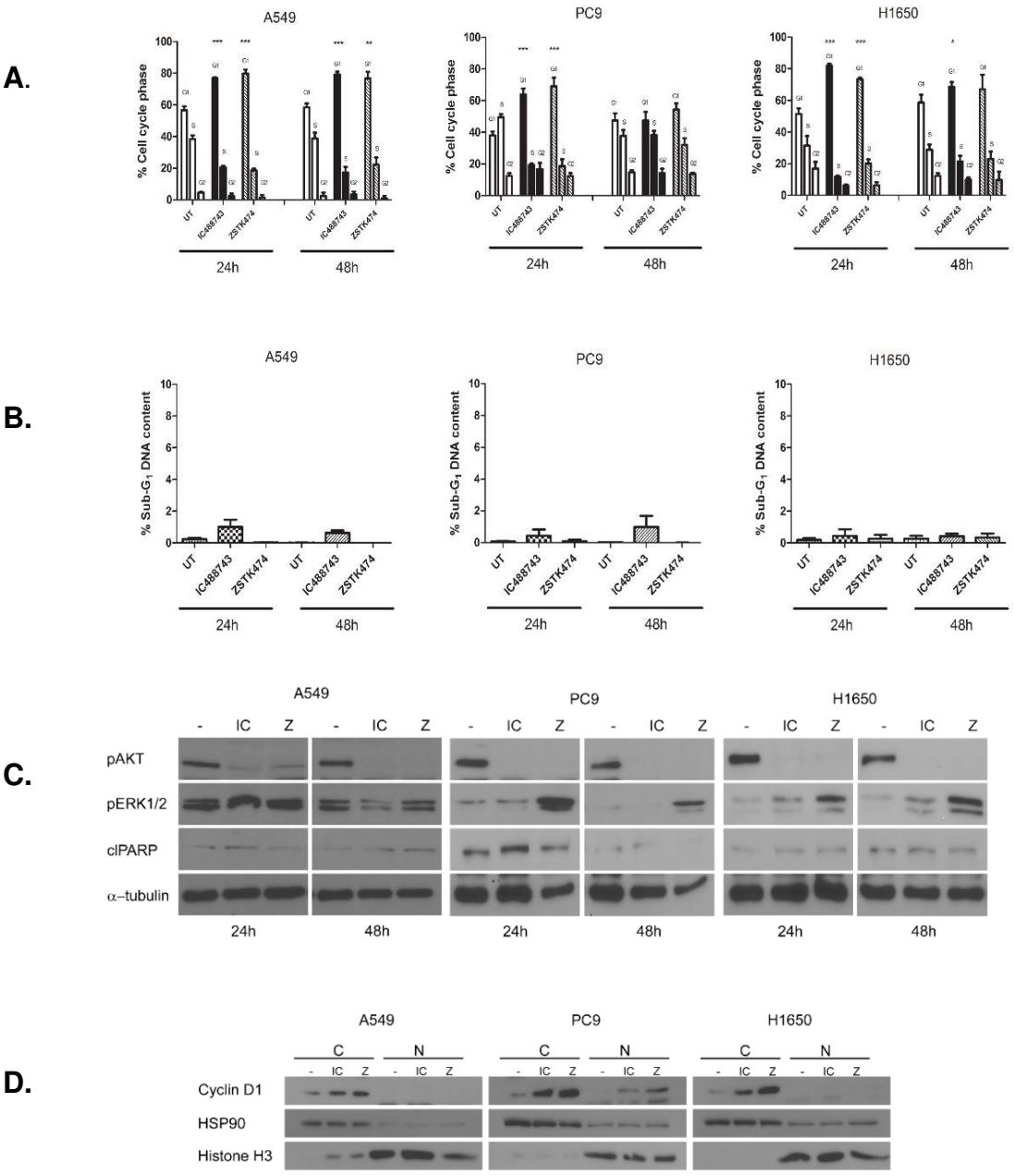


Figure 2.9. IC488743 and ZSTK474 treatment alters G_1 cell cycle progression without inducing apoptosis. (A) Control, IC488743 and ZSTK474 treatment at GI_{75} concentrations for A549 (IC 65μM and Z 1μM), PC9 (IC 100μM, Z 1.3μM), and H1650 (IC 55μM, Z 1.6μM) cells were assessed for relative cell cycle distributions by propidium iodide (PI) staining of DNA following 24 hr and 48 hr of treatment. Mean percentages of cells in G_0/G_1, S, and G_2/M are

indicated. Error bars represent standard deviation, (n=3) and Student t test showed significant difference (*p<0.05). **(B)** Control, IC488743, and ZSTK474 treated A549, PC9 and H1650 cells were analyzed for % sub-G_1 population indicating apoptosis. Mean percentages of sub-G_1 cells are displayed. Error bars represent standard deviation, (n=3). **(C)** Markers for pathway activation and apoptosis assayed in A549, PC9, and H1650 cells treated at GI_{75} concentrations of IC488743 (IC) or ZSTK474 (Z) for 24 and 48 hr then harvested for western blot analysis. **(D)** Cells treated for 48 hr with GI_{75} concentrations of IC488743 or ZSTK474 were subjected nuclear and cytosolic fractionation then western blot analysis to determine the sub cellular localization of G_1 to S-phase regulatory protein cyclin D1.

2.4. DISCUSSION

Lung cancer is a disease characterized by extensive genomic changes that unfortunately lead to millions of deaths worldwide each year because patients do not achieve a sustained response to therapy [247]. Only recently have actionable mutations and mutated signaling pathways been identified and targeted therapeutically [248-251]. Our interests converge on the PI3K/AKT/mTOR signaling axis because it represents one of the most commonly activated pathways in cancer for which few targeted therapies have resulted in clinical use in lung cancer [252].

In lung cancers, mutations have been reported in multiple genes that control PI3K/AKT pathway activation, including EGFR, KRAS, HER3 and BRAF

[49, 159, 186], even though few adenocarcinomas of the lung demonstrate mutations in PIK3CA [163]. Together, these observations make the effector molecules of the PI3K pathway alluring targets for cancer therapy. First generation PI3K inhibitors targeted all class I PI3K isoforms, but were not suitable for clinical use due largely to toxicity and poor bioavailability [253, 254]. Although class IA PI3K isoforms possess similar protein structure, control of expression, and regulation of activity, recent literature reports non-redundant cellular functions that appear to be isoform specific [108, 255-260]. Importantly, to our knowledge, PI3K isoform-specific activities have not been dissected in lung cancers. Therefore, we chose to investigate the intersection of therapeutically-actionable mutations and deregulated activities of the PI3K/AKT signaling cascade in NSCLC cell lines.

We investigated the activity of a panel of PI3K inhibitory compounds in vitro and in cell lines. The IC_{50} values PI3K isoform specificity and selectivity have been previously published and further validated by this laboratory. Using these compounds as tools, we intended to evaluate the activity of each compound for inhibition of growth and/or cell killing in a panel of NSCLC cells and to independently assess the necessity of each PI3K class IA enzymes in NSCLC.

We investigated anti-proliferative responses using a range of drug concentrations overlapping our in vitro estimation of IC_{50} for each isoform. We found that several compounds have anti-proliferative activity against the cell lines tested at micromolar concentrations. Importantly, we found that cell lines

containing mutated PIK3CA were most sensitive to A66. Notably, H460 bears an activating mutation in PIK3CA (E545K) as does H1975 (G188D) which apparently sensitizes the cells to A66 (GI_{50} 8.1μM vs 1.59μM, respectively). CAL-101 (GS-1101) is a small molecule inhibitor of p110δ isoform that has been demonstrated to having promising activity against chronic lymphocytic leukemia (CLL) [173, 222, 236]. We found that CAL-101 has similar anti-proliferative activity as the other p110δ inhibitor tested, IC488743, each demonstrating best activity in H1975 and H460 cell lines. Although CAL-101 and IC488743 elicited similar anti-proliferative profiles they differed most in the EGFR mutant lines PC9 and H1650. It is important to note that the GI_{50} values for even the most sensitive cell lines were well above those estimated IC_{50} values for isoform selectivity, and pharmacologically unachievable. However the data also suggest that at micromolar concentrations we may be achieving dual inhibition of p110β and p110δ while sparing p110α, resulting in diminished cellular proliferation for several cells of multiple genotypes. Having similar potency for individual class IA isoforms as the isoform-selective inhibitors, the pan-PI3K inhibitor ZSTK474 was far more effective at blocking proliferation than any of the isoform-selective inhibitors tested having GI_{50} in nanomolar range for all NSCLC cell lines.

The dose-dependent pharmacodynamic experiments largely mirror the proliferation results. Pan-PI3K inhibition with ZSTK474 completely abrogated pAKT signaling at 0.5μM and >50% at 0.1μM for A549, H1650 and PC9 cell lines (Figure 2.5.). For isoform-selective inhibitors, impaired PI3K/AKT pathway signaling was observed at micromolar concentrations, and elicited an anti-

proliferative response in most cases. Whereas, treatment at concentrations suitable for on target single isoform selectivity had little effect on proliferative signaling with the exception of A66 in PIK3CA active H1975 and possibly H460. A noteworthy counter example was identified in the PTEN-null prostate cancer cell line PC3, where we found selective p110β inhibition with TGX-221 effective at blocking proliferation and pathway activation relative to p110α, and p110δ selective inhibitors (Figure 2.6.). However, among the NSCLC cell lines, TGX-221, CAL-101 and IC488743, to achieve proliferative control these drugs are acting like weak pan-class IA PI3K inhibitors, largely inhibiting only p110β and p110δ, and off-target activity on p110α at higher concentrations. Consistent with this experiment, time-dependent inhibition of PI3K/AKT signaling was achieved with partial off-target activity that had differential effects that were sustained among the NSCLC cell lines. From these observations it is evident that PC9 cells are resistant to targeting p110β and p110δ isoforms, whereas H1650 cells are resistant to targeting p110α.

Together, these data suggest that there may be inherent resistance mechanisms in place in these cells such that isoform-selective inhibition of individual PI3Ks has minimal impact on intrinsic cell proliferative and survival signals.

Interestingly, in the preclinical development of CAL-101, drug concentrations of 5-10μM were needed to achieve cytotoxic activity which is at least 1000x more than the predicted IC_{50} for p110δ, likely inhibiting both p110β and p110γ PI3K isoforms. Furthermore, clinical exposure to CAL-101 is greater

than 2μM at lowest doses, suggesting that partial inhibition of other PI3K isoforms such as p110β may contribute in part to clinical cytotoxic effects observed in patients [242, 243, 261, 262]. Thus, therapeutic resistance to PI3K isoform-specific inhibition might involve one or more of the following mechanisms: compensation by other PI3K enzymes, compensatory signaling from components within PI3K/AKT pathway and/or elements from other oncogenic pathways, or over-expression of efflux transporters.

We chose to investigate the possibility of compensating PI3K enzymes with respect to resistance to the PIK3CD inhibitors, CAL-101 and IC488743, each of which possess a different selectivity profile for PIK3CA, PIK3CB and PIK3CD. Using siRNA-mediated knockdown of p110β, we tested the activity of CAL-101 and IC488743 in three cell lines each having variable sensitivity to either drug. From this experiment, it seems likely that ablation of two of the three enzymes of interest (PI3KA/B/D) is necessary to eliminate the majority of the pAKT activity, thought to mediate cell growth and survival.

Given that inhibition of multiple PI3K enzymes results in reduced proliferation, we also investigated the mechanism by which proliferation is halted in the presence of IC488743 and ZSTK474. The concentrations needed to achieve GI_{75} were significantly lower for the pan-PI3K inhibitor (1 - 1.6μM) as compared to IC488743 (55 - 100μM), further suggesting that pan-isoform blockade may be therapeutically necessary to negate compensatory PI3K signaling from other class IA isoforms that allow for continued proliferation. Our results indicate that both inhibitors impaired PI3K/AKT pathway signaling leading

to G_1 cell cycle arrest, which corresponded with increased cytosolic sequestration of cyclin D1. Despite having potent effects on proliferation, there was no observable induction of apoptosis for either drug suggesting that targeted inhibition of only the PI3K pathway is insufficient to induce apoptosis in NSCLC cells bearing similar genetic aberrations. In line with this, we observed a reciprocal increase in pERK after treatment with IC488743 and ZSTK474, indicating these cells may be exploiting the extensive pathway cross-talk and feedback mechanisms between PI3K/AKT and RAS/MAPK pathways that allow for cell cycle re-entry and escape from apoptosis. Interestingly, this increase in pERK is not observed in our early 3 hr concentration responses to ZSTK474 treatment at similar concentrations. However, others have shown time dependent feedback activation in response to PI3K inhibition often occur through FOX3A-mediated transcriptional upregulation of ERBB family receptors such as HER2 and HER3 leading to increased pERK signaling [263].

2.5 CONCLUSIONS

In summary, we hypothesized that isoform-selective inhibition of PI3K class IA enzymes was a rational choice for lung cancer patient therapy. Importantly, we found that PIK3CA mutated cells responded to p110α inhibition. Inhibition of both p110β/δ was more effective than inhibition of individual p110β and p110δ isoforms alone, however none of these appear to have clinical relevancy among the NSCLC tumor subtypes we tested. Overall, our findings provide evidence that redundancy among class IA isoforms contributes to drug

resistance, and that pan-class I PI3K inhibitors may hold greater therapeutic promise. Given the challenges for identifying effective treatment of lung cancer and the need to overcome various forms of therapeutic resistance, the most effective use of PI3K inhibitors may ultimately be in combination with other targeted or cytotoxic therapies.

CHAPTER 3

3.1.1 OVERVIEW

The deregulated activation the PI3K pathway is a hallmark in human cancer, thus making PI3K a logical target for therapeutic exploitation. New inhibitors of the PI3K pathway having improved potency and fewer side effects compared to first generation drugs [201]. Of these, there has been increasing interest those inhibitors having selectivity for individual PI3K isoforms. Our lab as well as others report that class IA PI3K isoforms have both dependent and redundant activities that is partially determined by the genetic context [231]. Accrued knowledge from these studies suggests that p110α responds preferentially to activated receptor tyrosine kinases (TKIs) whereas, p110β is the dominant isoform in tumors having phosphatase and tensin homolog (PTEN) loss-of-function mutations [99, 173, 174, 219-221]. Our studies outlined in Chapter 2 characterized and compared the activity of class IA isoform-selective inhibitors with that of a pan-class I PI3K inhibitor and found that likely compensatory signaling among the isoforms reduced overall sensitivity to the inhibitors despite genetic drivers, thus limiting the therapeutic promise for these drugs.

The implementation of molecular-based targeted therapies should not be a “one size fits all” approach. The mutational heterogeneity of lung cancers have made difficult single-agent targeted therapeutics as compared to traditional chemotherapies. The inhibition of a single target or pathway essential for tumor proliferation is likely a temporary effect and does not produce sustained growth

arrest. Prominent examples are found for inhibitors targeting EGFR, estrogen receptor (ER), and androgen receptor (AR) [38, 264, 265]. The complexity of tumor signaling networks, particularly in advanced tumors, which often have multiple molecular aberrations and context-specific pathway crosstalk, that ultimately prevent or circumvent the therapeutic blockade. Therefore, adopting a strategy to achieve robust lasting therapeutic control of a cancer may require rational combinations of targeted anti-cancer agents. The use of combined targeted therapies may serve to impair compensatory mechanisms and overcome resistance to therapy, and will require a better understanding of the complex signaling in context with molecular drivers of disease to identify valuable drug combinations.

3.1.2 INTRODUCTION

In adenocarcinomas of the lung, activating mutations in EGFR and KRAS comprise approximately 40% of genetic alterations. EML4ALK translocations, loss/gain of function of BRAF, PIK3CA, AKT1 and MEK comprise another 10-15% of cases [36]. A unifying theme among all of these alterations is that they interact either directly or indirectly with the PI3K pathway signaling. Importantly, targeted inhibition of the epidermal growth factor receptor (EGFR) can be an important target for treatment of advanced non-small cell lung cancer (NSCLC) [249, 266]. These drugs are safe, maintain a good toxicity profile and are well tolerated at high doses [267]. Small molecule inhibitors that block the tyrosine kinase domain of EGFR have been approved for treatment of locally advanced or

metastatic NSCLC as a second- or third-line therapy [266]. Although, EGFR-TKIs are approved as second-line treatment for advanced NSCLC, nearly 50% of patients are unable to obtain second-line treatment (non-platinum single-agent cytotoxic) due to the rapid decline of their clinical condition [268]. This represents key clinical end-point, especially for patients with aggressive tumors, as they need to be treated with drugs that in a second-line setting that demonstrate prolonged survival and preserve quality of life [269]. Divergent clinical responses to EGFR-TKI treatments have been associated with EGFR mutations and KRAS mutations. Patients harboring EGFR mutations in exons 19 and 21 are sensitive to TKI, whereas presence of PTEN or KRAS mutations define innate resistance to TKI therapy. However, most who initially benefit from EGFR-TKI treatment eventually experience tumor regrowth after 12 months of progression free disease [270]. Approximately 75% of resistance on therapy are attributable to either the T790M mutations of EGFR or the amplification of MET oncogene. Each of these can lead to reactivation of PI3K survival signaling [43-45].

Unlike EGFR mutant tumors, there are no current targeted therapeutic options for KRAS-active cancers. Activating mutations in KRAS result in defective GTPase activity leading to constitutive activation of multiple pathways responsible for cell growth and survival such as PI3K/AKT and RAF/MEK/ERK pathways [271]. Efforts to directly target KRAS have proven unsuccessful to date. However, other routes currently being investigated are to target the downstream effectors of RAS such as RAF, MEK, and PI3K. Although KRAS-active cancers are generally thought to be unresponsive to EGFR-TKI therapy,

there is also a more complex model to consider. Others have demonstrated that genotypically heterogeneous subpopulations of tumors are common [272]. Assuming the TKI-sensitive EGFR mutant subpopulations are killed by the targeted therapy, the growth and maintenance of the remaining non-EGFR mutant cells are unaffected manifesting as therapeutic resistance. In the SATURN trial, a large phase III trial having 889 randomized patients having not progressed after first-line chemotherapy, received erlotinib or placebo as a maintenance treatment [273]. Of these, 493 (55.4%) were analyzed for KRAS mutations. The study found that, irrespective of KRAS status, patients treated with erlotinib had longer progression free survival (PFS), with a modest but not statistically significant in improvement in the KRAS mutant population. Meaning that in patients harboring KRAS mutations that do not respond to EGFR-TKIs, a minimal survival effect may occur. Although several studies do identify a higher prevalence of clinical responders among KRAS wild-type as compared to KRAS mutant, diagnostic KRAS testing is not recommended for precluding an EGFR-TKI therapy to any NSCLC patients [274-276]. One must also consider the possibility that wild-type EGFR may still play a role in modulating growth survival signaling in KRAS-active cancers.

As summarized above, KRAS-active cancers and other therapy resistant subpopulations may receive some, albeit minor clinical benefit from targeted EGFR therapy overall. Consequently, any undiagnosed mutant tumor subpopulations harboring PTEN or KRAS mutations may negatively impact tumor responses to single-agent EGFR directed therapies leading to tumor regrowth.

Studies have shown tumor subpopulations harboring KRAS mutations in lung and colon cancers to persist at higher levels relative to normal tissue, however remaining undetectable by standard DNA sequencing techniques [277, 278]. Furthermore, PTEN and KRAS mutation status often differ between primary and metastatic tumors [279, 280]. This knowledge provides rationale for combining therapies that impair oncogenic pathways deregulated by PTEN and KRAS mutations (PI3K inhibitor), with those that inhibit EGFR signaling (EGFR-TKI).

Therefore, we propose an accelerated therapeutic strategy intended to prolong survival of those with advanced NSCLC after failure to progress on first-line chemotherapy by treating with EGFR-TKI in combination with a pan-PI3K inhibitor as maintenance therapy subsequent to receiving first-line chemotherapy. We hypothesize that the combined treatment with an EGFR-TKI and pan-PI3K inhibitor will have clinical value, interacting synergistically to sensitize NSCLC cancers innately resistant to EGFR targeted therapy. Our study found that despite being inherently resistant to EGFR-TKI inhibitors, tumors harboring KRAS mutations or defective PTEN are sensitized to EGFR therapy when combined with PI3K inhibition to arrest cell proliferation.

3.2. METHODS

Cell Lines and Culture Conditions

Human NSCLC cell lines obtained from American Type Culture Collection were A549, H460, and H1650. The lines were frozen at low passage for future use and subsequently were confirmed by STR testing (Bio-synthesis Inc,

Lewisville, TX.). All cell lines were propagated as monolayer cultures at 37˚C in 5% CO_2 using growth media containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glucose, sodium pyruvate, HEPES buffer and penicillin-streptomycin (Life Technologies, Grand Island, NY).

Reagents

The inhibitors erlotinib (EGFR-TKI) and ZSTK474 (pan-PI3K) were purchased from Selleckchem (Houston, TX). All inhibitors were dissolved in DMSO (Sigma Aldrich Corp, St. Louis, MO; BP231-100) to a stock concentration of 10mM, stored at -20C, and diluted to indicated final concentration in RPMI 1640 containing 1% FBS at time of use.

Cell Proliferation Assessment

The growth inhibitory activity of each compound was tested on cells using the Alamar Blue viability assay [237, 238]. Cells were plated at 2 x 10^3 cells/well in 96-well cell culture plate (USA Scientific, Ocala, FL; CC7682-7596). After adherence, cells were treated 24 hr later with treated with indicated combinations of erlotinib and ZSTK474 for 72 hr diluted in RPMI 1640 containing 1% serum. Dose-response curves and GI_{50} values were calculated using Graphpad Prism software.

Statistical analyses

Cell proliferation assays were completed three times with a minimum of 2 replicates per experiment. The technical replicate values were averaged for the three experiments and plotted, mean ± standard deviation (SD). CompuSyn software was used to evaluate drug combination median effects and combination indices (CI). CompuSyn algorithm simulating drug interactions utilize Chou-Talalay method, where additive effect (CI = 1), synergism (CI > 1), and antagonism (CI > 1) [281].

3.3. RESULTS

Combined EGFR-TKI and pan-PI3K inhibitor act synergistically to impair proliferation of KRAS-active NSCLC

We investigated the growth inhibitory activity of the EGFR-TKI, erlotinib, and the pan-PI3K inhibitor, ZSTK474, as measured in the NSCLC cell lines A549 and H460 by Alamar Blue assay (Figure 3.1.) We found that both A549 and H460 were similarly insensitive to single-agent exposure to erlotinib, reaching 50% growth inhibition at concentrations >3μM. The pan-PI3K inhibitor was markedly more potent, achieving half maximal growth inhibitory concentration in the nanomolar range.

Studies were then performed to determine the effect of combined EGFR-TKI and pan-PI3K inhibitor treatment on cell proliferation over 72 hr. Each inhibitor combination was tested at concentrations spanning GI_{50} values. We found that the combinations of erlotinib and ZSTK474 were more growth

inhibitory than either compound alone. Although similar, H460 cells demonstrated greater growth inhibition in response to the drug combinations. Growth inhibition was evauated by median effect analysis (CompuSyn, Memorial Sloan-Kettering Cancer Center and MIT) to determine if the improved growth inhibitory activity of the combinations was additive or synergistic (Figure 3.2.). Combinations were synergistic at 1:1, 1:10, and 1:100 ratios (ZSTK474:erlotinib), but antagonistic effects were evident at 10:1 and 100:1 ratios (ZSTK474:erotinib) (Table 3.1.). Overall, while a synergistic interaction was observed with pan-PI3K and EGFR-TKI inhibitors that impaired cell proliferation, we identified modest cytotoxic effect in the H460 cell line occurring at highest tandem drug concentrations.

Synergistic combinations of EGFR-TKI and pan-PI3K inhibitor block proliferation in EGFR mutant/PTEN-null NSCLC

The proliferative effects of erlotinib and ZSTK474 drug combinations were evaluated in the NSCLC cell line H1650 by Alamar Blue assay (Figure 3.3. A) H1650 cells were also found to be insensitive to single-agent erlotinib treatment, whereas the singe-agent pan-PI3K inhibitor was more effective at impairing proliferation after 72 hr exposure comparatively. Median effect analysis was calculated for each drug combination in order to assess inhibitory drug interactions (Figure 3.3. B). Our results for H1650 were similar to those found for A549 and H460, where synergistic interactions for ZSTK474:erlotinib ratios occurred at 1:1, 1:10 and 1:100, and antagonism in combinations where ZSTK474 concentrations exceeded the erlotinib concentrations (i.e. at ratios of

10:1, 100:1). Interestingly, at highest concentrations of erlotinib (30 and 3μM) we see identical responses to ZSTK474 combinations, each resulting in the complete blockade of proliferation and minor cytotoxicity.

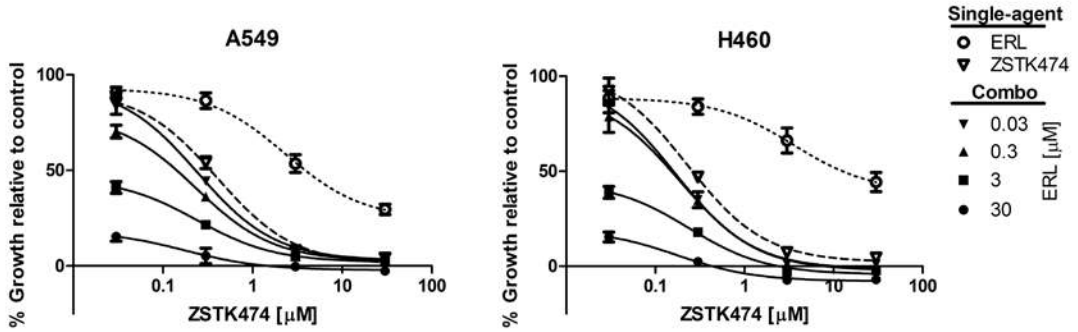


Figure 3.1. Growth inhibition in KRAS mutant NSCLC induced by EGFR-TKI, erlotinib (ERL), and the pan-PI3K inhibitor, ZSTK474, alone and in combination. A549 and H460 cells were treated with indicated concentrations of each inhibitor, as single-agent, or in combination. Combination data are presented as individual curves at fixed erlotinib concentrations (0.03 – 30μM) plotted against increasing ZSTK474 concentrations (0.03 – 30μM). Points represent mean of three independent experiments ± S.D., and lines were fitted using non-linear regression analysis.

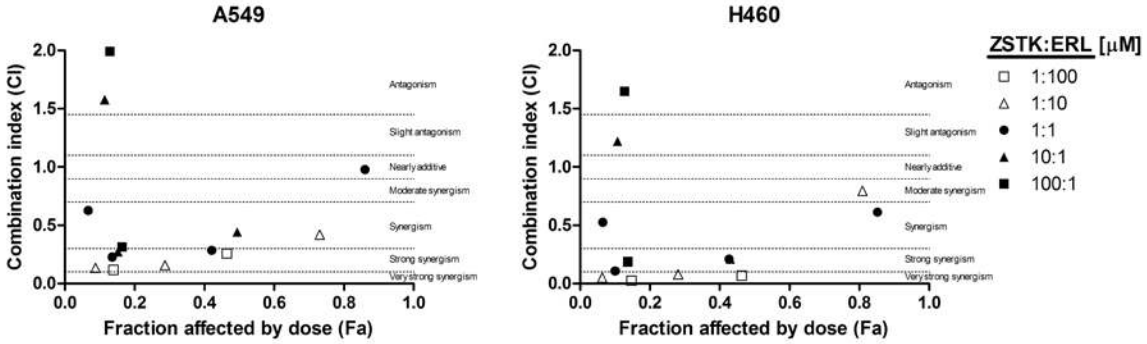
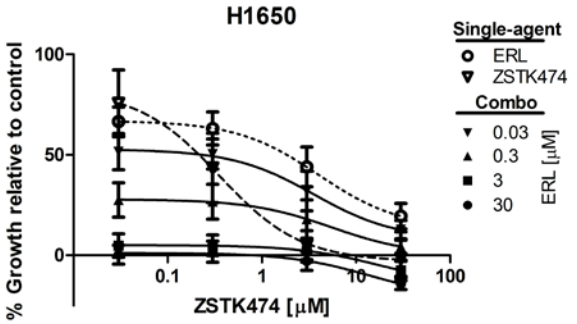


Figure 3.2. Interaction of EGFR-TKI and pan-PI3K inhibitors in KRAS mutant NSCLC. Median effect analysis was performed by CompuSyn software to study the interaction between the inhibitor combinations at the concentrations indicated in Figure 3.1. and Table 3.1. Points represent the mean of three independent experiments. Dotted lines mark range of interaction classification.

A.



B.

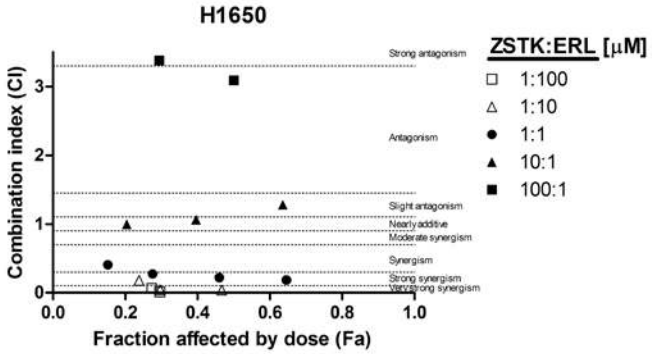


Figure 3.3. Proliferative responses and inhibitor interactions to combination EGFR-TKI and pan-PI3K inhibition in EGFR mutant, PTEN-null NSCLC. (A) Combination effects on proliferation in EGFR mutant NSCLC. Data presented as previously described in Figure 3.1. Points represent mean of three independent experiments ± S.D., and lines were fitted using non-linear regression analysis. **(B)** Interaction of EGFR-TKI and pan-PI3K in EGFR mutant NSCLC. Data are presented as previously described.

A549		ERL [µM]			
		30	3	0.3	0.03
ZSTK474 [µM]	30	0.62	1.58	1.99	2.08
	3	0.13	0.22	0.27	0.31
	0.3	0.11	0.15	0.28	0.44
	0.03	0.17	0.25	0.42	0.97

H460		ERL [µM]			
		30	3	0.3	0.03
ZSTK474 [µM]	30	0.52	1.22	1.65	1.84
	3	0.05	0.1	0.18	0.18
	0.3	0.02	0.08	0.2	0.211
	0.03	0.03	0.06	0.79	0.61

H1650		ERL [µM]			
		30	3	0.3	0.03
ZSTK474 [µM]	30	0.4	0.99	3.38	6.74
	3	0.18	0.27	1.06	3.09
	0.3	0.06	0.04	0.21	1.28
	0.03	0.03	0.01	0.03	0.18

Table 3.1. Summary of drug combinations with corresponding combination indices. Table summarizes calculated combination index value (CI) indicating individual concentrations of erlotinib (ERL) and ZSTK474 used in combination.

3.5. DISCUSSION

The intersection of signaling pathways involved in cancer has only recently been exploited for therapeutic gain. The PI3K/AKT pathway and RAS/MAPK pathway both signal downstream of receptor tyrosine kinases, and are considered central mediators of oncogenic signaling in solid tumors [107]. Numerous targeted small molecule inhibitors are currently under development targeting various elements of these cascades such as EGFR, PI3K, AKT, RAF and MEK [103, 214]. However, tumors often have molecular aberrations affecting multiple signaling pathways making single-agent inhibitors ineffective. Both pre-clinical evidence and early phase clinical trials suggest that combination therapy may be a more effective strategy as compared to single-agent treatments. Our experience with infectious diseases, such as HIV and tuberculosis, underscores the importance and success of drug combinations as therapeutic strategy [282] .

In the current study, we investigated the combined pharmacological inhibition of EGFR and PI3K in NSCLC cell lines resistant to EGFR-TKI therapy. Cells harboring KRAS activating mutations rarely coexist with mutations affecting EGFR, and are largely considered de-novo resistant to single-agent EGFR-TKI therapy because RAS lies downstream of EGFR, thus oncogenic signaling through PI3K/AKT and MAPK pathways can persist despite EGFR activation [223]. H1650 cells are found to contain both mutant EGFR (DelE746-A750) and PTEN deletion. Previous reports as well as observations in our lab have observed that cells harboring loss-of-function PTEN mutations are resistant to

EGFR inhibitors [172]. These studies demonstrate that in cells that are EGFR dependent, loss of PTEN may partially uncouple constitutive mutant EGFR signaling from downstream pathways PI3K/AKT and RAS/MAPK, thereby contributing to EGFR-TKI resistance. Together, these support targeting the PI3K pathway may be effective strategy to impede proliferation in EGFR inhibitor resistant tumors.

We have previously shown that all of the NSCLC cell lines (A549, H460, H1650) tested were sensitive to the single-agent pan-PI3K inhibitor ZSTK474. For KRAS-active A549 and H460, GI_{50} values 0.33μM and 0.15μM, and EGFR/PTEN mutant H1650 had GI_{50} at 0.69μM (Figure 2.3., Table 2.2.). Anti-proliferative responses correlated with pharmacodynamic signaling as indicated by complete loss of pAKT signaling (Figure 2.5.). However, single-agent pan-PI3K treatment did not induce apoptosis (Figure 2.9.). These data also suggest that oncogenic signaling through PI3K/AKT may not be limited to any specific cancer genotype and suggests that constitutive pathway activation may be limiting efficacy of PI3K inhibitors. We found that most EGFR-TKI and pan-PI3K inhibitor combinations tested in this study demonstrated marked synergistic growth inhibitory activity in all cell lines tested (Table 3.1.). These observations support and extend previous in vitro data demonstrating that PI3K inhibitors used in combination with cytotoxic agents, as well as anti-EGFR and MEK inhibitors have improved anti-tumor activity relative to single-agent treatments [159, 283-285].

When comparing dose response curves of drug combinations, we note distinct trend differences between the KRAS mutant and EGFR mutant NSCLC cell lines (Figure 3.1. and Figure 3.3.). Interestingly, among the KRAS mutant lines A549 are wild-type for PIK3CA, whereas H460 harbors (E545K) PIK3CA activating mutation, we do see an improved synergistic effect and cytotoxicity at higher drug combinations in H460 comparatively. Both KRAS mutant cell lines fail to respond to increasing erlotinib doses, thus reduction in proliferation is largely in response to PI3K inhibition. The opposite is observed in EGFR mutant H1650 cells. Proliferative capacity appears to be a function of EGFR-TKI concentration, not the PI3K inhibitor (Figure 3.3.).

Whereas all combinations were growth inhibitory, we observed best drug synergism at ZSTK474: ERL drug ratios where 1:10, 1:100 1:1000 (Table 3.1.), suggesting lower doses of PI3K inhibitor combined with erlotinib at concentrations at or above GI_{50} may be therapeutically most effective. Whereas, the combined treatments at reverse ratio had an antagonistic effect on cell proliferation (Table 3.1. and Figure 3.2.).

To date, targeting tumors with PTEN alterations or KRAS-active cancers with PI3K inhibitors has yielded mixed results. Neither of the two most studied pan-PI3K inhibitors, BKM-120 or GDC-0941, have demonstrated any preferential activity in vitro in PTEN-null cells [286, 287]. No responses to these drugs have been observed in patients bearing PTEN defective tumors in single-agent phase I trials [288]. Likewise, targeting KRAS mutant cancers with single-agent PI3K inhibitors have yet to demonstrate benefit [289]. A recent study identified tumors

having PIK3CA alterations or HER2 amplification were associated with sensitivity relative to those harboring PTEN or BRAF mutations, or tumors with concurrent PIK3CA and KRAS mutations which were all associated with resistance to the p110α selective inhibitor BYL719 [246]. For this reason, a potential strategy for treatment for KRAS-active cancers is largely focused on utilizing PI3K inhibitors in combination with MEK kinase inhibitors.

Additionally, the presence of KRAS mutations has also been proposed to be a negative predictor for response to anti-EGFR therapies in lung cancer patients [271, 276, 290]. This however, is disputed as evidenced by the phase III SATURN trial among others, whereby KRAS mutant patients had improved survival and no negative effects from single-agent EGFR-TKI therapy [273]. Inherent resistance to EGFR therapy is primarily associated with the absence of drug sensitizing mutations in EGFR, and to a lesser extent presence of other particular oncogenic molecules. Thus, the predictive value associated with EGFR mutational status for selection of patients for EGFR-TKI therapy outweighs KRAS status as a potential negative predictor of clinical benefit [274]. Along with the added difficulty of identifying minor tumor subpopulations, and genetic variability among primary and metastatic sites, clinically there may be rationale for combining EGFR-TKI with a PI3K inhibitor as a maintenance treatment regardless of tumor genotype.

3.5 CONCLUSIONS

From this investigation, we find that concurrent EGFR-TKI and pan-PI3K inhibition may be an effective therapeutic strategy sensitize tumors innately resistant to EGFR-TKI therapy. We show that combinations act synergistically to sensitize KRAS mutant and EGFR/PTEN mutant NSCLC human tumor cells lines that improve cytostatic responses. These effects also suggest that therapeutic resistance to PI3K kinase inhibitors may arise from upstream signaling through both wild-type and mutant EGFR. These preclinical data may offer new strategies for clinical progress accelerating ever more efficient and tolerable cancer therapies. Future study is needed to assess most effective scheduling of EGFR and PI3K inhibitors in order to better characterize and maximize observed synergistic effect on proliferation. This is particularly important because antagonism was observed in combinations at higher concentrations of PI3K inhibitor. Finally, additional controls representing normal lung epithelial cells should be included to provide early insight into issues of toxicity.

CHAPTER 4

4.1. SUMMARY OF RESULTS

The overarching hypothesis of this dissertation was that PI3K represents a clinically valuable molecular target for treatment of NSCLC.

The first hypothesis, outlined in Chapter 2, was tested using NSCLC cell line models to characterize the activity of isoform-selective pharmacological and genetic inhibitors of PI3K as compared to pan-class I PI3K inhibition. We identified that all class IA PI3K isoforms were expressed, albeit at differing levels among the cell lines tested, and having no correlation to known mutational status. We demonstrate that isoform-selective inhibitors of PI3K were found to be capable of inhibiting PI3K/AKT signaling necessary for proliferation in some genetic contexts or cell types such as mutant PIK3CA (with A66) or in PTEN deficient tumors of the prostate (with TGX-221). However, relative to pan-PI3K inhibition, all of NSCLC cell lines were resistant to isoform-selective PI3K inhibition, as reflected by micromolar GI_{50} values, and the inability to fully abrogate PI3K signaling. These results suggest that compensation among individual isoforms limit efficacy of isoform-selective inhibitors, and the presence of concurrent genetic aberrations further diminish any anti-tumor activity of PI3K inhibitors. Evidence for isoform compensation was further confirmed through studies using siRNA-mediated knockdown of p110β. Whereby the genetic loss of the p110β isoform alone had minor effects on PI3K/AKT signaling, and further signal attenuation was only partially achieved by addition of p110δ inhibitors. Finally, we identified that treatment with isoform-selective inhibitors and pan-PI3K

inhibitors are not cytotoxic, only producing a temporary cytostatic effect through G_1 arrest. This study highlights a fundamental dilemma for implementing isoform-selective inhibitors of PI3K, given that redundancy among the PI3K isoforms limit their effectiveness despite isoform expression levels and genetic context.

In Chapter 3, given that isoform-selective inhibition of PI3K in NSCLC was ineffective relative to pan-PI3K inhibition, we investigated the utility of combined pan-PI3K and EGFR inhibition to overcome resistance associated with single pathway inhibition. We hypothesized that simultaneously drugging the upstream surface receptor EGFR and PI3K may achieve more complete pathway control leading to improved anti-proliferative responses. The combination of pan-PI3K inhibitor, ZSTK474, and EGFR-TKI, erlotinib, resulted in synergistic blockade of proliferation in EGFR therapy resistant NSCLC cell lines. Thus, combined blockade of PI3K and EGFR may represent an effective therapeutic strategy in both wild-type and EGFR activated tumors, and may enhance anti-proliferative responses in tumors harboring concurrent mutations affecting PIK3CA or PTEN.

4.2. CONTRIBUTION TO THE FIELD

This work contributes to the fundamental understanding of the PI3K signaling cascade, and its role as an oncogenic pathway in NSCLC. The frequent activation of the PI3K/AKT pathway in cancer, and its critical role in tumor proliferation and survival make it a desirable target for drug discovery efforts. However, the recent boom in PI3K inhibitor development and

diversification has made difficult direct comparisons between drugs, models, and strategies for ideal clinical and genomic contexts. Our work addresses this important facet of targeted therapy, that being the ability for a drug (i.e. isoform-selective or pan-PI3K inhibitor) to achieve sufficiently complete pathway inhibition, and if that can translate into anti-tumor activity at tolerable patient doses. Through the characterization of in vitro activities of isoform-selective inhibitors we identified contexts whereby targeting particular isoforms may be clinically relevant and achievable, however isoform-selective inhibitors by design have poor selectivity for particular isoforms and consequently lack potency needed impede all PI3K signaling. Expanding upon this, our work goes on to describe an inherent mechanism of resistance to PI3K inhibitors resulting from compensatory class IA isoform signaling. Taken further, PI3K inhibitors have demonstrated promise when treated in combination with other therapies. In our study, we sought to explore the potential of combining an EGFR-TKI with a pan-PI3K inhibitor for treatment of EGFR therapy resistant NSCLC. As previously discussed, tumors harboring EGFR kinase domain mutations demonstrate favorable responses to EGFR-TKI. Because H1650 are EGFR (DelE746-A750) and PTEN deficient, targeting EGFR and PI3K in combination is a rational approach to overcoming PTEN mediated resistance to EGFR-TKI therapy. This combination strategy was also effective at blocking proliferation of KRAS-active cancers, which currently have no therapeutic options. There are a number of ways a cancer being wild-type EGFR and KRAS-active might be sensitized to combined EGFR-TKI and PI3K therapy. First being the interconnected nature of

EGFR signaling downstream to PI3K/AKT and RAS/MAPK pathways. Pharmacological blockade of EGFR likely reduces the overall mitogenic signaling downstream of EGFR receptors. Likewise, KRAS-active cancers signal downstream to both PI3K/AKT and MAPK pathways. Simultaneous inhibition of EGFR and PI3K, may reduce the overall oncogenic signaling downstream to both PI3K/AKT and RAS/MAPK pathways meanwhile the pan-PI3K inhibitor is able to fully block PI3K signaling. The interconnected feedback activation or repression between these two pathways remains an active area of research and may also contribute to the synergistic interaction. Finally, the PI3K blockade and potential reduction in RAS/MAPK signaling may negatively affect expression of ERBB family ligands, which may act to further reduce the overall contribution to proliferation by ERBB receptors. Although continued work must be done in order to identify and dissect the mechanism of this drug combination, we are encouraged by these findings which necessitate further investigation of this drug combination in a mouse xenograft model as a logical next step. Going forward further consideration must also be given to the optimal dosing conditions for each agent to achieve maximal therapeutic control while avoiding any associated toxicity.

4.3. TRANSLATIONAL AND CLINICAL RELEVANCE

The findings in this body of work hold substantial translational and clinical relevance as more PI3K inhibitors enter the clinical setting. A key question this dissertation addresses is whether compounds targeting single PI3K isoforms can

provide significant single-agent efficacy in NSCLC cells that express multiple isoforms, and how those responses compare to those observed with pan-PI3K inhibitors. We know that the PI3K/AKT pathway is important in cancer, and has great potential as a target for therapy. This work is the first to evaluate the individual PI3K isoforms as targets for therapy in genetically diverse NSCLC models.

Ultimately, for successful translation from cell culture models to the clinical implementation of rationally selected of single or combined targeted therapeutics, there must be characterization of pharmacodynamic and pharmacokinetic responses to single- and dual-agent therapy. Our work dissects the preclinical biomarkers for drug activity to assess the pharmacodynamic and kinetic responses to PI3K inhibition to better identify NSCLC subtypes sensitive to these drugs. Through this characterization we identified considerable redundancy among class IA PI3K isoforms for sustaining proliferation and survival. This represents a challenge for development of isoform-selective inhibitors, and calls into question the necessity for even more diversified PI3K inhibitor selectivity profiles. We speculate there may in fact be an advantage to developing a p110α/β selective inhibitors that target isoforms most highly expressed in solid tumor tissues of origin, and do not target the p110δ and p110γ isoforms, which would likely spare immune cells and avoid related toxicity. However, taken together we determined that isoform-selective inhibitors will likely fail in a clinical setting due to incomplete negative regulation of PI3K signaling.

Lastly, substantial evidence suggest single-agent inhibitors are ineffective as anti-cancer agents due to either lack of response or acquired resistance to drug [8,13-16]. Therefore, greater clinical responses may be realized through combinations of PI3K inhibitors with other targeted therapies. There is already emerging clinical support for this approach, for example the promising results combination study using the pan-PI3K inhibitor GDC-0941 with the HER2 directed therapy, trastuzumab, that was found also effective in trastuzumab-resistant cell models [291, 292]. Similarly, combining EGFR and PI3K inhibition could improve sensitivity in EGFR therapy resistant NSCLC. Utilizing two models for innate EGFR therapy resistance; we found concurrent blockade of EGFR and PI3K can synergistically block KRAS mutant NSCLC proliferation. This revelation is particularly important because KRAS driven tumors are the most common single-driver mutations found in advanced NSCLC, and currently there are no therapeutic options for these patients. The EGFR/PI3K inhibitor combination was also found to be effective in EGFR active NSCLC that are resistant to targeted EGFR inhibitors due to the functional loss of PTEN. This model underscores how in advanced cancers having multiple pathway defects, single-agent targeted therapies are ineffective due to uncoupled dependence ("loss of oncogene addiction") for a single driver such as EGFR. Thus permitting sustained pathway activation and the opportunity for pathway cross-talk. As a result of these studies, we hypothesize an overall initial increase in partial clinical responders to the combination, by sensitizing previously resistant NSCLC subtypes. We also predict that in EGFR-sensitive NSCLC, this combination

could also minimize or reduce the eventual selective pressure for the development and outgrowth of EGFR-TKI resistance by blocking reactivation of PI3K/AKT pathway. Slowing tumor reinitatiation even by a single month would represent a significant clinical breakthrough in NSCLC patients having acquired resistance to EGFR therapy.

As summarized above, the translational promise of this combination strategy targeting PI3K and EGFR are manifold. Foremost being those patients who currently have little or no therapeutic options, such as those harboring KRAS-active cancers. Secondly, as compared to front line cytotoxic therapies, the optimized use of orally available targeted inhibitors combination will likely represent a more convenient and tolerable treatment regimen. Finally, for patients receiving benefit from EGFR directed therapy, the addition of a PI3K inhibitor may improve duration of response relative to current single-agent therapy.

4.4. CONCLUSIONS

In conclusion, we were unable to prove our hypothesis that activated PI3K is a good single-agent target for treatment of NSCLC. We find that isoform-selective PI3K inhibitors lack potency relative to pan-PI3K inhibitors due to redundancies among isoform signaling. Although superior to isoform selective inhibitors, treatment with the pan-PI3K inhibitor alone had only temporary cytostatic anti-tumor activity. Importantly, we identified that targeting PI3K in combination with an EGFR inhibitor may offer potential benefit in both KRAS

active, EGFR wild-type, and in EGFR mutant NSCLC. We conclude that the value of PI3K as a therapeutic target will not be realized through monotherapy, but rather when PI3K inhibitors are utilized in combination with other rationally selected targeted agents.

APPENDIX

APPENDIX I: SUPPLEMENTARY FIGURES FOR CHAPTER 2

GI_{50} values of PI3K inhibitors for NSCLC cell lines					
GI_{50} (µM)	**A549**	**H460**	**H1975**	**PC9**	**H1650**
Isoform-selective PI3K inhibitors					
A66	5.2	1.27	2.61	7.55	42.08
TGX-221	>100	30.41	25.93	>100	3.58
CAL-101	16.9	35.94	3.49	3.70	6.04
IC488743	4.2	3.86	7.22	13.23	7.84

Table 2.3. Measurement of cell proliferation in synchronized NSCLC cell lines after PI3K inhibitor treatment. Cell lines A549, PC9, H1650, H1975 and H460 cells were treated with increasing concentrations (0.3 - 30 µM) of isoform specific inhibitors A66, TGX-221, CAL-101 and IC488743. Cell proliferation was determined by trypan blue exclusion assay after 72 hr treatment. Data is representative of 2 independent experiments (n=2). Experimental results were normalized to cells serum starved for 24 hr prior to treatment cells and divided by untreated control to determine (% growth relative to control). Non-linear curve fitting and GI_{50} values were generated using Graphpad Prism.

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PUBLICATIONS:

a. **Journal Publications**

1.) Stamatkin, CW**.**, Westendorf, C., Ratermann, K.L., Lannutti, B. and E.P. Black. Response of NSCLC cells to isoform selective inhibition of PI3K uncovers functional specificities of and compensations by class IA enzymes [Submitted]. Journal of Pharmacology and Experimental Therapeutics (JPET).

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6.) Shaaban KA, Stamatkin C, Damodaron C, Rohr J. 11-Deoxylandomycinone and landomycins X-Z, new cytotoxic angucyclin(on)es from Streptomyces cyanogenus K62 mutant strain. Journal of Antibiotics. 2011 Jan;64(1):141-50.

b. **Abstracts/Presentations**

1.) Stamatkin CW, Westendorf CL, Biliter R, Black EP. Targeting PI3K isoforms as a novel treatment for NSCLC. Molecular Therapeutics of Cancer Research conference (CMTRA). Boulder, CO (2013).

2.) Stamatkin CW, Westendorf CL, Biliter R, Black EP. Targeting PI3K isoforms as a novel treatment for NSCLC. Markey Cancer Center Research Day. University of Kentucky. (2013).

3.) Stamatkin CW, Westendorf CL, Biliter R, Black EP. Novel treatment strategies for inhibiting RAS mutant NSCLC. Markey Cancer Center Research Day. University of Kentucky. (2012).

4.) ER Barnea, Kirk D, Ramu S, Rivnay B, Sathiyasslan T, Stamatkin C, Roussev R, Paidas MJ Preimplantation Factor (PIF*) orchestrates systemic anti-inflammatory response by immune cells: A direct effect on peripheral blood mononuclear cells (PBMC). Society for Maternal Fetal Medicine. 32st Annual Scientific Meeting. Dallas, TX (2012).

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7.) Stamatkin C, Kumar R, Damodaron C. Induction of miR-1290 inhibits Akt-mediated cell proliferation in prostate cancer. AACR 102nd Annual Meeting, Orlando, FL (2011).

8.) Stamatkin **C**, Ko CE, El-Amouri I, Oakley OR. Rapid Recruitment of Novel Subsets of Splenic Monocytes to the Periovulatory Ovary. 30th Annual Conference of American Society for Reproductive Immunology. Nemacolin, PA (2010).

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