Genomic predictors of drug response to the alpha-specific phosphoinositol 3-kinase (PI3Kα-alpha) inhibitor BYL719 in head and neck cancers

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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GENOMIC PREDICTORS OF DRUG RESPONSE TO THE ALPHA- SPECIFIC PHOPHATIDYLINOSITOL 3-KINASE (PI3Kα) INHIBITOR BYL719 IN HEAD AND NECK CANCERS

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by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Background: PIK3CA is the only frequently mutated, druggable oncogene in head and neck squamous cell cancer (HNSCC), with PIK3CA point mutations and gene amplification rates of 17.5% and 40% respectively, with higher rates in HPV-positive disease. The objective of this research was to determine the effects of BYL719, an α-specific PI3K inhibitor in HNSCC cell lines.

Hypothesis: All cell lines with PIK3CA hotspot point mutations or gene amplifications will be sensitive to BYL719.

Methods: Twenty-eight HNSCC cell lines were subjected to increasing concentrations of BYL719 and cell viability was measured over time. Cell lines were screened for activating PIK3CA hotspot mutations and amplifications by real time PCR. Activity of PI3K pathway members was determined by immunoblot.

Results: All PIK3CA cell lines with hotspot point mutations were sensitive to BYL719 treatment, whereas all PIK3CA amplified cell lines were resistant. Moreover, resistant cell lines showed persistent protein activation of mTORC1, as determined by continued phosphorylation of ribosomal protein S6 following BYL719 treatment. Combination drug therapy with rapamycin, an mTORC1 inhibitor, reduced cell viability in BYL719-resistant cell lines. Nearly all PIK3CA amplified cell lines showed co-amplification of PIK3CA with SOX2 genes. Theses findings will help determine which population of patients will benefit most from BYL719 treatment.

Keywords

PIK3CA, head and neck cancer, head and neck squamous cell carcinoma, oropharyngeal squamous cell carcinoma, human papillomavirus (HPV), PI3K/AKT signaling, mTORC1, drug response, BYL719, real time PCR, copy number variations, SOX2
Co-Authorship Statement

Dr. Laurie Ailles performed all cell line-derived and patient-derived xenografts at her laboratory at the University Health Network in this study. Dr. Anthony Nichols, Dr. John Barrett, and I designed the protocol for these studies (Figure 6,7).

Dr John Barrett also assisted with PIK3CA amplification studies (Figure 1).

Dr John Barrett performed combination therapy of trametinib (MEK1/2 inhibitor)-BYL719, mentioned in the discussion.
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List of Common Abbreviations

4E-BP- eIF4E-binding protein
ABD- adaptor binding domain
AKT- protein kinase B, Ak (mice) thymoma
AMP- adenosine monophosphate
AMPK- 5’AMP-activated protein kinase
ANOVA- analysis of variance
ATCC- American Type Culture Collection
ATM- ataxia telangiectasia mutated kinase
BSA- bovine serum albumin
C-RAF- protein kinase-C Raf
CCLE- Cancer Cell Line Encyclopedia
CEF- chicken embryonic fibroblast
CNV- copy number variations
CO2- carbon dioxide
COSMIC- Catalogue of Somatic Mutations in Cancer
DHFR- dihydrofolate reductase
DMEM/F12- Dulbecco’s Modified Eagle Medium with Ham’s F12 1:1 mixture
DMEM- Dulbecco’s Modified Eagle Medium
DMSO- dimethyl sulfoxide
DNA- deoxyribonucleic acid
DNA-PK- DNA-dependent protein kinase
EGFR- epidermal growth factor receptor
eIF4E- eukaryotic initiation factor 4E
EMT- epithelial to mesenchymal transition
ERK- extracellular-signal-regulated kinase
FAM- Carboxyfluorescein
FBS- fetal bovine serum
FKBP12- FK506-binding protein of 12 kDa
FRB- FKBP12-rapamycin binding
GαR- goat anti-rabbit
GαM- goat anti-mouse
GAP- GTPase activating proteins
GPCR- G-protein coupled receptor
HIF-1α- hypoxia-inducible factor 1-alpha
HNSCC- head and neck squamous cell carcinoma
HPV- human papillomavirus
HRP- horseradish peroxidase
hVps34- human vacuolar sorting protein 34
IGF-1R- insulin-like growth factor receptor 1
IRS1- insulin receptor substrate 1
LOF- loss of function
LRCP- London Regional Cancer Program
Mbp- megabase pairs
MDM2- mouse double minute 2 homolog
MEK- mitogen-activated protein kinase kinase
MET - MNNG Hos transforming gene
mSIN1 - mammalian stress-activated protein kinase interacting protein
mTOR – mammalian target of rapamycin
NFκB - nuclear factor κB
NGS- next-generation sequencing
NGS- NOD/SCID/IL2-receptor-γ
nSH2- N-Terminal Src Homology 2
OCP database- Oropharyngeal Cancer Patient database
OICR- Ontario Institute for Cancer Research
OSCC- oral squamous cell carcinoma
PAK- p21-activated kinase
PCR- polymerase chain reaction
PDK1- phosphoinositide-dependent protein kinase
PDX- patient-derived xenograft
PH- pleckstrin homolog
PI3K – phosphatidylinositol 4,5-bisphosphate 3-kinase
PIP2- phosphatidylinositol-4,5-bisphosphate
PIP3- phosphatidylinositol-3,4,5-trisphosphate
PP2A- protein phosphatase 2A
pRB- Retinoblastoma protein
PTEN- phosphatase and tensin homolog
PVDF- polyvinylidene fluoride
PX- phox homology
RAG- RAS-related GTPase
RBD- RAS binding domain
RHEB- ras binding domain in brain
RPMI- Roswell Park Memorial Institute
rpm- revolutions per minute
ROX- 6-carboxyl-X-rhodamine (dye)
RTK- receptor tyrosine kinase
S6K1-p70 ribosomal S6 kinase 1
SCC- squamous cell carcinoma
SFM- serum-free media
SGK3- serum/glucocorticoid-regulated kinase 3
SH2- Src Homology 2
siRNA – small interfering RNA
shRNA- short hairpin RNA
SOX2- SRY (sex determining region Y) box-2
STR- short tandem repeats
TBS- Tris-Buffered Saline
TBST- TBS-Tween
TCGA- The Cancer Genome Atlas
TSC1/2- tuberous sclerosis complex
TSC- tuberous sclerosis
VEGF- vascular endothelial growth factor
WT- wild type
INTRODUCTION – Genomic predictors of drug response to the alpha-specific phosphoinositol 3-kinase (PI3Kα) inhibitor BYL719 in head and neck cancers

1.1 Introduction to Head and Neck Cancer

Head and neck squamous cell cancer (HNSCC) includes malignant squamous cell lesions arising in the oral cavity, nasal cavity, larynx, and pharynx. It is the sixth most common cancer by incidence worldwide, with approximately 600,000 new diagnoses per year (1). Despite significant advances in our understanding of the disease, the mortality rate has remained relatively high, with an estimated annual death toll of 355,000 (2).

Head and neck cancers have long been known to be caused by heavy tobacco and/or alcohol use (2,3,4). Government initiatives aimed at curbing smoking use were established in the 1960s and have been largely successful (4). Paralleling this decline in smoking was a decrease in smoking-related head and neck cancers (4), and as such, the number of patients presenting with tobacco or alcohol-induced HNSCC declined (5). These patients were older individuals with poorer overall health, and tumors most commonly found in the oral cavity and larynx (5). More recently, however, there has been a dramatic rise in the number of oropharyngeal cancers, now recognized to be caused by oral infection with human papillomavirus (HPV). This increase in incidence of HPV-related HNSCC has been described by some as a slow epidemic (3).

Tumors of the head and neck possess a large degree of heterogeneity with few consistent mutations across patient cohorts, underscoring the difficulty in identifying effective molecular targeting agents that will benefit a substantial population of HNSCC patients (6-11). The majority of HNSCC tumors have loss of function (LOF) mutations in tumor suppressor genes, thereby making effective drug targeting challenging (6,8,9-11). This is because it is much more difficult to reintroduce a gene product that has been lost, rather than target a gene product that is overactive (6,7). Compounding the problem is the rising incidence of HPV-driven HNSCCs, as HPV-induced and traditional HPV-negative HNSCCs are very distinct diseases with divergent clinical manifestations and treatment outcomes (6,12).
Tobacco and alcohol act as mutagenic agents by damaging cellular DNA, and introducing mutations in vital DNA repair genes. In contrast, HPV-related tumors express viral oncoproteins (e.g. E6 and E7), which function to inhibit and degrade critical tumor suppressor genes (e.g. p53 and the retinoblastoma protein (pRb) respectively), thereby overriding these critical cellular control mechanisms without the introduction of cellular gene mutations (12). Consistent with this mechanism, HPV-negative HNSCCs have been found to harbor more than twice as many mutations as HPV-positive HNSCC tumors (4.83 mutations/Mbp and 2.28 mutations/Mbp, respectively) (6). Many of the mutations in both types of HNSCC tend to occur in key tumor suppressor genes, particularly TP53, which is almost ubiquitously mutated in all HPV-negative HNSCCs (6,8,9-11, TCGA www.cbioportal.org). High incidences of CDK2NA loss (p16INK4A, p14ARF) and/or CCND1 gain (Cyclin D1) are also very common, and provide a means for aberrant cell cycle control (6,9,10).

HPV-positive patients fare better than HPV-negative patients as shown by increased progression-free survival and overall survival when treated with platin-based concurrent chemoradiation (13, 14). HPV-positive HNSCC patients also had significantly lower rates of distant metastases as well as lower incidences of secondary primary tumors (13). Moreover, tobacco smoking on its own, independent of the causative nature of a patient’s HNSCC, has a significant impact on both variables in that the risks of death or cancer relapse increase by 1% for each additional pack-year of tobacco smoking (13). The lack of LOF mutations in key cell cycle regulators, particularly TP53, in HPV-positive patients (6-11) may explain why HPV-positive HNSCC patients fare better than HPV-negative HNSCC. Poeta and colleagues published a large multicenter study analyzing TP53 status in 420 HNSCC patients and found patients with TP53 mutations showed a 1.5-fold decrease is survival compared to patients with wild-type TP53 (15). Tumors with LOF TP53 mutations also tend to respond significantly more poorly to cisplatin and 5’fluorouracil-based chemoradiation (16), and are associated with local recurrence after radiotherapy (17) as compared to TP53-WT tumors.

Generally, HPV-negative tumors have a higher frequency of transversions at CpG sites, which is characteristic of smoking malignancies (6). In contrast HPV-positive
tumors have more transitions at TpC sites based on TCGA data, and despite having fewer overall mutations, tend to have higher mutations in the PIK3CA (p110α) oncogene compared to HPV-negative tumors (6-10).

Our knowledge of head and neck cancer etiology and progression has significantly increased over the past thirty years, yet mortality rates have remained consistent (2,12). Despite a favorable prognosis for most HPV-positive patients, approximately 20% fail therapy, with more than half due to distant metastases (18). In contrast, HPV-negative patients are at a higher risk of local, regional and distant relapse, with approximately 50% succumbing to the disease (1).

In addition to mortality, both the disease as well as its treatment can often have marked patient morbidity. Indeed, HNSCC is unique as treatments often impact the most personal characteristics of what defines an individual, including facial appearance, and the ability to eat and speak. Other frequent side effects include pain, fibrosis, hearing loss, renal impairment and neurotoxicity (19). Thus, there are tremendous clinical challenges to deliver effective therapy that maximizes cure, while minimizing these sequelae.

1.2 Next-Generation Sequencing of HNSCC

The advent of next-generation sequencing (NGS) has revolutionized the field of genomics. It is now possible to routinely extract an organism’s nucleic acids, and perform large-scale, cost effective and highly accurate genome-scale sequence data acquisition. In cancer research, the clearest advances have been obtained by sequencing tumors along with matched normal DNA from patient blood. This strategy allows exclusion of germline variants to report solely somatic changes. To date, there have been no reported germline mutations that predict HNSCC susceptibility (6,9,11). Moreover, it has furthered our appreciation of the small number of true “driver” mutation events masked in a large population of “passenger” mutations (20). Bioinformatic analysis of patient tumors will allow medical professionals to identify gene mutations in key signaling pathways. It then becomes important to determine which mutations are responsible for carcinogenesis (20).
A mutation is defined as a change in the nucleotide sequence of the genome (20). Point mutations are the replacement of one nucleotide for another, or the insertion or deletion of nucleotides (20). These changes can either be synonymous, where no change in the amino acid sequence is observed, or nonsynonymous, where the amino acid sequence is altered (20). With nonsynonymous mutations, the resulting protein can either be misfolded, truncated, or maintain proper function in the case of conserved amino acid changes (20). Moreover, by the definition above, gene amplifications or deletions, as well as chromosomal translocations are also considered mutations. Gene amplifications are copy number gains above the normal diploid number, and deletions are losses of one gene copy (heterozygous) or both copies (homozygous). Chromosomal translocations are the rearrangement of parts between nonhomologous chromosomes (20).

The first NGS screens of HNSCC patient tumors described mutational events in key cell cycle components, as well as downstream effectors of mitogenic signaling (6,11). Additional discoveries included mutations in cell differentiation pathways, particularly NOTCH1 and FXBW7 (11). Recent studies have also identified incidences of mutations in pathways that influence terminal differentiation, cell proliferation, as well as apoptosis (6-11). A full, detailed summary of HNSCC NGS studies can be found in a review recently published by our group (Rizzo et al., Oral Disease 2014). Many of the mutations found in HNSCC are in tumor suppressors (6,9-11, TCGA www.cbioportal.org), which makes effective drug targeting challenging as there are no current effective strategies to target these agents (6,7). However two oncogenes, PIK3CA and HRAS, were found to be altered in a major subset of both HPV-positive and HPV-negative HNSCC tumors, of which only PIK3CA is amenable to drug targeting (6-10).

Indeed several groups have confirmed high incidences in HNSCC of mutations in not only PIK3CA but also in other members of the PI3K/AKT pathway, albeit the rate of PIK3CA mutation/amplification is markedly higher (7,9,10). This is suggestive of a high dependency on this pathway for oncogenesis (22-25). One study showed that PIK3CA mutation/amplification, as well as PTEN inactivation by gene copy loss or mutation occurred at high frequencies in both aggressive HPV-positive and HPV-negative tumors (9,10). This has been confirmed in an additional study that demonstrated multiple
mutations in the PI3K signaling axes are only found in advanced stage (Stage IV or higher) HNSCC (7). Considering the high rate of PI3K/AKT pathway alterations in locoregional disease, coupled with reports of additional mutations (e.g. *PTEN* loss, *HRAS* mutations) as the disease progresses, implies that the PI3K/AKT pathway plays an important role across the whole timeline of cancer development.

1.3 PI3K/AKT Pathway

The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT (protein kinase B) signaling cascade is the most commonly mutated pathway in cancers (6-11,22-25). Class I PI3Ks phosphorylate phosphatidylinositol to its more highly phosphorylated derivatives, generating second messengers that control proliferation, survival, motility and morphology (26). Class I PI3Ks are further subdivided into two classes, IA and IB (27). The class IA PI3Ks consist of PI3Kα, PI3Kβ, which are expressed in most tissues, and PI3Kδ, which is expressed in leukocytes (27). The class IB PI3K is the PI3Kγ, which is also found in leukocytes (27). Structurally, they are composed of a regulatory subunit (p85), which contains a src-homology (SH2) domain, and a catalytic subunit (p110), which has a RAS-binding domain (28). This complex is pre-formed but inactive in the cytoplasm of resting cells, waiting for appropriate cues for activation (28). The binding of the p85 SH2 domain to particular phosphotyrosine residues, often in response to receptor tyrosine kinase (RTK) activation, localizes the PI3K complex to the plasma membrane where it phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to its active phosphatidylinositol-3,4,5-triphosphate (PIP₃) form (Figure 1). Activation of PI3K-alpha (PI3Kα) is postulated to occur by one of two mechanisms: first, the localization of the PI3K complex to the plasma membrane places p110α in close proximity with its lipid substrates (28), or the RTK-p85 subunit binding interaction changes the conformation of the complex allowing activation of kinase activity (28-30). PIP₃ localizes and activates phosphoinositide-dependent protein kinase (PDK1) which then phosphorylates AKT at threonine 308 (29,31). Eventually, mammalian target of rapamycin (mTOR) inhibition is alleviated by AKT phosphorylation of the tuberous sclerosis complex (TSC1/2) proteins. TSC1/2 is a GTPase-activating protein, which catalyzes the conversion of GTP to GDP in
RHEB thereby suppressing mTOR activity (31). TSC1/2 complex phosphorylation inactivates the complex, allowing RHEB to bind GTP and activate mTOR (32).

mTOR kinases can be found in two distinct complexes, mTORC1 and mTORC2. mTORC1 is a multi-subunit complex composed of mTOR, the regulatory-associated protein of TOR (Raptor), the Gβ-like protein GβL, the proline-rich AKT substrate 40 kDa (PRAS40), and the DEP-domain-containing mTOR-interacting protein (Deptor). mTORC2 is composed of mTOR, rapamycin-insensitive companion of TOR (Rictor), GβL, mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), and Deptor (29,31). Individually, these complexes have distinct targets, with mTORC1’s role in the cell being particularly widespread, with important roles in cell growth and metabolism (24,31,33,34). Only mTORC1 is sensitive to rapamycin (sirolimus) treatment (33). This is because inhibition of mTORC2 lacks the FK506-binding protein of 12 kDa (FKBP12)-Rapamycin Binding (FRB) domain of mTOR, and therefore is unresponsive to FKBP12-rapamycin binding and inhibition (33).

In terms of relevancy for this thesis, mTORC1 plays an important role in protein synthesis (33). mTORC1 activation leads to the phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) and the p70 ribosomal S6 kinase 1 (S6K1), inhibiting the former and activating the latter (31,33). 4E-BP inhibition promotes cap-dependent translation by allowing the formation of the eIF4F complex (31,33,35). Activation of S6K1 activates in turn the S6 ribosomal subunit thereby allowing proper ribosomal assembly and the translation complex to form (33). Both 4E-BP and S6 phosphorylation are surrogate markers of mTORC1 activity (31,33,39). mTORC1 has also been shown to play important roles in regulating autophagy (36), lipid synthesis (37), and mitochondrial function (38).
Figure 1 PI3K AKT pathway signal transduction in protein synthesis. RTK activation and interaction with the SH2 domain of the PI3K regulatory subunit p85 leads to the activation of the catalytic p110α subunit and the subsequent phosphorylation of PIP$_2$ to PIP$_3$. As a result, both PDK1 and AKT are recruited to the plasma membrane where AKT is phosphorylated at Threonine 308 by PDK1. Full activation of AKT is postulated to occur by the phosphorylation of a secondary site at Serine 473 by mTORC2. AKT phosphorylates the TSC1/2 complex, thereby inhibiting its GTPase function and allowing RHEB to bind GTP and activate mTORC1. mTORC1 then phosphorylates the 4E-BP and the S6K, inhibiting the former and activating the latter, concurrently with S6 phosphorylation. As such, the eIF4F complex allows 5’ cap-dependent translation and the S6 phosphorylation permits proper ribosomal and translational machinery assembly.
The role of mTORC2 in the cell is much less well characterized than mTORC1, yet it is known to potentially facilitate the full activation of AKT by phosphorylating a secondary site at serine 473 (Figure 1) (33,39), however DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated kinase (ATM) are also postulated to play a similar role (40). Loss of mTORC2 however does not affect TSC1/2 phosphorylation (33). The primary role of mTORC2 is thought to be in actin cytoskeleton remodeling (31).

In normal physiology, PI3Ks are negatively regulated by the tumor suppressor phosphatase and tensin homolog (PTEN), which catalyzes the reverse reaction from active PIP$_3$ to inactive PIP$_2$ (41). In some cancers, loss of $PTEN$ either through focal deletion or loss of function mutation causes aberrant activation of the PI3K/AKT signaling axis, evident by high levels of AKT phosphorylation (31-34,39,41).

1.3.1 PI3K/AKT Pathway in Cancer

Aberrant PI3K/AKT signaling is activated through somatic mutations, RTK stimulation, particularly the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor 1 (IGF-1R), PTEN loss, or G-protein coupled receptors (GPCR) (33,43,44). This pathway has even been suggested to be so important to oncogenicity that cancer cells will actively maintain a certain homeostatic level of PI3K/AKT signaling in face of therapeutic intervention (45). Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by the formation of hamartomas due to LOF mutations in either TSC1 or TSC2, the key negative regulators of mTORC1 activity (46). Moreover, germline mutations in $PTEN$ result in Cowden’s disease, a multi-organ disease characterized by benign hamartomatous growths (47,48). Both of these syndromes lead to uncontrolled cell growth caused by uncontrolled activation of the mTOR (46-48). The importance of this signaling axis in cancer is evident by the large degree of crosstalk with adjacent pathways (28,31,33,41,43,45). The p110α subunit contains a RAS-binding domain, which upon RAS-GTP binding augments PI3K/AKT activity under certain cellular conditions. Mutations in RAS lead to the persistence of the RAS-p110α interaction, resulting in the stabilization, and thus constitutive activation of the PI3K complex (28).
PTEN loss of function, either through homozygous deletion, LOF mutation, or epigenetic silencing occurs in roughly 20% of all HNSCC (TCGA www.cbioportal.org) and has been shown to regulate AKT activation (46). Moreover, secondary PTEN loss has been shown to facilitate a more aggressive phenotype in HNSCC cells (42,47). Short hairpin RNA (shRNA) knockdown of PTEN in HNSCC cells results in decrease in cell adhesion, reduction in E-cadherin expression, and enhanced cell migration, all of which are characteristic of dedifferentiation and epithelial to mesenchymal transition (EMT) (47). PTEN loss more recently, has been shown to occur in distant metastasis in a patient with PIK3CA-driven breast cancer (42). All treatment refractory, secondary tumors in this patient that were resistant to BYL719, an alpha-specific PI3K inhibitor harbored a PTEN mutation, while other metastases that were drug responsive had wild type PTEN (42). These results were further confirmed in vitro using shRNAs against PTEN in BYL719-sensitive breast cancer cell lines, where PTEN loss led to BYL719 resistance (42).

AKT activation plays a role in tumor–induced angiogenesis through the interaction with the hypoxia-inducible factor 1-alpha (HIF-1α), and the vascular endothelial growth factor (VEGF) (28). Moreover, AKT is known to contribute to cancer cell immortalization through the indirect activation of nuclear factor kappa B (NFκB) and the sequestering of p53 through mouse double minute 2 homolog (MDM2) phosphorylation (41,48-50). Moreover, RAS signaling through the RAF/mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathway, as well as nutrient and oxygen sensing pathways (33) can also activate downstream mTOR leading to aberrant activation of key downstream components (51).

1.3.2 PI3K/AKT Pathway and HPV

Activation of the PI3K/AKT pathway is also essential for the infection, maintenance, and progression of HPV, as well as other DNA viruses, in the host cell (31). Viral-mediated activation of the PI3K/AKT pathway helps circumvent stress-mediated inhibition of mTORC1 during HPV infection through favorable interactions with TSC1/2 (31). Additionally, the viral oncoproteins E6 and E7 have been shown to interact with key members of the pathway: E7 can directly inhibit the action of protein phosphatase 2A
(PP2A), which modulates AKT signaling through the removal of phosphorylated residues at T308 and S473 (52,53). E6, conversely, binds to tuberin in the TSC1/2 complex and targets it for proteasomal degradation (54). Finally, constitutive maintenance of mTORC1 activation will maintain active 5’ cap-dependent translation through the phosphorylation of the 4E-BP, which relieves the negative inhibition on the eIF4F complex, and the S6 kinase (S6K), which phosphorylates S6 leading to the formation of translation initiation complexes (31,50,55). Taken together, a proposed feedback loop in which active HPV E6 and E7 proteins promote PI3K/AKT signaling can be imagined. This would lead to and the constitutive activation of mTORC1, which in turn maintains 5’ cap-dependent translation of more E6 and E7 proteins to further abrogate cell cycling as well as insert into the host’s genome (31,56-58).

1.3.3 PIK3CA

Through NGS efforts, PIK3CA has been identified as a key potential target in the treatment of HNSCC patients due to its high frequency of mutation, its oncogenic nature as well as its amenability to being targeted by small molecule inhibitors (54). HNSCCs lack activating mutations in oncogenes, yet have high rates of mutations in tumor suppressors (6-11,TCGA www.cbioportal.org). Amongst the select few activating mutations, clinical targeting of these components in HNSCCs tends to present with great challenges.

Point mutations in the HRAS gene have also been identified in HNSCC, with roughly 5% of all tumors possessing mutations in hotspot residues (G12, G13, Q61) (6-11,TCGA www.cbioportal.org). These mutations reduce GTPase activity, allowing HRAS to remain in the active state (6,11) in turn endowing the cell with cancer hallmarks (58,59). To date there are no successful strategies that effectively target RAS despite significant drug development efforts (65). This could be because of the lack of effective molecular compound-binding pockets on the surface of the protein, and the low affinities at which these compounds bind, which makes them incapable of competing with the high Ka of Ras and GTP (63).
HPV-positive and HPV-negative tumors have activating \textit{PIK3CA} point mutations in roughly 30\% and 10\% of tumors respectively (6-11). High incidences of \textit{PIK3CA} mutations are also observed in HPV-positive cervical squamous cell carcinomas (61,TCGA, \url{www.cbioportal.org}), implying a viral preference for activation of the PI3K/AKT pathway, as discussed above.

The \textit{PIK3CA} locus is located on chromosome 3 and can be mapped to position 3q26.3 (22-25). It encodes the p110α catalytic subunit of the class 1A of the PI3K family (22-25). In normal physiology, p110α is found in complex with, and regulated by the p85 subunit. It is composed of five domains: an adaptor binding domain (ABD), and a C2 domain, which both interact with p85’s nSH2 domain, a RAS-binding domain (RBD), a helical, and a kinase domain (67). Upon activation, p110α phosphorylates PIP\textsubscript{2} to its active PIP\textsubscript{3}, which binds to the PH domains of AKT and PDK1 and recruits them to the plasma membrane. AKT is then activated to induce canonical PI3K/AKT signaling (Figure 1) (26,31,41). Ninety percent of \textit{PIK3CA} point mutations are found clustered at three hotspots: two missense mutations in exon 9, which alter amino acids 542 (E to K) and 545 (E to K) in the helical domain, and a missense mutation exon 20, which alters amino acid 1047 (H to R/L) in the kinase domain (44). Additional point mutations have also been identified in HNSCC, however their transforming capabilities are less potent than the three hotspot mutations (7,67).

Chang and colleagues were the first to show \textit{PIK3CA}’s transforming ability by retrovirally transducing chicken embryonic fibroblasts (CEFs) with mutant \textit{PIK3CA}, which lead to potent activation of downstream AKT targets (25). Furthermore, it was shown that mutant \textit{PIK3CA} has the capacity to cause hemangiosarcomas in chickens 1 to 2 weeks after retroviral infection (25). An independent study by Kang and colleagues showed similar findings with retroviral infection of CEFs with \textit{PIK3CA} hotspot mutations; stable expression of hotspot \textit{PIK3CA} mutants were able to cause aberrant cell growth and activation of downstream PIP\textsubscript{3} and AKT. \textit{PIK3CA} mutations also afford the cell the capabilities of anchorage independent growth (23), another hallmark of cancer generation (64). One study showed that retroviral infection of NIH-3T3 cells with E545K or H1047 mutants led to elevated lipid kinase activity through PIP\textsubscript{3} and AKT activation.
Moreover, when these PIK3CA-mutant expressing NIH 3T3 cells were seeded onto soft agar, normally impermissible to cell growth, multiple colonies were formed. More recently, Okudela and colleagues demonstrated this same trend with immortalized airway epithelium 16HBE14o- cells. Retroviral transduction of cells with PIK3CA E545K and H1047R mutations lead to significant anchorage independent growth when plated on soft agar, and significantly increased trans-well migration, as compared to mock treated controls (68).

Studies examining the binding partners of helical and kinase p110α domain mutations have revealed key differences in their interactomes, specifically in the context of PI3K/AKT signaling (69). Zhao and Vogt determined that both the E542K and E545K mutations require an intact RAS-binding domain (RBD) and therefore RAS-binding to induce oncogenic transformation (69). Then again, dual helical/H1047R mutants are able to facilitate similar transforming capabilities suggesting that either kinase domain mutations, or RAS-E542/545/K activation of kinase activity accomplish similar results (69). This dependency on RAS or the H1047R mutation probably causes a conformational change at the substrate-binding site leading to active signaling (70,71). The H1047R kinase domain mutations are dependent on p85 binding, probably in part because the p85 subunit contacts the kinase domain affording proper protein folding confirmation (67,69).

Given that mutations in the helical domain are oncogenic (22-25,69,72) and result in similar activation of PI3Kα as kinase domain mutations, this suggests that the E542K and E545K mutations must have an impact on maintaining the kinase domain’s activity. The glutamate to lysine transition, which results in charge reversal from negative to positive, still permits p110α to be recruited to the plasma membrane (72). In fact, it is the C2 domain that seems to play a larger role in membrane localization (67). In the p85-p110α complex, both the helical and kinase domain form the core of the complex, with the helical domain providing a bridge from the central C2 domain to the kinase domain (67). The 542 and 545 residues interact with the nSH2 domain of p85, which also contacts the kinase domain. Hotspot helical domain mutations may weaken p85 regulation of p110α, thereby allowing oncogenic activity of the protein (67). Moreover,
helical domain oncogenicity is dependent on RAS binding (69); RAS binding can then alter the conformation of the kinase domain and activate signaling (70). Conversely, the H1047R kinase domain substitution appears to confer oncogenicity by a different mechanism, either by altering the conformation of the kinase activation loop, or increasing the binding affinity of p110α for the phosphatidylinositol substrate (22,29,43).

Given the propensity for both helical and kinase domain mutations to elicit oncogenicity, it can be postulated that both sets of mutations mitigate regulatory signals from the p85 subunit, while maintaining active kinase function.

The role that proto-oncogene amplification plays in the generation of a cancerous phenotype has been well documented (73). Amplification refers to an increase in gene copy number above the normal diploid number (74). Gene amplification has been observed as far back as 1978, when Alt and colleagues observed selective amplification of the dihydrofolate reductase (DHFR) gene in a subset of culture murine cells (75). DHFR results in the production of tetrahydrofolate, the active derivative of folate in humans, which is essential for rapidly dividing cells, i.e. cancer cells, in order to produce thymine (75).

Many studies have confirmed the high incidence of amplification in three families of protooncogenes implicated in cancer: MYC, ERBB2, and RAS (either KRAS, NRAS, or HRAS) (74). MYC amplifications are found at high frequencies in breast and ovarian, as well as in squamous cell carcinomas (76). Huw and colleagues reported that MYC amplification could mediate resistance to PI3K pathway inhibition (77). The best example of ERBB2 amplification is in breast cancers, where ERBB2 amplification is a predictive marker for poor prognosis and advanced stage, predicts response to HER2 inhibition, and is found in 15-30% of all breast cancer tumors (78). In HNSCC, the ERBB-1 or EGFR gene is amplified in roughly about 10% of tumors and is an indicator of poor prognosis (9,79). Finally the RAS family is amplified sporadically across a large panel of cancers (74).

Amplification of PIK3CA occurs at a slightly greater frequency than PIK3CA point mutations in HNSCC. Multiple studies have reported high incidences of PIK3CA
amplification in both HPV-positive and negative HNSCC, with 28% incidence in HPV-positive disease, and 21% in HPV-negative disease (6,7,TCGA www.cbioportal.com). *PIK3CA* amplification is also common in cervical cancers (TCGA: www.cbioportal.com), which further implies an additional link between HPV and PI3K/AKT signaling. Moreover, the ability of *PIK3CA* amplifications to drive oncogenic progression is supported by the fact that *PIK3CA* amplifications have been identified in pre-cancerous colorectal adenomas (80), as well as early gastric cancer lesions (81). *PIK3CA* amplification is also a marker for poor prognosis and treatment failure across multiple cancer types and treatment modalities (79,81-85). Importantly, *PIK3CA* amplification can give rise to the same oncogenic phenotype elicited by *PIK3CA* hotspot point mutations. Okudela and colleagues retrovirally transfected overexpressed wild type (WT) *PIK3CA* into the human bronchial epithelial cell line 16HBE14o-, and showed a significant enhancement in anchorage independent growth and cell migration, although to a lesser degree than activating *PIK3CA* hotspot point mutations (68). While *PIK3CA* amplification probably confers oncogenicity by a different means than *PIK3CA* point mutations, it can be reasoned that at the very least 3q26.3 chromosomal amplification plays a significant role in HNSCC oncogenesis.

The exact mechanism in which *PIK3CA* amplification drives oncogenesis has been challenged. Some suggest that certain proximal genes at the 3q.26.3 chromosomal region are the actual driver alterations (85). CNV analysis of oral squamous cell carcinoma (OSCC) have shown that specific regions of the 3q chromosome are amplified in more than half of tumors (10). Moreover, nearly half of all tumors also demonstrated gains of regions on the 3q chromosomal arm coupled with losses of regions on the 3p arm (10), which was more common in HPV-positive tumors (56). Multiple potential oncogenes are present near the 3q26 loci, particularly *SOX2*, (discussed below) *TP63, TERC, RAB7A,* and *PRKCI* (10). A recent review by our group outlines the roles of the last 4 genes in HNSCC mentioned above (Rizzo et al., *Oral Disease* 2014).

*SOX2* (sex determining region Y box-2) has long been recognized as a key transcription factor in development and cancer, with an important function in the maintenance of pluripotency in undifferentiated stem cells (87,88). Studies have shown
that *SOX2* is amplified in up to 21% of all HNSCC tumors (9, TCGA www.cbioportal.org). Similar findings have also been reported in other smoking-related malignancies such as lung (86) and esophageal squamous cell carcinomas (SCC) (87), as well as HPV-driven cervical cancers (TCGA). Importantly, *SOX2* is proximal to the *PIK3CA* gene in the often-amplified 3q26 chromosomal region (9,10). Analysis of the most recent TCGA data on HNSCC (www.cbioportal.com) showed that the *PIK3CA* and *SOX2* genes are both amplified in 21% of HNSCC patient tumors, with nearly 100% correlation. Multiple studies have shown an upregulation of SOX2 in cancer cells, including HNSCC stem cells (88), implicating SOX2 in EMT and metastasis (89,90). Moreover, SOX2 has been shown to impact PI3K, AKT, and mTORC1 signaling (89), while mediating resistance to pathway inhibition (90). Importantly, SOX2 amplification cannot singularly drive malignant transformation (85); considering the proximity and oncogenic abilities of *PIK3CA*, the two amplification events may work together to result in an aggressive cancer phenotype.

The functional consequences of *PIK3CA* aberration in HNSCC suggest an important role for this gene in cancer progression. Moreover, the strong interplay between HPV presence and *PIK3CA* would suggest an additional role for *PIK3CA*, or at least the PI3K/AKT pathway in viral function and progression to oncogenesis. More research is needed, which is outside the scope of this thesis. Nonetheless, the mode of action of *PIK3CA* point mutations and amplifications on clinical targeting is of great significance and merits significant attention.

### 1.4 Clinical Targeting

Many head and neck cancer patients, especially those with oropharyngeal tumors are frequently treated with non-surgical techniques (91). In patients with locally advanced disease, the addition of platin-based chemotherapy to radiation improved local control as well as overall survival as compared to radiation therapy alone (91-96). Chemotherapy is not curative in HNSCC when given alone, but rather is used to sensitize cells to radiation as well as prevent distant metastasis (96). As such concurrent chemoradiation is the standard of care for locally advanced HNSCC based on several prospective studies (93-95), while showing a more favorable response in HPV-positive HNSCC patients.
Despite moderate success, this treatment modality is associated with high toxicity as well as significant impacts on quality of life, irrespective of the burden of HNSCC disease alone. Moreover, in the case of HPV-positive HNSCC, patients tend to be younger (5) and more likely to survive the disease (2,13,14), leaving them to deal with the QOL burdens long after their treatments and cancer has subsided. It therefore becomes imperative to develop new therapies that not only maximize cure, as overall mortality rates are still roughly 50% (1,2,12,18), but also minimizes treatment and disease-associated morbidities. Targeted therapy of frequently mutated genes is an active area of clinical research and could provide a means for achieving these goals.

### 1.4.1 Mechanisms of Resistance

It is unlikely that one treatment modality will be effective in all HNSCC patients. Moreover, even patients that meet the criteria for selected treatment (e.g. presence of mutation, no previous radio or chemotherapy) will vary in their response to a given treatment (5,13,14). The advent of NGS has significantly improved our understanding of HNSCC biology in that it has identified key differences between HPV-positive and HPV-negative disease, while elucidating the mutational characterization of both subtypes (6-11, TCGA [www.cbioportal.org](http://www.cbioportal.org)). Importantly, it has identified a variety of molecular targets that may be amenable to targeted therapy (e.g. small molecule inhibitors or monoclonal antibodies). These agents have the potential to improve cure rates while reducing toxicity burdens associated with conventional HNSCC treatments (1,2,12,18,96). Despite great potential, numerous studies have demonstrated drug resistance in a setting where cell lines and/or xenografts were expected to respond (42,57,77,101,103-109). In these cases, resistance is mediated through activation of secondary pathways (103,104), acquired secondary mutations downstream of the targeted gene product (42,106-108), or *de novo* mutations in the targeted gene (77,109). Moreover, the upregulation of P-glycoprotein or multidrug-resistance (MDR)-associated proteins (MRP) expression, both ATP-binding cassette (ABC) transporter family, have long been known to drive resistance to many treatment modalities across many cancer subtypes (*See ref 110 for a comprehensive review*).
It then becomes paramount to know the tumor’s mutational profile to explain resistance, and why observations are in discordance with initial hypothesis. As such, an appreciation of tumor evolution and the consequencial heterogeneity that it causes. The presence of convergent mutations could afford tumor cells phenotypic similarities but with distinct genotypes, and thus potentially different drug responses (111). It is the duty of clinicians and scientist to appreciate this evolutionary pattern and account for this heterogeneity by acquiring multiple biopsies from a patient tumor and properly identifying the oldest phylogenic driver mutation in these tumors (111).

1.4.2 Cetuximab

Cetuximab is a monoclonal antibody against EGFR, a member of the homolog of the erythroblastic leukemia viral oncogenes (HER) family of receptors, with RTK function (91). In 2006, the FDA approbed the treatment of cetuximab with combination radiotherapy in HNSCC patients with locally or regionally advanced disease (www.cancer.gov). Bonner and colleagues determined that adjuvant cetuximab and radiation therapy compared to radiation alone, improved locoregional control in patients with advanced stage HNSCC (57). Cetuximab has also been approbed as a single agent for patients with recurrent or metastatic HNSCC who have previously failed platinum-based therapy (National Cancer Institute, www.cancer.gov).

EGFR alterations are frequent in epithelial cancers, with HNSCC being no exception (57). Multiple studies have reported EGFR gene amplification in approximately 10% of all HNSCC (6,7,9-11, TCGA www.cbioproject.org), while being overexpressed in 80-90% of all HNSCC (79,112). However, in contrast to other cancers, particularly lung cancer, activating point mutations in EGFR are extremely rare (9, TCGA www.cbioproject.org). Cetuximab treatment has been shown to afford patients 9.1% survival advantage at 5 years when used in combination with radiotherapy compared to radiotherapy alone (57), however these findings are underwhelming in comparison to response rates in other cancers (59). Amplification of the EGFR gene might explain this poor response, and could account for poor responses also observed with small molecule inhibitors against EGFR, particularly erlotinib and gefitinib (58,59).
1.4.3 PI3K/AKT pathway

The PI3K/AKT pathway and its spanning influence on multiple cellular pathways and processes, suggests that it is logical set of targets for the treatment of not just HNSCC, but also many other cancers. The presence of activating mutations in PIK3CA provides an avenue for targeted therapy in HNSCC, as it is far more practical to design inhibitors than to restore function to mutated tumor suppressors. Much of our preliminary understanding of PI3K inhibition comes from the Penicillium funiculosum metabolite Wortmannin, and the synthetic inhibitor LY294002, The former binds irreversibly, while the latter binds in a reversible manner (97). Both bind to the ATP binding pocket, like more recently developed PI3K inhibitors, however they are not isoform specific leading to a high toxicity that makes them inappropriate for clinical use (97). Nonetheless, they have provided a valuable template for the design of new generation PI3K inhibitors that are in clinical use today.

The lowest level of conservation among the PI3Ks occurs at a helix in the activation domain between residues 1032 and 1048 (27,67), thereby providing optimal avenues for drug specificity. Studies comparing p110γ and p110α provide insight into the oncogenic action of H1047R mutations; in p110γ the R1073 residue is equivalent to the H1047 residue in p110α (67). H1047 normally forms a hydrogen bond with the carbonyl oxygen of L956, something that does not occur at equivalent residues in p110γ. Instead, R1073 forms a hydrogen bond further along the helix, resulting in a shift in the activation loop structure (67). A similar change in residue interactions is thought to occur in H1047R PIK3CA mutations, thereby altering folding of the kinase domain (67).

Currently, the pan-isoform PI3K inhibitor BKM120 (Novartis) is the furthest along in development for the treatment of HNSCC. Ongoing Phase I and Phase II clinical studies have looked at BKM120 alone, and in combination with cetuximab with reported improved survival in patients with recurrent, metastatic or incurable, progressive HNSCC (41). BEZ-235 (Novartis) is a dual mTOR/PI3K inhibitor, which has high affinity for the PI3K isoforms p110α (IC50=4nM), p110γ (IC50=5nM), and p110δ (IC50=7nM) (98). One study has shown that HNSCC cell lines harboring PIK3CA H1047R mutations are highly sensitive to BEZ235 compared to HNSCC cell lines with wild-type PIK3CA (7).
This data was confirmed in murine xenograft models using cell lines and patient-derived HPV-positive PIK3CA-mutated (E545K) tissue (7). Moreover, the combinatory treatment of BEZ235 and cetuximab was more effective than cetuximab alone in these models. It is important to note however that drugs that inhibit strictly the alpha subunit of PI3K have not been available until recently. Despite these encouraging findings, ongoing developments with BEZ235 have been stalled due to reformulation of the drug (99). Additionally two novel dual PI3K/mTOR inhibitors, the orally administered PF-502 and intravenously administered PF-384 (Pfeifer), have shown promising preliminary results (41,99), yet more research is needed.

A major drawback of pan-PI3K inhibitors however is whether a combination inhibitor can sufficiently target all cancer-driving mutations at levels that are not toxic to cell lines in vitro or, more importantly, in patients (114). The close link between PI3Ks and glucose homeostasis would imply that wide-spanning PI3K inhibition would induce hyperglycemia in patients, probably because of the overlapping roles PI3Ks, in this context PI3Kα and PI3Kβ, play in insulin signaling (40,102,114). This dependency is exemplified by studies where the pancreas and muscles of PTEN-deficient mice are protected against diet-induced insulin resistance (40,115-120). In the case of HNSCC, mutations in other members of the PI3K family are few (6,9,10,11,TCGA www.cbioportal.org), and therefore multi-targeted therapy seems redundant. Moreover, the low specificity of pan-PI3K inhibitors can cause inhibition of other vital cellular enzymes, particularly DNA-PK, essential for the repair of double stranded DNA breaks (120). Nevertheless the use of pan PI3K inhibitors in cancer treatment does have some merit: Castel and colleagues found that secondary BYL719-resistant breast tumors had a loss of heterozygosity (LOH) event in PTEN, which did not occur in the primary tumor (42,43,46,47). PTEN-deficient models have been shown to rely on p110β, rather than p110α (42), thereby implying a potential use for pan PI3K inhibition, or in this case combinatory p110α-β or p110β-specific inhibition in these tumors.

The AKT inhibitor MK-2206 (Merck) has shown promising effects when used concurrently with paclitaxel in preclinical and Phase I trials, and is currently being evaluated in Phase II trials as a single agent for recurrent metastatic HNSCC (100).
Rapamycin, as well as several analogs, have also shown promise in early clinical, as well as pre-clinical, models with regards to targeting mTOR (41). As mentioned previously, rapamycin only inhibits the mTORC1 complex, yet there are many drugs that inhibit both mTORC1 and mTORC2 complexes (31,99). A major problem in targeting both AKT and mTOR however is the negative feedback loop that ensues, whereby decreased AKT and mTOR signaling recycles RTKs, and the insulin receptor substrate 1 (IRS1) to the cell membrane. This reactivates PI3K/AKT and adjacent pathway signaling, and leads to the reinstitution of cell growth (50). This observation stresses the need for the investigation and development of dual PI3KCA-mTOR inhibitors, or the implementation of combinatory use of PI3K and AKT or mTOR inhibitors.

The PI3K/AKT signaling axis is a very logical area to focus our attention for targeted therapies in HNSCC. This is based in the fact that PI3KCA, particularly in HPV-positive cancers, has significantly higher mutation and amplification rates and can be targeted due to its oncogenic nature. Despite HRAS also being oncogenic and often mutated in HNSCC, understanding the complexities of this small GTPase and how to overcome its remarkable affinity for GTP remains an unresolved challenge. PI3KCA on the other hand, has such a fundamental role in cancer progression; being able to control this pathway as well as its upstream and downstream components may be the key to unlocking the proper treatments for HNSCC.

1.4.4 BYL719

BYL719 (Novartis) is an orally administered PI3K inhibitor that strongly and selectively inhibits the gene product of PI3KCA, p110α. It preferentially binds to the p110α catalytic subunit and its hotspot mutations with a 50-fold smaller Kd, as compared to other PI3Ks, or mTOR (101). Moreover, its ability to potently inhibit AKT phosphorylation was also significantly better than either PI3Kβ or PI3Kδ isoforms (102).

BYL719, from the 2-aminothiazole classes of inhibitors (27), binds exclusively to p110α and its mutational derivatives with equal affinity (Kd ~4-5nM). The drug was designed to interact with PI3K p110α specific residues in the ATP binding pocket of the kinase domain (27,113). The primary amino group of BYL719 forms three hydrogen
bonds with PI3Kα residues, two of which are bidentate to the Q859 residues, which is only found in PI3Kα thereby providing the high degree of specificity (27). As a result of this hydrogen bonding, the BYL719-pyrrolidine moiety neatly resides in the catalytic pocket, which further explains the compounds high affinity for the p110α isoform (27).

In the case of pan-PI3K inhibitors, one of the greater areas of concern focuses on the high concentrations and therefore high toxicity required for effective therapeutic benefit (27,50,101,102,113,121). The rationale for the alpha-specific PI3K inhibitor BYL719 is that singular targeting of the key mutational event may not only be more effective, but will also minimize off target toxicity events, such hyperglycemia from insulin resistance, and weight loss (100-102,114,121). These same shortcomings are observed with dual PI3K-mTOR inhibitors, as the majority of these compounds inhibit the p110α, β, and δ isoforms, as well as mTORC1 and mTORC2 (99). In cancers where most of these targets aren’t mutated, it raises the questions as to whether doses required to inhibit the oncogenic isoform are attainable without unacceptable toxic effects (114). In terms of HNSCC, only PIK3CA point mutations and amplification occur in a significant subset of patient tumors (6-11).

Preliminary in vitro and xenograft studies with a panel of breast cancer lines and cell line-derived xenografts have shown BYL719 to be highly specific in targeting PIK3CA and inhibiting proliferation. Additionally, multiple groups have analyzed BYL179 drug response in a panel of cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE), and found the best responses were observed in head and neck cancer and luminal breast cancer cell lines (97,100). In terms of safety profile, BYL719’s toxicity appears limited, with reversible hyperglycemia and mild GI discomfort reported as the only adverse effects (101,121). Additionally, it was found that the therapeutic index, the amount of drug required to produce 50% toxicity in a population over the minimum effective dose (122), was estimated to be 3.25 for BYL719, as compared to 1.1 for a pan-PI3K inhibitor (101).

The primary focus of this thesis is to define which HNSCC genotype benefits most from BYL719 drug treatment. As such, the end goal is to be able to clearly define which
patients will respond and which patients will not in order to maximize efficacy of treatment.
1.5 Hypothesis and Specific Aims

Hypothesis: HNSCC cell lines harboring *PIK3CA* point mutations and gene amplifications will be sensitive to BYL719, whereas cell lines lacking hotspot mutations or gene amplifications in *PIK3CA* will be more resistant to this inhibitor.

Specific Aims:

1) To determine rates of *PIK3CA* hotspot mutations and amplifications in a large cohort of HNSCC primary tumor samples
2) To test the sensitivity of a panel of head and neck cell lines to the alpha-specific PI3K inhibitor BYL719
3) To measure the efficacy of BYL719 on tumor growth in cell line and patient-derived murine xenografts
4) To determine the oncogenic dependency of HNSCC cell lines to key PI3K/AKT pathway components using siRNA knockdown, and determine if *PIK3CA* is the oncogenic driver in *PIK3CA* point mutant or amplified cell lines
5) To analyze the differences in BYL719 response in sensitive and resistant cell lines by analysis of the PI3K/AKT pathway
6) To determine and test combination drug therapies in BYL719 resistant cell lines according to the differences observed in Objective 4
2 MATERIALS AND METHODS

2.1 Deparaffinization of Patient Samples

The formalin fixed paraffin embedded blocks from each patient’s primary tumor site were sectioned (5 µm thick) and mounted on slides. The slides were then deparaffinized with 3 minute washes in xylene (100%) twice, followed by a 1:1 xylene:ethanol mix, then ethanol (100%) twice, followed by single washes in ethanol at 95%, 70% and 50%. Lastly, the slides were washed in water for 5 minutes.

2.2 DNA extraction

Deparaffinized tissue was scraped into a 1.5 ml eppendorf tube containing 50–100 µl (depending on the amount of tissue) of TE and proteinase K (final concentration 2 mg/ml) and incubated overnight at 65°C. Following proteinase K treatment, the samples were heated at 95°C for 10 minutes and allowed to cool to room temperature. Two hundred nanolitres of each sample was used directly in the qPCR reactions.

2.3 Cell culture

Detroit562, JHU006, JHU011, JHU029, SCC4, SCC9, SCC15, SCC25, HMS001, 93-VU-147T, UM-SCC47, UPCI:090, UPCI:154, PCI6A, PCI6B, PCI13, PCI22B, PCI30, RF15A, RF15B, RF22B, RF37A, RF37B, and FaDu cell lines were grown in Dulbecco’s Modified Eagle Medium with Ham’s F12 1:1 mixture (DMEM/F12) supplemented with 10% native fetal bovine serum (FBS), 1% penicillin/ streptomycin, and 2% hydrocortisone. The Cal33 cell line was grown in DMEM supplemented with 10% heat inactivated FBS, 1% penicillin/ streptomycin, 1% L-glutamine, and 2% hydrocortisone. The HSC-2 cell line was grown in Roswell Park Memorial Institution (RPMI) media supplemented with 10% native FBS, 1% penicillin/ streptomycin, A summary of all cell lines used with their HPV, PIK3CA, and HRAS mutation/CNV status as well as the sources of all cell lines can be found in Table 1.
Table 1 Summary of HNSCC cell lines with HPV-status, *PIK3CA* hotspot mutation or amplification, PTEN loss, and HRAS hotspot mutation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HPV status</th>
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<td>-</td>
<td>-</td>
<td>UPMC</td>
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Cal33 | Negative | H1047R | - | - | Centre Antoine-Lacassagne
HSC2 | Negative | H1047R | - | - | Tokyo Medical and Dental University

ATCC denotes American Type Culture Collection
UPMC denotes University of Pittsburg Medical Center
RF denotes Dr. Robert Ferris, University of Pittsburg Cancer Institute

2.4 STR profiling of HNSCC cell lines

All cell lines had 50ng of DNA extracted using the AllPrep DNA/RNA Kit (Qiagen Cat No 80204) according to the manufacturers protocol. Samples were sent to The Center for Applied Genetics (TCAG) in Toronto for short tandem repeat (STR) profiling. All HNSCC cell lines were compared to a reference genome in order to ensure proper identity.

2.5 PIK3CA-hotspot mutational and PIK3CA, SOX2, PTEN CNV status, using real time-PCR

2.5.1 PIK3CA hotspot mutations

Primers were designed for 4 specific PIK3CA mutations, E542K, E545K, H1047R, and H1047L, for real time-PCR analysis. Taqman probes and primer sequences are summarized in Table 2. E542K and E545K were conjugated to 6-carboxyl-X-rhodamine (ROX) fluorophores; H1047L and H1047R were conjugated to Carboxyfluorescein (FAM) fluorophores; and GAPDH, which served as the control, was conjugated to the Cy5 fluorophore. Experiments were performed in 96 well plates divided in half, so that E542K (ROX), H1047L (FAM), and GAPDH (Cy5) were detected in the first set of reactions, and E545K (ROX), H1047R (FAM), and GAPDH (Cy5) were detected in the second set of reactions.

PCR reactions (20µl) were prepared with 200ng of patient DNA, 10µl of Quantitech Multiplex PCR NoROX Master Mix (Qiagen), 7µl of water, 0.04µl of FAM probe, 0.04µl of ROX probe, 0.04µl of Cy5 probe, and 2.44µl of TE buffer (Invitrogen). 0.04µl (0.24µl total volume of primer) of each forward and reverse primer sequences for the appropriate mutational analysis and GAPDH control were also added. PCR reactions
underwent a 15 minute denaturation step at 95°C, followed by 60 seconds at 94°C for annealing, and 90 seconds at 60°C for extension for 40 cycles.

Mutational analysis was performed in Excel, where ∆Ct was calculated according to the formula ∆Ct = Ct (sample) – Ct (GAPDH control). Mutations were detected by comparing ∆Ct of patient samples to the ∆Ct of known, previously sequenced controls. For E542K, patient 91 was used as a positive control; for H1047L, JHU029 was used as a positive control; for E545K, PCI6A was used as a positive control; and for H1047R, HSC2 was used as a positive control. Patient samples were removed from the study if their Cy5 (GAPDH) Ct score was greater than 35.0 after three replicates. It was deemed that these samples had insufficient DNA and therefore were excluded from the study. Seven HPV-positive samples were excluded from the study. All results were transcribed into the Oropharyngeal Cancer Patient (OCP) Database and checked for HPV status (Nichols et al, unpublished).

Table 2 Taqman probe and primer sequence for RT-PCR

<table>
<thead>
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<th>Target</th>
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</thead>
<tbody>
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<td>GAPDH</td>
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</tr>
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<td></td>
<td>F-GCTCATTTGCCAGGGGGAGGCC</td>
</tr>
<tr>
<td></td>
<td>R-CTGATGATCTTGGGCTGGTGG</td>
</tr>
<tr>
<td>PIK3CA E542K</td>
<td>5’-[ROX]GCAATTTTCTACAGAGATCCTCTC[BHQ2]</td>
</tr>
<tr>
<td></td>
<td>F-GGGAAAATGACAAAAGAAGCAGCTC</td>
</tr>
<tr>
<td></td>
<td>R-CTTCTCTCCTGTCAGTGATTCT</td>
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<td>PIK3CA E545K</td>
<td>5’-[ROX]GCAATTTTCTACAGAGATCCTCTC[BHQ2]</td>
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<tr>
<td></td>
<td>F-GGGAAAATGACAAAAGAAGCAGCTC</td>
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<tr>
<td></td>
<td>R-ACTCCATGAAAAATCTCTTCTC</td>
</tr>
<tr>
<td>PIK3CA H1047L</td>
<td>5’-[6FAM]GCAAGAGGCTTGGAGTATT[BHQ1]</td>
</tr>
<tr>
<td></td>
<td>F-CCCTAGCCTTAGATAAAAACTGA</td>
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<tr>
<td></td>
<td>R-TGTTGTCCAGCCACCATGCA</td>
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<tr>
<td>PIK3CA H1047R</td>
<td>5’-[6FAM]GCAAGAGGCTTGGAGTATT[BHQ1]</td>
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<td>F-CCCTAGCCTTAGATAAAAACTGA</td>
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<tr>
<td></td>
<td>R-TGTTGTCCAGCCACCATGCA</td>
</tr>
</tbody>
</table>

*F denotes forward primer sequence
*R denotes reverse primer sequence

2.5.2 PIK3CA amplification

Primers to detect PIK3CA gene amplification have been described previously, and are summarized in Table 3. SYBR PCR reactions were performed in 96 well plates
 divided into halves where one half detected *TRAT1* controls, and the second half detected *PIK3CA* amplifications. *TRAT1* and PIK3CA are located on opposite arms of chromosome 3, and *TRAT1* was assayed in order to control for ploidy number, as the gene is found at an infrequently amplified locus on the 3p chromosomal arm (127). PCR reactions (15µl) were prepared with 200ng of either cell line, or patient DNA, 7.5µl of Power SYBR Green (2x) PCR Master Mix, 0.1µl of both Forward and Reverse *PIK3CA* or *TRAT1* primers, and 7.1µl of water. PCR reactions were subjected to 10 mins of initial denaturation at 95°C, followed by 40 cycles of 30 secs of denaturation at 95°C, 60 secs of annealing at 55°C, and 60 secs of extension at 72°C.

Amplification analysis was performed in Excel, where ∆∆Ct was computed using the standard formula (123). Amplifications were detected by comparing ∆∆Ct of samples to ∆∆Ct of TCGA-defined controls. FaDu and SCC15 are amplified cell lines and were used as positive controls; 93-VU-147T has high levels of *PIK3CA* amplification and also served as a positive control, and Cal27 has been shown to have normal putative diploid gene copy number and served as a negative control (CCLE). All samples were normalized to the average of three germline controls taken from the blood of three different HNSCC patients in order to control for diploid copy number. Patient samples were removed from the study if their Ct score was greater than 35.0 as that sample was considered to have insufficient DNA. After 3 replicates, 4 HPV-negative patient samples were excluded from the study. All results were transcribed into the Oropharyngeal Cancer Patient (OCP) Database and checked for HPV status.

### 2.5.3 *PTEN* CNV

*PTEN* PCR primers were designed to detect CNV, and are summarized in Table 3. SYBR PCR reactions (15µl) were prepared with 200ng of cell line DNA, 7.5µl of Power SYBR Green (2x) PCR Master Mix, 0.1µl of both Forward and Reverse of either GAPDH or PTEN primers, and 7.1µl of water. PCR reactions were subjected to 10 minutes of initial denaturation at 95°C, followed by 40 cycles of 30 seconds of denaturation at 95°C, 60 seconds of annealing at 55°C, and 60 seconds of extension at 72°C.
CNV analysis was performed in Excel, where ΔΔCt was computed using the standard formula (123). *PTEN* loss was detected by comparing the ΔΔCt of cell lines to the ΔΔCt of the PC3 prostate cancer cell line, which harbors a homozygous deletion at the *PTEN* locus (124).

### 2.5.4 SOX2 amplification

Primers were designed for *SOX2* and are described in Table 3. All reactions were performed in duplicate in a 96-well plate, divided in three. The first four columns (32 wells) were used to detect *TRAT1* status, the next four for *PIK3CA* amplification, and the final four for *SOX2* amplification. *PIK3CA* amplification was assayed to confirm *SOX2* amplification, as the TCGA ([www.cbioportal.com](http://www.cbioportal.com)) reported nearly 100% co-amplification of the two genes.

SYBR PCR reactions (15µl) were prepared with 200ng of cell line DNA, 7.5µl of Power SYBR Green (2x) PCR Master Mix, 0.1µl of both Forward and Reverse primers of *TRAT1*, *PIK3CA*, or *SOX2*, and 7.1µl of water. PCR reactions were subjected to 10 min of initial denaturation at 95°C, followed by 40 cycles of 30 seconds of denaturation at 95°C, 60 seconds of annealing at 55°C, and 60 seconds of extension at 72°C.

*SOX2* amplification was computed in Excel in a similar fashion to *PIK3CA* amplification described above. FaDu, and SCC15 served as CCLE-defined positive controls, and Cal27 served as a CCLE-defined negative control. All cell lines were normalized to three germline controls from the blood of three different HNSCC patients. ΔΔCt was calculated using the formula previously described (98), and compared to *PIK3CA* amplification.

**Table 3 Primer sequences for SYBR RT-PCR**

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<th>Target</th>
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<td>R-GACAGTAAGATACAGTCTAC</td>
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<td>R-AGAGATTGGCATGCTGTGCA</td>
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<td><em>TRAT1</em> F</td>
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<tr>
<td></td>
<td>R-GGGTCTTTCTCGTTAGGACTTAG</td>
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</table>
2.6 Drug treatments and determination of cell viability

Cells were seeded at a density of 1.0-2.0x10^4 cells/well (20-40% confluency) in 96-well dishes. BYL719 was resuspended in dimethyl sulfoxide (DMSO) in order to achieve a 11.4mM stock concentration, and rapamycin was resuspended in DMSO to achieve a stock concentration of 10mM. The following day, cells were treated with escalating doses of BYL719 (0.5µM-8µM), or rapamycin (0.01µM-10µM). A well was left untreated (normal growth media), and another was treated with 0.09% DMSO to act as negative controls. Cells were incubated at 37°C with 5% carbon dioxide (CO₂) until needed. At 48, 72, 96, and 120 hours, cells were subjected to 10µl of PrestoBlue™ Cell Viability Reagent (Invitrogen) and incubated at 37°C with 5% CO₂ for 30 minutes. PrestoBlue™ detects cell viability by capitalizing on the fact that viable cells maintain a reducing environment in their cytosols. The resazurin-based solution contains a cell-permeable compound that is blue with minimal fluorescence. Upon entry to the cytosol of viable cells, that compound is reduced to fluorescent red compound which can be detected. Cell viability was measured by fluorescence using the Wallac Victor² 1426 Multilabel Counter with an excitation wavelength of 535 nanometers (nm) (25nm bandwidth) and an emission wavelength of 615nm (10nm bandwidth).

2.7 Determination of the half maximal inhibitory concentration (IC₅₀)

Decrease in cell viability was quantified using Excel and normalized to DMSO-treated controls in order to determine the half maximal drug concentration, IC₅₀. This is defined at the drug concentration required to inhibit 50% of cell growth. The upper limit of the dose range was 8µM, and therefore cell lines that did not reach an IC₅₀ were not given an experimental value for BYL719 response. IC₅₀ was determined in two ways:

| SOX2      | F-CACATGAAGGAGCACCCGGATTAT  \
| R-GTTCATGTGCCGTAACTGTCAT  |
| GAPDH     | F-GCTCATTGCGAGGGGGAGCC  \
| R-CTGATGATCTTGAGGCTGTGG  |

F denotes forward primer sequence  
R denotes reverse primer sequence
first, using the growth curves, the dose at which the curve reached 50% decrease in cell viability was determined on the x-axis. Second, non-linear regression was also performed in order to ascribe a predicted IC₅₀ for cell lines that responded outside the dose range of this study.

2.8 Non-Linear regression

Non-linear regression was performed in Graphpad Prism in order to ascribe IC₅₀ values to cell lines that didn’t reach a decrease of 50% cell viability at our highest dose used. Scatterplots were generated in Graphpad Prism, and a line of best fit was generated for the data points. Since most curves did not experience a linear response to BYL719, we chose non-linear regression for our analysis. An equation was generated using the function in Excel in the form of \( Y = A e^{-bx} \), where \( y \) is the proportion of cell viability (in our case we are looking at a 50% decrease in cell viability, so 0.5), \( A \) is the initial cell viability, \( b \) is the coefficient denoting decrease in cell viability (decay coefficient), and \( x \) is the drug concentration required to achieve ‘\( y \)’. In order to compute a predicted IC₅₀, the \( y \) value was denoted as 0.5 (for 50% decrease in cell viability), and the equation was solved for \( x \).

2.9 Determination of effective combinatory rapamycin dose

In order to determine the effective concentration of rapamycin to use in combinatory treatment with BYL719, the half maximal drug concentration of rapamycin was determined in a subset of HNSCC cell lines. Four PIK3CA-amplified or point-mutated cell lines (PCI6A, FaDu, 93-VU-147T, and Cal33 see Table 1) were selected in order to perform this study. Cell lines were treated with escalating doses of rapamycin (10\( \mu \)M, 20\( \mu \)M, 30\( \mu \)M, 40\( \mu \)M, and 50\( \mu \)M) and allowed to incubate for 72h at 37°C with 5% CO₂. At 72 and 96 hours, cells were assayed for cell viability as previously described. The IC₅₀ for the 4 cell lines was determined by methods described above (Materials and Methods 2.7). Previous studies, specifically using rapamycin, have deemed 20% IC₅₀ as an appropriate starting point for drug combination synergy (125,126). Three of the four cell lines tested had a rapamycin IC₅₀ between 30-40\( \mu \)M (FaDu was >50\( \mu \)M). Therefore, 6\( \mu \)M (20% of 30\( \mu \)M) was deemed appropriate for initial combination therapy studies.
2.10 Western blotting

Cells were washed with ice cold PBS and either scraped and collected into 1.5ml Eppendorf tubes, or incubated for 5 minutes on ice in their respective wells with ice-cold 0.5% NP40 Lysis Buffer (0.5% NP-40, 150mM NaCl, 50mM Tris-HCl pH 7.8) supplemented with 0.5% protease inhibitor cocktail (Sigma). Cells collected by scraping were centrifuged at 1000 rpms for 5 minutes in order to pellet the cells, followed by the addition of 75-100µl 0.5% NP40 Lysis Buffer supplemented with 0.5% protease inhibitor cocktail. Lysates were cleared by centrifugation at 10,000 rpm for 10 minutes at 4°C. Total protein concentration was measured by a modified Bradford assay (Invitrogen). Five micrograms of whole cell lysate was resolved on NuPage 4-12% continuous gradient bis-tris gels (Life Technologies) at 200V for one hour. Resolved proteins were transferred onto polyvinylidene fluoride (PVDF) (GE Healthcare) membranes using a wet transfer method at 1.1h at 20V with a limit of 25 A/membrane.

Membranes were blocked in either 5% BSA in tris-buffered saline (TBS)-Tween (TBST), or 5% nonfat dry milk in TBST for one hour at room temperature rocking according to the manufacturers guidelines or recommendations. Primary antibody (diluted in either 5% BSA-TBST for phospho-proteins, or 5% nonfat dry milk in TBST for detection of proteins independent of phosphorylation status) was placed on the membrane and allowed to incubate overnight at 4°C with rocking. A list of the primary antibodies and their working concentrations can be found in Table 4.

The following day, membranes were washed 3 times with TBST for 10 minutes. Secondary antibodies against either mice or rabbits where raised in goats and the resulting secondary antibodies were conjugated to horseradish peroxidase (HRP) (1:5000 GaM (Jackson Research Labs), or 1:2000 GaR (Jackson Research Labs)). Secondary antibodies were diluted in 5% nonfat dry milk-TBST and placed on the membranes and rocked at room temperature for 30 minutes. Protein-antibody complexes were detected using chemiluminescence with Luminata Crescendo Western HRP Substrate (Millipore) and resolved on Amersham Hyperfilm ECL (GE healthcare) or using the ChemoDoc Gel Imager (BioRad). B-Actin, α-Tubulin, eIF4E and/or Rab11 were used as loading controls.
Table 4 Comprehensive list of all primary antibodies used in this thesis

<table>
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<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Dilution Media</th>
<th>Company</th>
<th>Secondary antibody</th>
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<td>Sigma</td>
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</table>

## 2.11 Combination therapy of rapamycin and BYL719

Cells were plated into 96-well plates as described previously, and in 6-well plates at 1.0x10^5 cells/well to achieve 50% confluency the next day. Stock solutions of 6µM rapamycin, 10µM BYL719 with either 6µM or 30µM of rapamycin, and 10µM of BYL719 were prepared in the appropriate media. Eight cell lines, all resistant to singular BYL719 treatment, were used for this study. Their mutational profiles can be found in Table 1.
In the 96-well plates, cells were subjected to increasing doses of BYL719 (1µM, 2.5µM, 5µM, 7.5µM, and 10µM) at a constant rapamycin concentration of 6µM. A DMSO-treated control was administered in the first row of every plate. A second plate was also treated in parallel with identical dosage of BYL719 to serve for comparisons sake. Cells were incubated at 37°C with 5% CO₂ for 72 and 96 hours before cell viability using PrestoBlue™ Cell Viability Reagent (Invitrogen) was used to determine cell viability, as previously described above. Half maximal inhibitory concentrations (IC₅₀) for each cell line were determined as previously described, and BYL719 alone and BYL719 +6µM rapamycin were compared.

In the 6-well plates, cells were subjected to 8 different drug combination treatments: either BYL719 alone at 0µM (DMSO-treated control), 1µM, 5µM, or 10µM, or identical BYL719 concentrations but with 6µM of rapamycin. Cells were allowed to incubate at 37°C with 5% CO₂ for 36h, which was determined experimentally to be the timepoint at which AKT phosphorylation decreased considerably, then collected by cell scraping as described above. A Western blot was performed for each cell line (as described above) and each membrane was blotted for p-S6 (S235/6) (Pathscan #1, or #2), p-AKT (T308), p-AKT (Ser473) (Pathscan #1), and AKT (Table 2). Rab11 (Pathscan #1) or eIF4E (Pathscan #2) served as a loading control.

2.12 Densitometry

In order to determine the effects of BYL719, rapamycin, and BYL719-rapamcyin combination, densitometry was performed using ImageJ to quantify S6, and AKT phosphorylation. The pS6 band at 32kDa, and the Rab11 band at 25kDa for each dose were boxed using the ‘box tool’ in order to generate densitometry graphs. Background signal was eliminated using the ‘line tool’ and values were generated and exported to Excel for analysis. Densitometry readings for S6 phosphorylation were first normalized to their corresponding Rab11 loading controls, then normalized to the difference of the untreated control. S6 phosphorylation decrease was graphed as a percentage of the untreated controls.

ImageJ was also used to determine the effects of BYL719 on phospho-AKT levels
at Serine 473. Excel was also used for this analysis. Phospho-AKT levels were first normalized to endogenous AKT for each cell lines. This value was then normalized to the untreated control and multiplied by 100 in order to generate an AKT phosphorylation percentage for 5µM BYL719 treatment. Phospho-AKT (Ser473) was graphed as a percentage of the untreated controls.

2.13 siRNA study for oncogenic dependency

Cal33, Detroit562, PCI6A, and FaDu were selected in order to analyze whether siRNA knockdown of key PI3K/AKT signaling components in cells with PIK3CA mutations (Cal33 and Detroit562), amplifications (FaDu), or both (PCI6A) resulted in decreased cell viability. Cells were plated in a 60mm dish at a density of 5.6x10^5 cells/well in order to achieve 50% confluence the next day. We ordered a panel of siRNAs against different components of the PI3K/AKT signaling axis from ABM Good in order to analyze the effects of knockdown on cell viability: Two AKT siRNAs (cat. nos. 659 and 660), two mTOR siRNAs (cat nos. 603 and 604), and two PIK3CA (cat nos. 20 and 22). Seven and a half microlitres of siLentFect (Bio-Rad) in 250µl of serum-free media (SFM), and 20nM of siRNA into 250µl of SFM was added to cells in 2.5ml of fresh, complete media as suggested by the BioRad Protocol. Briefly, 250µl of siRNA-SFM mixture was added to the 250µl siLentFect-SFM mixture, vigorously mixed, and allowed to incubate at room temperature for 20 minutes in order to allow proper conjugation of siRNA and siLentFect for effective intracellularization. siRNA-siLentFect-SFM mixture was then added to 60mm dishes, rocked back and forth to ensure mixing, and incubated at 37°C with 5% CO₂ overnight. Twenty-four hours later, cells were trypsinized and placed equally into a 96 well dish. Presto Blue Reagent was administered to the wells at 48, 72, 96, and 120h after transfection.

2.14 Animals

NOD/SCID/IL2-receptor-γ (NSG) mice were bred internally at the OCI Animal Care facility and ranged from 4-6 weeks old. All animals were kept in a pathogen free environment on a standard 12h day/12h night cycle and were fed a standard sterilized pellet diet and water ad libum. Animals were treated under the ethical guidelines of the
Animal Care Committee at the OCI Animal Care Facility.

2.15 Generation of drug treatment dose and schedule

In vivo BYL719 dosing levels and scheduling were determined using cell line-derived xenografts. A cell line-derived xenograft was generated by injecting $10^7$ cells of a moderately sensitive (blue-shading in Figure 4A) cell line into the flanks of NSG mice in a volume of 100µl cell culture media and matrigel (1:1). Approximately 20 mice were then treated with placebo (2 mice) and increasing doses of BYL719 (2 mice per dose) by oral gavage. In this manner, a reasonable dose and schedule was established for further xenograft (cell line and patient-derived) studies.

2.16 Generation of cell line-derived NSG mouse xenografts

This protocol was designed by our group, but was performed at the University of Toronto in Dr. Laurie Ailles’ lab. Cal33 and 93-VU-147T cell lines were selected for in vivo xenograft studies with NSG mice. Cal33 cells were chosen because they were the most sensitive of all PIK3CA-mutant cell lines; 93-VU-147T cells were chosen because they are HPV-positive, harbor the highest degree of PIK3CA amplification (Figure 2), and were also highly resistant to BYL719 (Figure 4A). $10^7$ cells of either Cal33 or 93-VU-147T in a volume of 100µl cell culture media and matrigel (1:1) were injected into the flank of NSG mice. Animals were randomized into one of three groups (n=5): corn oil control, 25mg/kg of BYL719, or 50mg/kg of BYL719. Tumors were allowed to grow until they reached a volume of $200\text{mm}^3$, roughly 2 weeks after injections. At this volume, it was experimentally determined by Dr. Ailles that the tumors underwent log phase growth, which proved to be the ideal time point for drug delivery. Tumor volume was measured every other day with digital calipers and tumor volume was calculated by the formula $\text{Volume} = (\text{Length} \times \text{Width}^2) \times 0.52$. BYL719 was given daily by oral gavage. Corn oil was used because it solubilized BYL719. The endpoint of this study occurred on Day 26 when tumor diameters reached the maximum allowable size of 1.5cm (Animal Care Committee) in the control group mice. Mice were euthanized according to the ethical guidelines of the Animal Care Committee at the OCI Animal Care Facility.
2.17 Handling of fresh tumor specimens for xenograft generation

Tumor specimens were obtained either by cupped biopsy at the time of diagnosis or taken from the center of the resection specimen at the time of surgical management. A pathologist confirmed the presence of invasive SCC on a portion of the sample by frozen section. Tumor samples were then placed on ice and shipped overnight to the Animal Care Facility at the Ontario Institute for Cancer Research (OICR).

2.18 Tissue processing, implantation and tumor molecular characterization

Tumor tissue was cut into small pieces of ~1x1 mm using a sterile surgical blade. One representative piece was to be frozen at −80°C for further molecular and pathological analysis. As soon as possible (ideally within 24 h), pieces were implanted subcutaneous in the flanks of 5 NSG mice. Once palpable, tumors were measured twice weekly until a maximum diameter of 1.5 cm was reached over the course of 2-6 months. The tumors were then surgically removed and representative pieces were preserved in various fixatives for additional analysis. Hematoxylin and eosin (H&E) slides were made from formalin-fixed, paraformaldehyde embedded (FFPE) blocks in order to follow any changes in morphology and to confirm the epithelial nature of the tumor. DNA was extracted from the original patient’s blood, and DNA and RNA was extracted from a small portion (3x3 mm) of the first pass tumor. The rest of the tumor was sectioned into 1x1 mm pieces, and then re-implanted subcutaneously in a new generation of mice. This process was repeated for every passage. Tumors are considered engrafted if they are passaged at least once in vivo. Tumours are considered engraftment failures if tumour growth is not detected by the sixth month after implantation. Note that subsequent passages are of high efficiency, in that >90% of tumours engraft and up to 20 mice can easily be implanted from a single PDX tumour.

2.19 Generation of patient-derived NSG mouse xenograft

This protocol was designed by our group, but was performed at the University of Toronto in Dr. Laurie Ailes’ lab. Patient tumors were implanted into the flank of NGS
mice and allowed to grow until palpable. Mice were divided into two groups of 5 and given one of two treatments, corn oil control, or 50 mg/kg BYL719. Once tumors were palpable (on day 28), BYL719 was administered 5-days/week by oral gavage. Tumor volume was measured every other day with digital calipers and tumor volume was calculated by the formula $\text{Volume} = (\text{Length} \times \text{Width}^2) \times 0.52$. The endpoint of this study occurred on Day 48 when tumor diameters reached the maximum allowable size of 1.5cm (Animal Care Committee) in the control group mice, and all animals were euthanized according to the guidelines set forth by Animal Care Committee. Tumor volume was weighed at the end of the study in order to assess size.

2.20 Statistics

The data was analyzed in SigmaStat. Comparison of HPV-positive and HPV-negative tumors’ $PIK3CA$ mutational data was analyzed using a Fisher’s Exact test. All IC$_{50}$ comparisons, phospho-S6 levels, and phospho-AKT levels were analyzed using a Student’s T-test. Short hairpin RNA as well as combination drug therapy response was analyzed using a One-way analysis of variance (ANOVA) with a Tukey’s range test. P values less than 0.05 were considered significant (* $<$0.05).
3 RESULTS

3.1 *PIK3CA* hotspot point mutations and amplifications occur in a significant proportion of HNSCC tumors, with a proportion of tumors harboring both *PIK3CA* hotspot mutations and amplifications

Multiple NGS studies have confirmed the high incidence of both *PIK3CA* hotspot mutations (E542K, E545K, H1047R) and gene amplifications in HNSCC tumors (6,9-11, TCGA www.cbioportal.org). Therefore, we sought to determine whether similar frequencies would be observed in HNSCC patient tumor DNA taken from the London Regional Cancer Program (LRCP) tumorbank. Fresh patient samples were prepared as described above and stored at -80°C. If a patient sample had a threshold value (Ct) above 35.0 for its control (either *TRAT1* (amplification) or *GAPDH* (mutation)), the sample was deemed to have poor DNA content and was discarded from the study. After three experimental repeats of 191 oropharyngeal patient samples, 8 were excluded from the mutational analysis, and 5 from the amplification analysis. No non-oropharyngeal patient samples were excluded from the study.

One hundred and eighty-three patient oropharyngeal tumor, and 89 patient non-oropharyngeal HNSCC tumors DNA samples were subjected to real time-PCR analysis for mutation detection as described above. Four different point mutations were sought out: E542K, E545K, H1047L, and H1047R. Indeed, *PIK3CA* mutational rates determined agreed with those previously reported (6,7,9-11, TCGA www.cbioportal.org) with an overall mutation rate of 17.5%, with significantly higher point mutation rates in HPV-positive oropharyngeal tumors (28.2% in HPV-positive, compared to 8.2% in HPV-negative) (P<0.05) (Table 5). Non-oropharyngeal tumors had significantly lower incidence of *PIK3CA* mutations (4.4%) as compared to oropharyngeal tumors (P<0.05) (Table 6).

For *PIK3CA* gene amplification, oropharyngeal samples were tested using SYBR Green real time-PCR described above. One hundred and eighty-six patient samples were tested. The *TRAT1* gene, found on the 3p chromosome, (127), was used as a control for
Table 5 PIK3CA hotspot mutation frequencies in oropharyngeal patient samples from LRCP tumorbank. Real time-PCR results revealed high PIK3CA hotspot mutational rates in oropharyngeal tumors from the LRCP tumorbank. Bracketed numbers denote the number of tumors that had PIK3CA mutations. N=183 (N=85 HPV-positive, N=98 HPV-negative), *Denotes statistical significance between HPV-positive and HPV-negative PIK3CA mutation rates according to a Fisher’s Exact Test (P<0.05)

<table>
<thead>
<tr>
<th>PIK3CA status</th>
<th>HPV-positive</th>
<th>HPV-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>85</td>
<td>98</td>
<td>183</td>
</tr>
<tr>
<td>E542K</td>
<td>7% (8)</td>
<td>3.1% (3)</td>
<td>6% (11)</td>
</tr>
<tr>
<td>E545K</td>
<td>15.3% (13)</td>
<td>5.1% (5)</td>
<td>9.8% (18)</td>
</tr>
<tr>
<td>H1047L</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H1047R</td>
<td>3.5% (3)</td>
<td>0</td>
<td>1.6% (3)</td>
</tr>
<tr>
<td>Overall</td>
<td>28.2% (24)*</td>
<td>8.2% (8)</td>
<td>17.5% (32)</td>
</tr>
</tbody>
</table>

Table 6 PIK3CA hotspot mutation frequencies in non-oropharyngeal patient samples from LRCP tumor databank. Real time-PCR results revealed lower PIK3CA hotspot point mutational rates in non-oropharyngeal HNSCC tumors from the LRCP tumorbank compared to oropharyngeal tumors. Bracketed numbers denote the number of tumors that had PIK3CA mutations N=89 *Denotes statistical significance of PIK3CA mutations from oropharyngeal tumor rates (p<0.05)

<table>
<thead>
<tr>
<th>PIK3CA status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>89</td>
</tr>
<tr>
<td>E542K</td>
<td>1.1% (1)</td>
</tr>
<tr>
<td>E545K</td>
<td>2.2% (2)</td>
</tr>
<tr>
<td>H1047L</td>
<td>0</td>
</tr>
<tr>
<td>H1047R</td>
<td>1.1 (1%)</td>
</tr>
<tr>
<td>Overall</td>
<td>4.4% (4)*</td>
</tr>
</tbody>
</table>
the relative diploid number, and was used to standardize \textit{PIK3CA} amplification to normal diploid 3q chromosome levels. The results suggested that \textit{PIK3CA} amplification occurs at high frequencies in both HPV-positive and negative oropharyngeal tumors (Table 7), which correlated with the current literature (7, TCGA www.cbioportal.com) (HPV-positive 47.8%, HPV-negative 33%), despite our gene amplification rates being higher. Much like with hotspot point mutational analysis, \textit{PIK3CA} amplification occurs at a higher frequency in HPV-positive oropharyngeal tumors compared to HPV-negative oropharyngeal tumors, yet this difference was not significant (p=0.052) (Table 7).

We next sought to determine the number of oropharyngeal patient tumors that harbored both \textit{PIK3CA} hotpot mutations and amplifications. All patient samples that were excluded due to insufficient DNA did not have a \textit{PIK3CA} point mutation or amplification. Forty percent of all patient tumors that had \textit{PIK3CA} point mutations also had \textit{PIK3CA} amplifications, with more HPV-positive tumors (45.8%) having both events compared to HPV-negative tumors (27.3%) (Table 8). HPV-positive tumors with helical domain mutations both had 5 tumors with \textit{PIK3CA} gene amplification (E542K 5/8, and E545K 5/13), whereas HPV-positive H1047R mutant tumors only had one (1/3) (Table 8). Conversely, only HPV-negative tumors with E545K mutations showed co-amplification of the \textit{PIK3CA} gene (E545K 3/5, E542K and H1047R both 0/3) (Table 8). In both types of HNSCC, dual mutational events were almost equally frequent in both helical domain point mutations (E542K 5/11, E545K 8/18) and much less common in tumors with H1047R point mutations (1/6) (Table 8). Whether multiple mutations in the \textit{PIK3CA} gene results in a different phenotype than singular \textit{PIK3CA} point mutation or amplification remains to be determined.

3.2 \textit{PIK3CA} amplification occurred in a subset of HNSCC cell lines, whereas \textit{PTEN} loss did not

Eleven of the 28 HNSCC cell lines, two being HPV-positive (93-VU-147 and HMS001) had at least one extra copy of \textit{PIK3CA} relative to the control gene (Figure
Table 7 *PIK3CA* amplification frequencies in oropharyngeal patient samples from LRCP tumorbank. Real-PCR results revealed *PIK3CA* amplification rates from oropharyngeal tumors from the LRCP tumor databank agreed with the amplification rates reported in current NGS literature. N=183 (N=85 HPV-positive, N=98 HPV-negative). Statistical analysis using a Fisher’s Exact Test revealed that the difference between HPV-positive and HPV-negative amplification rates approached significance (P=0.052)

<table>
<thead>
<tr>
<th><em>PIK3CA</em> status</th>
<th>HPV-positive</th>
<th>HPV-negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>N</td>
<td>92</td>
<td>94</td>
<td>186</td>
</tr>
<tr>
<td>Amplification</td>
<td>47.8% (44)</td>
<td>33% (31)</td>
<td>40.3% (75)</td>
</tr>
</tbody>
</table>

Table 8 *PIK3CA* hotspot mutation and amplification frequencies in oropharyngeal patient samples from LCRP tumorbank. Real time-PCR results revealed that dual *PIK3CA* point mutations and gene amplification occur in oropharyngeal tumors.

<table>
<thead>
<tr>
<th><em>PIK3CA</em> status</th>
<th>HPV-positive</th>
<th>HPV-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp/ E542K</td>
<td>62.5% (5/8)</td>
<td>0/3</td>
<td>45.4% (5/11)</td>
</tr>
<tr>
<td>Amp/ R545K</td>
<td>38.5% (5/13)</td>
<td>60% (3/5)</td>
<td>44.4% (8/18)</td>
</tr>
<tr>
<td>Amp/ H1047R</td>
<td>33.3% (1/3)</td>
<td>0/3</td>
<td>16.7% (1/6)</td>
</tr>
<tr>
<td>Overall</td>
<td>45.8% (11/24)</td>
<td>3/11</td>
<td>40% (14/35)</td>
</tr>
</tbody>
</table>
This finding was relevant because it provided us with representation of PIK3CA amplification for BYL719 testing. Moreover, we also identified one cell line that had both a PIK3CA hotspot mutation (E545K) and gene amplification (PCI6A). Considering the amount of tumors with co-insult events, the PCI6A cell line afforded us a model to determine the sensitivity of these tumors to BYL719.

In normal physiology, PTEN antagonizes the action of PI3Kα signaling (39), and latent, secondary mutations in PTEN have been shown to facilitate a more aggressive phenotype, as well as mediate BYL719 resistance (42,47), I therefore sought to characterize PTEN status in all our HNSCC cell lines. The PC3 cell line has been reported to have a homozygous deletion at the PTEN locus (124) and was therefore used as the positive control for PTEN loss. None of the HNSCC cell lines used in this study harbored homozygous PTEN deletions (Figure 3A,B).

3.3 PIK3CA hotspot mutant cell lines are significantly more sensitive to BYL719 than both PIK3CA-amplified and PIK3CA-non-hotspot mutant cell lines

Twenty-eight cell lines were treated with increasing doses of BYL719, and sensitivity was determined by calculating the half maximal inhibitory concentration (IC\(_{50}\)). A cell line was deemed sensitive if it responded to BYL719 at a dose below 2µM because above this concentration BYL719 begins affecting the other PI3K isoforms (105). The upper range of our study was 8µM. If a cell line did not experience a 50% decrease in cell viability at this dosage, it was deemed resistant and grouped into the red-shaded area of Figure 4A. Non-linear regression was performed in order to ascribe a predicted-IC\(_{50}\) for cell lines outside the dosage limit and allow proper statistical analysis (Figures 4B,C,D).

Contrary to our initial hypothesis, only cell lines that harbored H1047R PIK3CA-hotspot mutations (Detroit562, Cal33, and HSC2) were sensitive to BYL719 treatment (2.06+/−2.40 µM, green shading in Figure 4A), which was significantly different from PIK3CA amplified (P=0.003), and PIK3CA non-hotspot (P=0.011) cell lines (Figures 4A, B, D). The JHU029 cell line, which harbors a non-conventional H1047L PIK3CA
Figure 2 *PIK3CA* amplification status in HNSCC cell lines. A, B) Real time-PCR was performed as described above on HNSCC cell lines in order to determine CNV of the *PIK3CA* gene. The CCLE had previously characterized a subset of our cell lines by copy number arrays. This information was used as our gold standard to which we could compare our real time-PCR copy number results. In order to determine ∆∆Ct, cell lines were normalized to germline controls with diploid *PIK3CA* gene status (not shown on graphs).
Figure 3 PTEN loss in HNSCC cell lines. A, B) Real time-PCR was performed as described above on HNSCC cell lines in order to determine whether loss of the PTEN gene occurred in any of our cell lines. Previous publications (described above) have demonstrated that PTEN is homozygously deleted in PC3 cells, a prostate cancer cell line. We therefore used that cell line as a control to validate our experiment. In order to determine ∆∆Ct, cell lines were normalized to germline controls with normal PTEN gene dosage (only shown on Figure B).
mutation that is not observed in patients (Tables 4) had an IC_{50} of ~4 \mu M. Surprisingly, all cell lines with PIK3CA gene amplification, regardless of mutation status (see PCI6A, Figure 4), were resistant to treatment with BYL719 within the range of the study (12.12+/−4.58 \mu M). Not surprisingly then, the overall half maximal inhibitory concentration for this group was not significantly different from the PIK3CA WT cluster (P=0.248) (Figure 4B).

The p110α catalytic subunit has a RAS binding domain, which can both activate and augment PI3K/AKT signaling (29,33,41). RAS activation may also leads to alternative downstream activation of mTORC1 activity through the mitogen-activated protein kinase (MAPK) pathway (33), and mutations have shown to predict resistance to PI3K inhibition in other cancers (33,41). Unsurprisingly then, it was predicted that cell lines containing an HRAS mutation would be resistant to BYL719 treatment. Our lab has shown that three HNSCC cell lines had HRAS hotspot mutations (Nichols et al., unpublished), however none of these had a concomitant PIK3CA alteration. As predicted, all three were resistant to BYL719 treatment (Figure 4A).

Finally, cell lines devoid of PIK3CA hotspot mutations or gene amplification varied in their response to BYL719, with the majority being resistant (9.24+/−5.69 \mu M). One cell line was deemed sensitive, JHU-011, and two others HPV-positive cell lines (UM-SCC47 and UPCI:090) had moderate sensitivity (denoted by blue shading in Figure 4A). HPV-positivity did not seem to affect BYL719 sensitivity. This can be deduced primarily because HPV-positive cell lines, denoted by bolding and asterisks, did not respond with any visible trend. Instead, cell lines tended to cluster according to PIK3CA status (Figure 4).

### 3.4 PIK3CA point mutant, but not PIK3CA amplified cell lines, are oncogenically dependent on PIK3CA, but not AKT1 or mTOR

Four HNSCC cell lines, 2 with PIK3CA hotspot mutations (Detroit562, Cal33), one PIK3CA-amplified (FaDu), and one with both a point mutation and amplification (PCI6A), were subjected to a panel of siRNAs against three key members of the
Figure 4 The effects of BYL719 on HNSCC cell lines at 72h. A) Cell lines were treated with increasing doses of BYL719 and assayed for cell viability at 72h with the PrestoBlue Cell Viability assay. Mutational analysis was performed (see above) and is reported on the X-axis. Cell lines that did not respond below 8µM are denoted in the red-shaded area. Cell lines that responded below 2µM were deemed sensitive, as denoted by the green shading; Cell lines that responded at a dose above 2µM were deemed moderately sensitive, as denoted by the blue shading. B-D) Mean difference +/- SD computed using non-linear regression; Statistical analysis for each data set was computed using a Student’s t-test: B) PIK3CA mutant vs. non-mutant (P=0.011), C) PIK3CA amplified vs. non-amplified non-mutant (P=0.248), and D) PIK3CA mutant vs. PIK3CA amplified (P=0.003).
PI3K/AKT signaling axis, *PIK3CA*, *AKT1*, and *mTOR*. The rationale of this study was to determine whether *PIK3CA* points mutations and/or amplifications were the oncogenic driving factors in these cell lines. Immunoblots were performed in parallel to demonstrate the effectiveness of siRNA knockdown on respective components.

Both cell lines with a *PIK3CA* point mutation (Detroit562, Cal33) showed significant reduction in cell viability compared to scrambled siRNA treated controls when treated with siRNAs against *PIK3CA* (p<0.05) (Figure 5A,B). In contrast, both cell lines with *PIK3CA* amplifications showed no decrease with similar siRNA treatment (Figure 5C,D). Moreover, siRNA targeting of either AKT1 or mTOR showed no difference in any of the cell lines in terms of cell viability as compared to siRNA-scrambled treated controls (Figure 5). This is interesting because AKT1 and mTOR are the two major effectors of canonical PI3K/AKT signaling (31); as such, the persistent cell viability in cell lines with singular knockdown of both of these two key components is suggestive that they play a less prominent role in *PIK3CA*-mediated oncogenesis. On the other hand, AKT and mTORC1 inhibition (in this case through mTOR knockdown) initiates feedback loops that aim to reactivate PI3K signaling through the upregulation of RTK expression and recycling maintaining PI3K/AKT signaling and could explain my results (103,104).

Interestingly, knockdown of *PIK3CA* in the FaDu and PCI6A cell lines was ineffective at reducing viability, despite both cell lines harboring *PIK3CA* amplifications. This would imply that *PIK3CA* amplification in these cell lines is not in fact the oncogenic driver, or at least not the only one. These results moreover show a similar result to what was observed with BYL719 in that *PIK3CA*-amplified cell lines are not responsive to *PIK3CA* inhibition alone. These findings suggest that different *PIK3CA* mutations, in this case hotspot point mutation and amplifications, have different responses to small molecule inhibition.
Figure 5 Effects of siRNA targeting of key members of the PI3K/AKT axis in 4 HNSCC cell lines. A) Cal33, B) Detroit562, C) FaDu, and D) PCI6A cell lines were subjected to siRNA-mediated knockdown of PIK3CA, AKT1, and mTOR and assayed for cell viability at 96hrs post transfection. E) Corresponding immunoblots to show effective siRNA targeting. All siRNA cell viability effects were normalized to untreated controls (not shown). *Denotes statistical significance from si-scrambled treated controls with P<0.05 using a one-way ANOVA with Tukey’s Range Test.
3.5 BYL719 is effective at preventing tumor growth in \textit{PIK3CA}-hotspot mutant cell line-derived and patient-derived xenografts

Two cell line-derived NSG mouse xenografts were established to test the effects of BYL719 in an \textit{in vivo} model and determine whether the findings correlated with \textit{in vitro} findings presented above. The H1047R \textit{PIK3CA}-mutant BYL719-sensitive Cal33 cell line, and the \textit{PIK3CA}-amplified, HPV-positive, BYL719-resistant 93-VU-147T cell line were chosen for the study. Cal33 was chosen because it was the most sensitive cell line to BYL719 (Figure 4A); conversely, 93-VU-147T was chosen not only because it was highly resistant, but also because it had the highest CNV of the \textit{PIK3CA} gene (Figure 2A).

After 13 days, mean tumor volumes reached 200 mm$^3$ and two doses of BYL719, 25mg/kg and 50mg/kg (105, as well as calculations performed by Dr. Ailles and our lab), and a corn oil-treated control, were administered to mice randomly divided into three groups of 5. Tumor volume was measured every other day, as described in the materials and methods. After 13 days on BYL719 treatment (Day 26), Cal33 xenograft mice showed significant decreases in tumor growth and volume at both BYL719 treatment doses as compared to the corn oil-fed controls (p=0.01 for both doses). Moreover, on average at the 50mg/kg treatment dose, tumor volume did not increase (=200mm$^3$) over the 13-day course of the study (Figure 6A), which demonstrates that BYL719 was effective in tumors with \textit{PIK3CA} point mutations. Conversely, the 93-VU-147T xenograft mice showed no difference at either of the two BYL719 doses (Figure 6B), which agreed with our \textit{in vitro} findings.

We also sought to determine the effects of BYL719 on patient-derived xenografts (PDX). One \textit{PIK3CA} E545K mutant tumor (London 26) successfully engrafted into NGS mice, and was subjected to two treatment groups, 50mg/kg of BYL719 or corn oil placebo control (Figure 7). BYL719 treatment commenced in PDXs once tumors were palpable, roughly around 28 days. By day 44, a difference could be observed between the BYL719-treated and untreated mice groups (Figure 7A). The study was terminated once tumors in the control group reached the maximum allowed size of 1.5 cm (Day 48).
Figure 6 The effects of BYL719 on tumor growth in cell line-derived mouse xenografts. A) Cal33 (H1047R) and B) 93-VU-147T, HPV-positive PIK3CA amplified, cell lines were injected into the flank of NGS mice to generate tumors. Once tumors reached a volume of 200mm³, one of three treatments was administered (listed above). Tumor volume was measured daily. Statistical analysis revealed a significant difference for both doses as compared to untreated control in the Cal33-derived xenografts (p<0.05). Conversely, the 93-VUT-147-derived xenograft did not observe a difference at any dose as compared to the untreated control. Results generated by Dr. Laurie Ailles
Figure 7 The effects of BYL719 on tumor growth in patient-derived mouse xenografts. London 26 was injected into the flank of NGS mice to generate tumors. A) Average tumor volume (in mm$^3$) of corn oil control and BYL719 at 50 mg/kg over 48-day period of treatment. BYL719 was administered at day 28 when tumors became palpable. Drug was administered by oral gavage 5 days/week for 48 days when control mice reached their end point. * Denotes statistical significance from corn oil control using a two-tailed T-test (p<0.05, N=5). B) Average tumor weight at 48 days with or without BYL719 treatment at 50 mg/kg. *Denotes statistical significance from corn oil control using a one-tailed T-test (p<0.05, N=5)
BYL719-treated tumors were on average about half the size of untreated tumors. This was also the case with tumor weight, as on average, BYL719-treated tumors weighed half as much as untreated tumors, which was significantly different (Figure 7B). These findings seemingly validated the observation that BYL719 was effective at targeting \textit{PIK3CA} point mutations, but not \textit{PIK3CA} amplifications in cell line-derived HNSCC tumors. Additional studies are needed to determine the effects on \textit{PIK3CA}-amplified and WT PDXs. The encouraging results have demonstrated the effectiveness of BYL719 in preclinical models. Moreover, our xenograft studies validate our \textit{in vitro} work with 28 HNSCC cell lines and have demonstrated a preferential response in \textit{PIK3CA} point mutant systems.

3.6 Persistent mTORC1 activity as evident by S6 phosphorylation was consistent in all BYL719 resistant \textit{PIK3CA}-amplified cell lines

The results from Figures 4, 6 and 7 demonstrate that \textit{PIK3CA} amplification, regardless of mutation status, is a predictive marker of BYL719 resistance. We therefore sought to compare the effects of BYL719 inhibition on downstream effects of activated AKT (p-AKT T308, S473), and mTORC1 in sensitive and resistant \textit{PIK3CA}-altered cell lines (Figure 8). The mTORC2 complex, while able to phosphorylate AKT at Serine 473 (31), to our knowledge plays a much less prominent role in PI3K/AKT signaling compared to mTORC1. It is postulated instead to be more actively involved in the organization of the actin cytoskeleton (39,132). For this reason, preliminary studies focused on AKT-mTORC1 signaling.

Four cell lines, Detroit562, Cal33, PCI6A, and FaDu, were treated with BYL719 for 36h to observe and compare the effects of drug inhibition on PI3K/AKT pathway signaling (Figure 8A). In sensitive cell lines, BYL719 was effective at drastically reducing the phosphorylation of AKT at threonine 308 and serine 473, and also subsequent mTORC1 activation, as measured by S6 phosphorylation at both serine 235 and 236 (11.25+/-0.9 % of control). Conversely however, resistant cell lines showed persistent activation of p-S6 (S235/6), albeit reduced from the untreated control, despite
Figure 8 The effects of BYL719 on downstream components of the PI3K/AKT signaling pathway A) Resistant lines PCI6A (amplified, E545K) and FaDu (amplified), and sensitive lines Detroit562 and Cal33 (both H1047R), were treated with BYL719 for 36h to assess the effects of drug inhibition on downstream pathway components. ImmunobLOTS reveal decreased phosphorylation AKT (Thr308, Ser473) in all cell lines; pS6 (235/6) was maintained in resistant cell lines. B) Percentage of S6 phosphorylation (Ser235/6) in PIK3CA amplified (PCI6A), BYL719 resistant (n=5), and PIK3CA mutant,
BYL719 sensitive (n=2) cell lines. ImageJ was used to compute densitometry. A paired T-test demonstrated a trend towards significance between the two groups (P=0.0831)
reduced p-AKT (T308, S473) (Figure 8A). Quantification of knockdown by densitometry clearly shows reduced S6 phosphorylation levels (S235/6) at 5µM in BYL719 sensitive cell lines (Figure 9B). This trend of maintained mTORC1 activity as a means for resistance through S6 phosphorylation was evident for 3 additional cell lines harboring PIK3CA amplifications (49.96±/−12.06 % of control) (Figure 8B). A paired T-test demonstrated a trend towards a significant difference between the sensitive and resistant cell line populations (P=0.0831), and the means of each group demonstrated greater levels of S6 phosphorylation in the BYL719 resistant population (49.96±/−12.06 % of control, for resistant vs. 11.25±/−0.9 % of control, for sensitive).

3.7 AKT phosphorylation at Serine 473 is maintained after BYL719 treatment in HPV-positive HNSCC cell lines

HPV-positive and HPV-negative HNSCC are two very distinct disease states as has been previously discussed (6-14). Considering this, we sought to determine if HPV infectivity in HNSCC cell lines showed a different phenotypic response to BYL719. Eleven HNSCC cell lines were treated with increasing doses of BYL719 (0,1,5,10µM) for 36h. All cell lines showed effective knockdown of AKT phosphorylation at threonine 308. Moreover, mTORC1 activity as determined by S6 phosphorylation depended more so on PIK3CA amplification status rather than the presence of HPV (Figure 8). However, it was observed that HPV-positive cell lines showed persistent AKT phosphorylation at the secondary AKT phosphorylation site, Serine 473, after BYL719 treatment (Figure 9).

Four HPV-positive, and 7 HPV-negative cell lines were studied in order to analyze the observation that AKT phosphorylation at Serine 473 was maintained after BYL719 treatment. Cell lines were chosen to represent the full panel of PIK3CA insults examined in this thesis (Table 1). The HPV-positive cell lines were 93-VU-147T, HMS001, UPCI:154, and UM-SCC47. The HPV-negative cell lines were FaDu, PCI6A, Cal33, Detroit562, Cal27, JHU006, and SCC15. The levels of AKT phosphorylation in all HPV-positive cell lines were assayed after 5µM BYL719 treatment (Figure 9A). Five micromolar of BYL719 was the dose chosen for analysis because all sensitive cell lines responded to doses considerably below this concentration, and all resistant cell lines showed no response at this dose.
Figure 9 The effects of BYL719 on phospho-AKT (Ser473) levels in HPV-positive cell lines. Eleven HNSCC cell lines, 4 HPV-positive and 7 HPV-negative, were treated with escalating dose of BYL719 in order to observe the effects of drug inhibition on AKT phosphorylation at Serine 473. A) Immunoblotting of 4 HPV-positive cell lines and B) of 4 HPV-negative cell lines; for the HPV-positive cell lines, 3 out of 4 had persistent AKT

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57kDa

P=0.0157

HPV+   HPV-
phosphorylation at Serine 473. C) Quantification and comparison of phospho-AKT (Ser473) levels at 5µM BYL719 in HPV-positive and negative cell lines. Densitometry was computed using ImageJ. Statistical analysis using a One-tailed Student’s t-test revealed that HPV-positive cell lines had significantly higher levels of pAKT(Ser473) as compared to HPV-negative cell lines (p= 0.0157, Mean ± SD: HPV-, 19.764 ± 13.216 densitometry units, N=7; HPV+, 84.990 ± 58.150 densitometry units, N=4).
Three of the four HPV-positive cell lines had maintained levels of AKT phosphorylation at Serine 473 that was similar to untreated controls after 5µM treatment with BYL719 (Figure 9A). Conversely, AKT S473 phosphorylation in HPV-negative cell lines was dramatically reduced after similar treatments with BYL719 (see Figure 9B). Quantification of phospho-AKT levels as a percent of untreated controls demonstrates the great discrepancy in BYL719’s effect at reducing AKT phosphorylation in HPV-positive cell lines as compared to HPV-negative cell lines (Figure 9C). Statistical analysis using an unpaired Student’s t-test revealed that levels of phospho-AKT (S473) were significantly higher in HPV-positive cell lines as compared to HPV-negative cell lines (p=0.0157, Mean ± SD: HPV+, 19.764 ± 13.216 densitometry units; HPV-, 84.990 ± 58.150 densitometry units) (Figure 9C). The only cell line that didn’t show decreased phosphorylation of AKT phosphorylation at Serine 473 in the HPV-positive group was UPCI:154. Interestingly, the UM-SCC47 cell line also maintained AKT S473 phosphorylation despite being moderately sensitive to BYL719. This would imply that maintained AKT S473 phosphorylation does not mediate resistance to BYL719 in HPV-positive cell lines.

3.8 HNSCC cell lines are resistant to rapamycin, an mTORC1 inhibitor

All PIK3CA-amplified cell lines showed persistent mTORC1 activity evident by S6 phosphorylation (Figure 8) suggesting that mTORC1 was in fact the major effector to target in these cell lines with PIK3CA gene amplification. Therefore, we sought to determine whether targeting mTORC1 with rapamycin in PIK3CA-amplified, BYL719 resistant cell lines would be effective.

Rapamycin (sirolimus) is a macrocyclic antibiotic first isolated from the bacterium Streptomyces hygroscopicus (30). It is the first known inhibitor of mTORC1, and preceded other mTOR inhibitor compounds such as temsirolimus (CCI779), everolimus (RAD001), and deforolimus (AP23573) (32). Rapamycin functions by binding to a hydrophobic pocket in the FKBP12, a protein folding chaperone for proteins with proline residues (133). This protein-drug complex further binds to the FRB domain of mTOR, thereby inhibiting its activity (103). Rapamycin is only truly effective at
inhibiting mTORC1, as previously alluded to (see Introduction 1.3 PI3K/AKT Pathway) and is an FDA approved cancer therapeutic.

Nine HNSCC cell lines, 93-VU-147T, HMS001, UPCI:090, UPCI:154, FaDu, PCI6A, UM-SCC47, Detroit562, and Cal27, were subjected to increasing doses of rapamycin (Figure 10A). Cell lines were chosen to represent H1047R point mutants, PIK3CA amplifications, and PIK3CA WT clusters (Table 1). I also sought to determine if two of the PIK3CA WT cell lines that were sensitive to BYL719, UM-SCC47 and UPCI:090, were also sensitive to rapamycin treatment. Only one of the cell lines (UPCI:090) reached an IC$_{50}$ at the upper most dosage of 10µM after 72h of treatment (Figure 10A). It can be deduced that for most HNSCC cell lines, singular mTORC1 targeting is ineffective at decreasing the viability of cell lines with PIK3CA insults.

Effective decrease in mTORC1 activity was determined by performing immunoblot analysis of S6 phosphorylation. Three cell lines, Cal27, 93-VU-147T, and FaDu (Figure 10B-D), were chosen and treated with increasing doses of rapamycin. Immunoblot analysis revealed that two cell lines, FaDu and 93-VU-147, both experienced drastic decrease in S6 phosphorylation at 100nM of rapamycin, whereas Cal27 experience considerable reduction at 1nM (Figures 10B-D). It can be inferred then that mTORC1 activity was sufficiently decreased in the cell viability studies in Figure 10A, thereby demonstrating that alone, rapamycin is unable to thwart growth in HNSCC cell lines.

Since all PIK3CA-amplified cell lines were resistant to both separate treatments of BYL719 and rapamycin, despite having a known oncogenic driver mutation and persistent mTORC1 activity, I postulated that combination therapy of both drugs in these cell lines would effectively reduce PI3K/AKT pathway signaling, and therefore reduce cell viability. In order to do this, the half maximal drug concentration of rapamycin had to first be determined. Four HNSCC cell lines, Cal33, FaDu, PCI6A, and 93-VU-147T, were treated with increasing concentrations of rapamycin that were above the maximal dose in Figure 10A. Three of the 4 cell lines responded to rapamycin treatment at a concentration between 30 and 40µM (Figure 11). FaDu responded to treatment above
Figure 10 The effects of rapamycin in a panel of HNSCC cell lines at 72h. A) Nine HNSCC cell lines were treated with increasing doses of rapamycin and assayed for cell viability using the Presto Blue Cell Viability Reagent. B) Cal27, C) 93-VU-147T, D) FaDu, Immunoblots showing knockdown of mTORC1 signaling evident by S6 phosphorylation with increasing doses of rapamycin.
Figure 11 Determination of the effective rapamycin dose for combination studies with BYL719. The half maximal inhibitory concentration (IC$_{50}$) of 4 HNSCC cell lines all harboring $PIK3CA$ alterations (FaDu, PCI6A, Cal33, and 93-VU-147T) to rapamycin treatment was determined by generating growth curves to extrapolate a proper dose to use in synergy with BYL719.
50µM. In order to perform combination therapy, a lower dose of rapamycin that had no effect on HNSCC cell line growth had to be chosen in order to potentially observe a synergistic effect with BYL719. Studies have shown that in order to observe a synergistic effect in combination therapy studies, independent of drug toxicity, a dose considerably below the IC$_{50}$ needs to be used (125-128). Studies using rapamycin specifically have suggested a dose that is 20% the IC$_{50}$ from in vitro cell line testing is sufficient for combination therapy (125,126). Therefore, 6µM of rapamycin was used for combination therapy with BYL719, which was 20% the IC$_{50}$ of 3 of 4 cell lines tested in Figure 10.

3.9 HNSCC cell lines with $PIK3CA$ amplifications that were resistant to BYL719 treatment are sensitive to combination treatment of BYL719 and rapamycin

HNSCC cell lines with $PIK3CA$ amplification did not respond to singular treatment of BYL719 nor rapamycin. BYL719 treatment revealed effective knockdown of AKT phosphorylation in these cell lines, however mTORC1 activity, as determined by S6 phosphorylation, was maintained (Figure 8). mTORC1 initiates many important functions in the cell (31,33,35,36-38), and is therefore a logical candidate for targeted therapy. Targeting of mTORC1 with rapamycin in a panel of HNSCC cell lines was largely ineffective, with the exception of one cell line (UPCI:090), which was also moderately sensitive to BYL719, yet does not contain any known $PIK3CA$, or PI3K/AKT pathway alterations. I sought to determine whether combinatory therapy of HNSCC cell lines with BYL719 and 6µM of rapamycin was more effective (Figure 12).

$PIK3CA$-amplified cell lines 93-VU-147T, SCC15, PCI6A, FaDu, and HMS001, and $PIK3CA$ WT cell lines Cal27, UPCI:154, and JHU006 were treated with increasing doses of BYL719, while maintaining a constant rapamycin concentration of 6µM. Cell viability was measured at 72h (Figures 12) and 96h (data not shown) in order to determine the effects of combinatory drug therapy on BYL719 resistant cell lines. All $PIK3CA$-amplified cell lines responded to combination treatment, which resulted in a BYL719 half maximal inhibitory concentration that was significantly lower than BYL719...
Figure 12 The effects of combination therapy on a panel of HNSCC cell lines at 72h. A) PCI6A, B) FaDu, C) with corresponding immunoblots with BYL719 in combination with 6µM and 30µM rapamycin; D) 93-VU-147T, E) SCC15, F) HMS001, G) Cal27, H) JHU006, and I) UPCI:154. HNSCC cell lines were subjected to combination therapy of BYL719 and 6µM rapamycin and compared to BYL719 alone. Cell viability was determined using PrestoBlue Reagent at 72h. Immunoblots were performed in order to compare decreased signaling (as determined by phosphorylation) of PI3K/AKT pathway between the two treatments. Phospho-AKT (T308, S473), and phospho-S6 (S235/6) were used as markers to determine effectiveness of drug treatment. Statistics were performed using a one-way ANOVA with Tukey’s range test (*denotes P<0.05, and **P<0.001 in order to appreciate the significance of the effect of combination therapy)
Figure 13 Predicted IC$_{50}$ using non-linear regression of PIK3CA amplified HNSCC cell lines after 72h treatment with BYL719 and 6µM rapamycin. Five HNSCC cell lines (FaDu, PCI6A, SCC15, 93-VU-147T, and HMS001) harboring PIK3CA gene amplifications were subjected to combination therapy of BYL719 and 6µM rapamycin for 72h. Cell viability was measured using PrestoBlue Reagent. Non-linear regression was performed to ascribe an IC$_{50}$ to cell lines whose IC$_{50}$ fell outside the dose range of this study. Cell lines with an IC$_{50}$ above 15 were ascribed a value of 15µM for statistical purposes denoted by *. A one-tailed t-test was performed to determine significance. The mean IC$_{50}$ of the BYL719 + 6µM rapamycin treatment was significantly different then the mean IC$_{50}$ of the BYL19 alone treatment (p=0.0002, N=5).
treatment alone (Figure 12). PCI6A and FaDu, both of which were shown to be resistant to BYL719, and not solely oncogenically dependent on \textit{PIK3CA} (Figure 5), were sensitized to BYL719 when used in combination with rapamycin (Figure 12A-C). However, PCI6A, which harbors both an E545K point mutation and gene amplification showed the least dramatic response to combination therapy (~5\(\mu\)M in combination vs. 10\(\mu\)M with BYL719 alone) (Figure 12A,C). The HPV-positive cell line, 93-VU-147T, showed the greatest difference in response, as BYL719 treatment alone yielded an IC\(_{50}\) considerably above the 8\(\mu\)M limit (Figure 12D) (non-linear regression extrapolated IC\(_{50}\) of 40.27\(\mu\)M), whereas combination therapy with rapamycin lowered the IC\(_{50}\) to 0.89\(\mu\)M at 72h (Figure 12D), and >1\(\mu\)M at 96h (data not shown). All other \textit{PIK3CA}-amplified cell lines tested also showed increased sensitivity to BYL719 when used in combination with rapamycin (Figure 12E-G).

Quantification of combination therapy IC\(_{50}\) compared to BYL719 alone IC\(_{50}\) was performed using non-linear regression to ascribe values to responses outside the 10\(\mu\)M study limit (Figure 13). Cell lines that had a half maximal inhibitory concentration above 15\(\mu\)M were ascribed a value of 15 for statistical analysis (denoted by * in Figure 13). The mean IC\(_{50}\) of \textit{PIK3CA}-amplified cell lines after combination therapy (4.37±1.60 \(\mu\)M) was significantly lower (p=0.0002) to the IC\(_{50}\) of the same cell lines under BYL719 treatment alone (11.74±2.05 \(\mu\)M) (Figure 13) or rapamycin alone (Figure 10).

None of the three \textit{PIK3CA} WT cell lines showed a response to combination treatment of BYL719 and 6\(\mu\)M rapamycin, which was not significantly different from BYL719 alone treatment (Figure 12G-I). Statistical analysis revealed no significant difference at any of the doses in either treatment (Figure 12G-I). This would imply these cell lines are not oncogenically dependent on PI3K/AKT signaling for survival. This is in contrast to the \textit{PIK3CA}-amplified lines, which require both mTOR and PIK3CA inhibition, and the \textit{PIK3CA} mutant lines that require solely \textit{PIK3CA} inhibition. These findings provide valuable information in understanding the differential response of \textit{PIK3CA} mutations to BYL719 treatment.
3.10 **PIK3CA** and **SOX2** co-amplification was common in many HNSCC cell lines

siRNA knockdown of PI3K/AKT pathway components demonstrated that **PIK3CA**-amplified cell lines were not oncogenically dependent solely on **PIK3CA** signalling (Figure 5). This is supported by the fact that all **PIK3CA**-amplified cell lines were resistant to singular BYL719 treatment (Figure 4). We sought to query the 3q26 chromosomal region for alternative proto-oncogenes that may explain resistance to BYL719. This region, which includes the **PIK3CA** gene, is frequently amplified in head and neck cancer (6, TCGA, www.cbioportal.org).

A retrospective analysis of the most recent TCGA CNV data from 10 different cancers revealed that HNSCC had the second highest overall rate of **PIK3CA** amplifications, with ovarian cancer having the greatest increase in copy number (Figure 14A). Furthermore, HPV-driven cervical cancer and smoking-induced lung SCC had the third and fourth most **PIK3CA** amplifications (Figure 14A). We next queried the 3q26 amplicon and identified 4 genes that are proto-oncogenic or tumor suppressive, **SOX2**, **TP63**, and **TERC**. A review of these genes and their potential roles in HNSCC can be found in a recent review published by our group (Rizzo et al., Oral Disease 2014). These three genes are proximal to **PIK3CA** (178.87-178.96 Mb), with **SOX2** being most proximal (181.43 Mb), followed by **TP63** (189.35-189.62 Mb) downstream, and **TERC** further upstream (169.48 Mb). The **RAB7A** gene located further upstream (128.44-128.5 Mb) (TCGA, [www.cbioportal.org](http://www.cbioportal.org)) was also queried. All co-amplification values were normalized to the overall rate of **PIK3CA** amplification in each cancer in order to show an overall rate of co-amplification in all tumors. HNSCC showed the second highest rate of **PIK3CA** co-amplification with **SOX2** (20.58%), **SOX2** and **TP63** (18.83%), and **SOX2**, **TP63** and **TERC** (17.74%), and the highest co-amplification rate with **SOX2**, **TP63**, **TERC**, and **RAB7A** albeit at much lower rates (4.59%) (Figure 14B). Ovarian cancers had the highest overall rates of **PIK3CA** co-amplification with three of the four genes (**SOX2**, **TP63**, **TERC**). More importantly, **PIK3CA** amplifications occurred nearly 100% of the time with **SOX2** amplifications, in HNSCC with only one tumor not having the co-amplification event (TCGA, www.cbioportal.org) (Figure 14C).
Figure 14 *PIK3CA* co-amplification in HNSCC. A-C) Retrospective analysis of the TCGA CNV data at the 3q26 locus in 10 different cancers. A) *PIK3CA* amplification rates in 10 different cancers. B) Co-amplification of *PIK3CA* (normalized to *PIK3CA* amplification rates for each cancer) with different genes on the 3q chromosome (*RAB7A, TERC, SOX2, and TP63*) in 10 different cancers. C) Co-amplification of *PIK3CA* with regions of the 3q chromosome in HNSCC. D) Co-amplification of *PIK3CA* and *SOX2* in HNSCC cell lines by real-time-PCR. Red line denotes cut-off for amplification, as normalized to patient germline controls (labeled Germ).
Considering the nearly 100% correlation between PIK3CA and SOX2 co-amplification (Figure 14C), we sought to determine whether this phenomenon occurred in a panel of HNSCC cell lines. Furthermore, SOX2 has been shown to influence PI3K/AKT pathway, particularly mTORC1 activity, in cancer (90) therefore affording us a possible explanation for why PIK3CA-amplified cell lines are resistant to BYL719 treatment. TRAT1 and germline controls were used to control for ploidy. As was the case with the TCGA data, nearly all cell lines with reported PIK3CA amplification also had concomitant SOX2 amplification (Figure 14D). Only two of the cell lines, RF15A and RF15B, showed PIK3CA amplification without concurrent SOX2 amplification. Interestingly, five cell lines previously reported to not have amplifications in PIK3CA, JHU006, JHU029, SCC4, SCC9, and UM-SCC47, increases in copy number above the threshold set by the germline controls (Figure 14D). UM-SCC47 had considerably higher levels of both PIK3CA and SOX2. A logical explanation for this is either a drastic change in ploidy number in this cell line, 3p arm deletion, as has been reported in a portion of HNSCC (7). We are currently characterizing our entire panel of cell lines with Oncoscan copy number array chips to provide definitive copy number data.
4 DISCUSSION

This thesis work did not support our initial hypothesis that HNSCC cell lines with PIK3CA alterations (i.e. point mutation or gene amplification) would be responsive to drug treatment with the alpha specific PI3K inhibitor BYL719. Instead, a preferential response to BYL719 was observed in cell lines and xenografts carrying PIK3CA hotspot point mutations. Cell lines carrying an amplification of the PIK3CA locus, regardless of the mutation status of PIK3CA, were refractory to BYL719 treatment. We found that cell lines and xenografts with PIK3CA amplifications were resistant to BYL719 treatments, with SCC25 being the only PIK3CA-amplified cell line that partially responded to drug treatment within the study range (IC₅₀ ~8µM) (Figure 15). Moreover, the only cell line that harbored both a PIK3CA point mutation and amplification, PCI6A, was resistant to BYL719, with a profile that mimicked other cell lines with PIK3CA amplifications. This is suggestive that resistance to BYL719 is caused by gene amplification rather than the activity of the helical domain mutation.

Point mutations in PIK3CA occur most commonly at three hotspots: transitions (G>A) at both 1781 and 1790 result in non-synonymous substitutions at amino acids 542 (E>K) and 545 (E>K) in the helical domain. Similarly, a transition (A>G) at 3140 results in a non-synonymous substitution at amino acid 1047 (H>R) in the kinase domain (Catalogue of Somatic Mutations in Cancer (COSMIC), cancer.sanger.ac.uk). These mutations account for greater than 90% of all PIK3CA point mutations (COSMIC, cancer.sanger.ac.uk). Helical domain mutations are thought to acquire oncogenic ability by weakening key interactions in the N-terminal SH2 (nSH2) domain of p85 thereby limiting p85’s negative regulatory effect on the PI3K complex (67). Conversely, the H1047R kinase domain mutation occurs at an alpha helix at the end of the activation loop. Interestingly, the region of p85 where both the 542 and 545 amino acids of the p110α subunit interact also comes into contact with p110α’s kinase domain (67). It is therefore possible to hypothesize that helical domain mutations can impact kinase domain function.
Figure 15 Summary of the effects of BYL719 on PIK3CA point mutant, amplified, and WT HNSCC cell lines. Cell lines or xenografts with PIK3CA hotspot point mutations (E545K, H1047R) were sensitive to BYL719 treatment and showed decreased AKT and mTORC1 activity thereby resulting in a decrease in cell viability. PIK3CA WT cell lines (i.e. no hotspot point mutations or gene amplifications) also showed decreased AKT and mTORC1 activity, however no decrease in cell viability was observed. Finally, PIK3CA-amplified cell lines showed persistent mTORC1 activity when treated with BYL719 and therefore no noticeable decrease in cell viability despite decreased phosphorylation of AKT (T308, S473). However combination treatment of BYL719 and rapamycin in PIK3CA-amplified cell lines reduced the activity of both AKT and mTORC1, and therefore showed a decrease in cell viability.
PI3K/AKT pathway activation even at basal levels appears so important to oncogenicity that certain cancer cells will actively maintain a basal homeostatic level of PI3K/AKT signaling, probably due to the large degree of crosstalk it has with adjacent pathways (28,41-43,46-48,51). Therefore it might not be surprising that our results showed that all PIK3CA-amplified, BYL719-resistant cell lines showed persistent mTORC1 activity as evident by the maintenance of phosphorylated S6 (Figure 15). Conversely, this was not observed in PIK3CA-point mutated, BYL719-sensitive cell lines. Interestingly, the JHU029 cell line, which harbors a H1047L mutation, had a higher IC$_{50}$ than all three H1047R mutants. Huang and colleagues have suggested that amino acid substitutions of the 1047 histidine residue shift the hydrogen bonding slightly, altering the conformation of the kinase domain (67). The H1047L mutant however is incapable of forming proper hydrogen bonds due to the substituted leucine, instead only forming weak Van der Waals interactions. This change in kinase domain may explain the difference in JHU029’s response to BYL719 (IC$_{50}$~4µM) as compared to H1047R mutants.

All PIK3CA WT cell lines were non-responsive to BYL719, and showed a detectable reduction in p-AKT (T308, S473) and p-S6 (S235/6). These findings are not surprising as PIK3CA WT cell lines are not oncogenically dependent on PI3K/AKT signaling for their cancerous phenotype, and therefore inhibition of this pathway has no effect on these cell lines (Figure 15). Despite the majority of PIK3CA WT cell lines being resistant to BYL719, we did identify 3 cell lines that showed preferential response to BYL719; JHU011, UM-SCC47, and UPCI:090. The latter two are HPV-positive, and HPV’s use of the PI3K/AKT pathway for constitutive maintenance of viral-protein translation could help explain this sensitivity (31). UM-SCC47 and UPCI:090 have also been shown to be sensitive to radiation in an AKT-dependent manner compared to HPV-negative cell lines (136). JHU011, on the other hand, may harbor a non-hot spot mutation with oncogenic potential (7,29,67), which would explain its response to BYL719.

Immunoblot analysis of HPV-positive HNSCC cell lines after 36h BYL719 treatment revealed maintained AKT S473 phosphorylation, despite decreased phosphorylation at AKT T308 (Figure 9). This could be explained by E7 inhibition of the
AKT phosphatase PP2A causing a negative effect on the turnover of the phosphate at Serine 473 (31,52,53). This explanation seems more plausible than persistent mTORC2 activity because treatment of these cell lines with BYL719 and rapamycin was able to decrease AKT S473 phosphorylation (Figure 12D and F). Furthermore, this maintained AKT S473 phosphorylation did not appear to impact BYL719-response as HPV-positive cell lines tended to cluster according to PIK3CA mutational status (Figure 4A).

Different PIK3CA hotspot point mutations most likely cannot explain BYL719 response in HNSCC cell lines. This is supported by studies by Furet and colleagues that showed BYL719 has nearly equal dissociation propensities (denoted by Kd values) for p110α WT (Kd=5nM), E545K (Kd=4nM), and H1047R (Kd=5nM) (27). All three PIK3CA H1047R mutants (Detroit562, Cal33, HSC2) were sensitive to BYL719 treatment in vitro (Figure 4), with an additional cell line-derived xenograft (Cal33) (Figure 6A), and a PDX harboring a PIK3CA E545K mutation (London26) (Figure 7), both being sensitive to BYL719 treatment as well (Figure 15). Only the PCI6A cell line, which has both a PIK3CA E545K mutation and gene amplification, was resistant to BYL719 treatment suggesting that PIK3CA amplification is the predictive feature of BYL719 resistance. Additionally, sequencing data from our lab has shown that only 20% of the PCI6A cell line DNA contains the PIK3CA E545K mutation implying that the mutation is phylogenically young, and may not constitute a primer oncogenic driver event in this cell line (Nichols et al., unpublished). Additional studies will be needed to determine whether PIK3CA gene amplification and E545K point mutation in PCI6A occur on the same allele.

Few studies have suggested that PIK3CA H1047R mutants have a preferential response to PI3K/AKT/mTOR pathway inhibitors, as compared to the helical domain mutations (131,137). Conversely, and in-line with the data presented, studies with BYL719 in breast cancer cell lines in culture and cell line-derived xenografts have shown that a significant subset of both helical and kinase domain PIK3CA-mutated cell lines and tumors were sensitive to BYL719 treatment with IC_{50} values and tumor responses similar to my study (105). MCF7 and BT483 cell lines, harboring an E545K and E542K PIK3CA mutation respectively, were both highly sensitive to BYL719 at doses below 1µM (105).
with decreased AKT and mTORC1 signaling (105), which was consistent with our data. This trend of BYL719 effectiveness irrespective of mutation type was also observed in other cancers (101,138). Studies by Fritsh and colleagues reported a 64% response rate to BYL719 of PIK3CA mutant cell lines from all cancers (N=22/34), as well as significant reductions in tumor growth in cell line-derived xenografts (101).

The rates of PIK3CA hotspot point mutations in patient oropharyngeal tumors from the LRCP tumorbank correlated with the rates reported in the current literature (6,9,10,11,TCGA www.cbioportal.org). HPV-positive oropharyngeal tumors also had significantly higher rates of PIK3CA hotspot point mutation as compared to HPV-negative tumors. PIK3CA point mutations in non-oropharyngeal tumors were far less common than in oropharyngeal tumors, which were also expected (Table 6). The heavy dependency of HPV on PI3K/AKT signaling in order to carry out infection and basic viral function is suggestive of a commentialistic relationship between the virus and the pathway (31,52,53). It has also been reported that the E5 viral protein upregulates and maintains the expression of the epidermal growth factor receptor (EGFR), which in turn activates PI3K/AKT signaling (139).

PIK3CA gene amplification occurred in a major subset of both HPV-positive and HPV-negative oropharyngeal tumors. These findings match previous findings reported by several groups in which a high incidence of PIK3CA amplifications occurred across both types of HNSCC (6,9,10,11,TCGA www.cbioportal.org). Moreover, most of these studies also remarked on the propensity of dual PIK3CA point mutation and amplification in the same tumor, albeit at lower rates than our study (9,TCGA www.cbioportal.org). Indeed, our rates of PIK3CA amplification were higher than reported in recently published sequencing papers, with HPV-positive HNSCC tumors harboring between 25-30% amplification, and HPV-negative tumors slightly lower (20%) (7,TCGA www.cbioportal.org). This could be explained by our relatively low cut off ∆∆Ct value of 1 (normalized to patient germline controls), where all tumors with a ∆∆Ct above 1 were categorized as PIK3CA-amplified. Studies have defined one extra gene copy as an amplification event (140, TCGA www.cbioportal.org), yet the significance of one extra gene copy is debatable as an oncogenic driver event. Multiple studies however have
shown that absolute, rather than relative, increase in \textit{PIK3CA} gene copy correlated with increased expression and therefore AKT phosphorylation (129,131,141,142). Conversely, expression of the \textit{TP63} locus found upstream of \textit{PIK3CA} on the 3q chromosomal arm was more so dictated by relative copy number changes (130). Future studies should seek to understand the effects of relative and absolute \textit{PIK3CA} copy number gains, and assess the impact on oncogenicity.

The impacts of \textit{PIK3CA} amplification or point mutation, while both being able to drive oncogenesis (22-25,68), in HPV-positive and negative HNSCC are likely different. In HPV-negative HNSCC, carcinogenic insult introduces many different chromosomal abnormalities (6,12) implying that \textit{PIK3CA} alterations can occur just as commonly as any other gene mutations or amplifications. This could explain why \textit{PIK3CA} point mutations and amplification rates do not stand out from other mutations or amplifications in HPV-negative HNSCC (TCGA). Conversely, PI3K/AKT pathway activation is strongly tied to efficient HPV function (31,52,53). The acquisition of high viral E6 and E7 expression would drive the cell into S phase without appropriate checkpoint controls, thereby permitting HPV genome replication (143). Moreover, HPV genome fragments expressing E6 and E7 can insert into the host’s genome, and indirectly facilitate the amplification of proximal genes (56-58,82). However, E6 and E7 insertion seems to occur at random (144), therefore viral-directed targeting and subsequent amplification of the \textit{PIK3CA} gene seems unlikely. Nonetheless, amplifications and point mutations in \textit{PIK3CA} could be beneficial to the fitness of HPV in that it dampens stress response, promotes viral protein translation, and suppresses apoptosis (31). This relationship is further supported by the high propensity of 3q24-29 chromosomal amplifications and \textit{PIK3CA} mutations in cervical cancers (145,TCGA www.cbioportal.org). It is important to note however that the purpose of HPV is not to cause cancer; the E6 and E7 viral oncoproteins accelerate the differentiation of squamous cells so that infectious HPV-progeny may be released at the surface (31,143,146,147). This is supported by the fact E6 and E7 can immortalize keratinocytes (146), but cannot cause oncogenic transformation on there own and require additional oncogenes (147). It is the oncogenic nature of \textit{PIK3CA}, rather than the presence of HPV, that leads to oncogenic transformation (143). It is possible then to see an increased reproductive fitness, and therefore a selection bias, for HPVs that have
infected cells with acquired \textit{PIK3CA} mutations. Given that \textit{PIK3CA} amplifications and point mutations occur at roughly equal rates in HPV-positive HNSCC (6,7,9-11,TCGA www.cbioportal.org), it can be assumed that both types of mutations are sufficient in driving oncogenesis, and offer an explanation as to why there is an apparent close tie between HPV and \textit{PIK3CA} mutation rates.

The observation that all \textit{PIK3CA}-amplified cell lines and xenograft were resistant to BYL719 went against our initial hypothesis and remains a topic of debate. Most studies across different cancers appear to agree that \textit{PIK3CA} amplification is a marker for treatment resistance and poor patient outcomes (81,106,148-150). Yet, some studies have shown the opposite (101,129,151). Interestingly, studies by Fritsch and colleagues demonstrated in cell lines and cell line-derived xenografts that \textit{PIK3CA}-amplified models were sensitive to BYL719 treatment, however only a few HNSCC cell lines were used in that study (101). Instead our results demonstrated in a panel of 29 cell lines, all 11 with \textit{PIK3CA} amplifications and in one cell line-derived xenograft, that amplification predicted resistance to BYL719. Resistant cell lines that were HPV-negative but still possessed \textit{PIK3CA} amplifications showed significant reduction of AKT phosphorylation at threonine 308 and serine 473 after treatment with BYL719. This decrease in phospho-AKT (T308, S473) mirrored the response of BYL719-sensitive \textit{PIK3CA} mutant cell lines.

Mechanistically, amplification-driven resistance likely occurs due to insufficient drug targeting of the increased numbers of \textit{PIK3CA} gene product, with the amount of drug acting as the limiting factor (148). This principle would agree most convincingly with our findings, based on the fact that the 93-VU-147T cell line, as determined by our group, has the highest number of \textit{PIK3CA} gene copies and therefore correspondingly, the highest predicted IC\textsubscript{50} for BYL719 of 40.27\(\mu\)M. Since the affinity of BYL719 for both point mutant and WT p110\(\alpha\) is identical, it is unlikely that resistance is explained by insufficient drug binding to the WT form (67), as is the case with certain MEK1 inhibitors (107). Singular targeting of the two key nodes of the PI3K/AKT axis, i.e. AKT and mTOR, initiates feedback mechanisms whereby RTKs are re-cycled to the plasma membrane and PI3K and/or ERK activity is reinstated (103,104,107,108,152,153). In the
context of this study, singular mTORC1 inhibition results in receptor recycling, independent of Forkhead box (FOXO) proteins, thereby reactivating PI3K, ERK, and AKT signaling (103,104). Mitogen-activated protein kinase (MAPK) signaling (RAS-RAF-MEK-ERK pathway) moreover is activated by the S6 kinase (S6K)-PI3K-RAS feedback loop when faced with mTORC1 inhibition in order to maintain oncogenic processes (104). This might provide a means as to why rapamycin inhibition in HNSCC cell lines was largely ineffective. Furthermore, it stresses the importance and validity of complete and selective PI3K\(\alpha\) inhibition in PIK3CA-altered tumors; not only is it the driving force behind the oncogenic phenotype (22-25,68), but it is also the primary node of the feedback mechanisms induced by AKT and mTORC1 (104,130).

Amplification as a means for acquired resistance and therefore poor prognosis has been well documented. Two independent studies have shown that acquired \(BRAF\) or \(KRAS\) amplifications in colorectal cancer cells with previous \(BRAF\) mutations become resistant to MEK1/2 inhibition (107,109). Moreover, acquired \(PIK3CA\) amplification has also been shown to mediate a resistant phenotype in breast cancers. Specifically, acquired \textit{de novo} \(PIK3CA\) amplification in the H1047R-mutated KPL-4PR cell line are resistance to the PI3K inhibitor GDC-0941 (77). While we did not look at novel amplification events in this study, we did observe treatment resistance in the only cell line that had both a \(PIK3CA\) point mutation and gene amplification, PCI6A (Figure 4A). When considering amplification as a singular means for treatment resistance, Turke and colleagues showed that MNNG Hos Transforming gene (\textit{MET}) amplification, a hepatocyte growth factor receptor that functions upstream of ERBB3/PI3K/AKT signaling, conferred resistance to EGFR inhibition in non-small cell lung carcinomas (106). Moreover, and consistent with amplification as a means for poor prognosis, Suda and colleagues identified copy number alterations in \(PIK3CA\) as a poor prognostic factor in non-lymph node metastatic HNSCC (149).

In order to explain amplification-mediated resistance, an analysis of possible secondary mutations in two of the more proximal proteins to PI3K\(\alpha\), PTEN and RAS, was performed based on their direct involvement in PI3K\(\alpha\) function and their roles in driving PI3K/AKT signaling. Previous studies have implicated \textit{PTEN} loss as a
mechanism for resistance to PI3K inhibition (42,101), with more recent work reporting that PTEN loss was present at secondary sites refractory to BYL719 treatment in PIK3CA-driven breast tumors (42). Moreover RAS mutations, specifically HRAS mutations, occur in 5% of HNSCC tumors (6,9-11,TCGA www.cbioportal.org) and have strong influence on PI3Kα, particularly with helical domain mutations (69). A strong interdependency between the two pathways for effective drug treatment has also been reported, as PIK3CA mutation or PTEN loss mediate resistance to MEK inhibitors (154), and activating mutations in RAS significantly reduce the efficacy of PI3K inhibitors (70,103). HRAS mutations have also been shown to mediate resistance to EGFR tyrosine kinase inhibitors (155). Unfortunately none of the cell lines tested showed concomitant PIK3CA amplification or mutation with either PTEN loss or HRAS mutations (data not shown) and this therefore cannot explain the discrepancy in our results. The three cell lines that did have HRAS mutations, RF22B, 37A, and 37B, unsurprisingly were resistant to BYL719 treatment. As with PTEN loss, RAS mutations in secondary tumor sites refractory to PI3K inhibition could function as a means for resistance, as a positive association has been reported between PIK3CA and RAS (NRAS, KRAS, HRAS) mutations in treatment refractory tumors across multiple cancers (137).

The large degree of crosstalk between the PI3K/AKT and MAPK pathway has been alluded to in this report in terms of the interdependency required for effective drug treatment (103,154). One study showed that in pituitary gland tumors, PIK3CA amplification and RAS mutation rarely occurred together, suggesting an overlapping oncogenic role (156). Additionally, another study showed that PI3Kα inhibition in PIK3CA-mutated, HER2-amplified breast cancer cell lines resulted in a RAS-independent decrease in RAC1/p21-activated kinase (PAK)/protein kinase-C Raf (C-RAF)/MEK/ERK signaling (157). Our results clearly show potent maintenance of mTORC1 signaling in BYL719-resistant cell lines in the absence of AKT phosphorylation. Moreover, the significant decrease in IC₅₀ observed when BYL719-resistant cell lines are treated in combination with BYL719 and rapamycin implies that resistance is mediated through maintenance of PI3K/AKT pathway integrity and is separate from p-AKT activity (Figure 15). It was initially postulated that pathway signaling integrity was maintained through activation of the RAS/RAF/MEK/ERK pathway. Activation of this axis can be
achieved, much like PI3K/AKT pathway, by growth factor binding to RTKs, which leads eventually to phosphorylation and inactivation of the TSC1/2 complex and activation of mTORC1 by RHEB-GTP (33,41). Moreover as has been previously discussed, RTK recycling also occurs with both AKT and mTORC1 inhibition in FOXO-dependent and independent manners, respectively (33,103,158). Instead, a study conducted by our lab revealed combination therapy of BYL719 and trametinib, a potent MEK1/2 inhibitor, did not have an observable affect on \textit{PIK3CA} amplified cell lines (Nichols et al, unpublished). Moreover, pathway integrity in HNSCC cell lines was not maintained by PI3Kβ activity based on no changes in cell viability observed when treated with the pan-PI3K inhibitor PX-866 (Nichols et al., unpublished).

An alternative means of explaining sustained mTORC1 activity during BYL719 treatment is by AKT-independent mechanisms of mTORC1 activation. mTORC1 activity is closely tied to amino acid availability and does not require AKT input (159). Inhibition of glucose transport by BYL719 occurs in a two-fold fashion: insulin receptor (IR)-insulin receptor substrate-1 (IRS1) signal transduction, as well as FOXO-dependent recycling of IR-IRS1 signal transduction, both dependent on PI3Ka, are inhibited by BYL719 (103,104). This is evident by abrogation of AKT phosphorylation. The inability to shuttle glucose into the cell results in a low AMP: ATP ratio, sensed by 5’ AMP-activated protein kinase (AMPK), which inhibits mTORC1 function by phosphorylating and activating the GAP activity of the TSC1/2 complex (33). A primary function of mTOR proteins is to integrate signals from nutrient and energy sensing with cell proliferation (158). Amino acids can be used as fuel by the cell (160), which is especially important for cancer cells with an intrinsically high metabolic rate (161). The RAS-related GTPase (Rag)-family maintains mTORC1 signaling through Raptor interaction in an amino acid-dependent manner, and thus affords the cell the ability to undergo cell proliferation processes (33,159). Despite explaining an alternative means for mTORC1 activation, this model is incomplete because it fails to explain the relationship between Rag proteins and different \textit{PIK3CA} mutations, especially when considering that \textit{PIK3CA} amplification and point mutations exert different modes of action. Moreover, the TCGA reports only a small percentage of tumors that have both Rag and \textit{PIK3CA} co-
mutation/amplification events, suggesting that a Rag mutation across all PIK3CA amplifications is unlikely to explain resistance (TCGA www.cbioportal.org).

A second mean of AKT-independent mTORC1 activation for explaining resistance in PIK3CA-amplified models is through PI3K activation of PDK1 activity. Mutations in the PI3K/AKT pathway in HNSCC, outside of PIK3CA and PTEN are rare and therefore do not provide an explanation for our results (6,9-11,TCGA www.cbioportal.org). Canonical activation of AKT phosphorylation, particularly AKT1, by PI3Kα activation alleviates TSC1/2 GAP-activity on RHEB, which activates mTORC1 and drives oncogenic functions (31,32,162). One method for oncogenic PI3Kα signaling independent of AKT activation is through PI3Kα activation of PDK1 (163). Sequestering of PDK1 to the plasma membrane independent of AKT in response to lower levels of phosphatidylinositol accumulation, or RTK stabilization leads to the activation of serum/glucocorticoid-regulated kinase 3 (SGK3) (33,163). The SGK family of proteins shares a high degree of functional and structural similarity to AKT, with growing roles in normal and cancer cell signaling (164). PDK1-phosphorylated SGK3 is recruited to endosomes in an inter-dependent manner by the nutrient signaling, class III PI3K human vacuolar sorting protein 34 (hVps34) (164). hVps34 generates phosphatidylinositol 3-phosphate (PI(3)P), which localizes phospho-SGK3 through a N-terminal phox homology (PX) domain (165) and is required for full activation of SGK3 (166). hVps34 may in turn be modulated by SGK3 (164). Most importantly, hVsp34 activates mTORC1 signaling, and complete loss of hVsp34 blocks insulin-dependent phosphorylation of S6K and eIF4F activation (167). The proposed phospho-SGK3-hVps34 relationship could promote mTORC1-dependent growth through activation of improper nutrient sensing (164). Moreover, SGK3 loss, rather than AKT, has been shown to contribute significantly to cancer phenotype regression in breast cancer (168), ovarian endometrioid adenocarcinoma (169), and prostate cancer studies (170). In terms of this study, incomplete knockdown of PIK3CA-amplified gene products, while sufficient to decrease AKT signaling, may activate mTORC1 signaling in an SGK3-dependent manner. Further studies are needed to elucidate the precise overlap of AKT and SGK family proteins in cancer, as well as the precise roles, if any, of SGK3 activation on mTORC1. It would also be valuable to look at the effects of singular AKT and SGK3, as well as combination
knockdown of these components with BYL719 treatment in order to carefully analyze whether resistance is mediated through this mechanism.

The final proposed explanation of PIK3CA amplification as a means for BYL719 resistance is through the co-amplification event of other PI3K/AKT pathway activating or associated proto-oncogenes within or near the 3q26 amplicon. Retrospective analysis of the TCGA data revealed that head and neck, and for that matter, HPV-associated tumors, i.e. head and neck and cervical, show the highest prevalence of PIK3CA amplification, as well as amplification of the proximal proto-oncogenes SOX2, and TERC, and the TP63 tumor suppressor gene (10,155,TCGA www.cbioportal.org). Additionally, the proto-oncogene RAB7A, located just distal to the aforementioned region, at the 3q28 locus, also showed high levels of co-amplification in these cancers, yet at lower rates than SOX2, TERC, or TP63 (Figure 14). While site-directed E6 and E7 genome integration at this locus is unlikely (144), the advantage of PI3K/AKT pathway activation, in this case by PIK3CA amplification, to HPV function has already been discussed; this observation suggests that either HPV cancers frequently require activation of the PI3K/AKT signaling to progress to malignancy, or that an HPV-related mechanism leads to genetic instability in this gene (8,32,33,41). The presence of PIK3CA amplification and also 3q amplification in early malignant lesions has been shown to promote progression to malignancy in cervical (83) and head and neck cancers (84).

Of the candidate genes within the 3q26 amplicon, SOX2 is of particular interest because of its potential impact on PI3K/AKT pathway activity (90). The other candidate oncogenes or tumor suppressors, while also found within or distal to the 3q26 amplicon, do not appear to have a direct effect on PI3K/AKT signaling and therefore are outside the scope of this study. A detailed analysis of these genes can be found in a recent review published by our group (Rizzo et al., Oral Disease 2014). MYC amplification, found at the 8q24 chromosomal region (171), is also appealing, but does not afford an explanation for the differences in BYL719 response observed between PIK3CA-amplified and mutated cell lines and xenografts. This is primarily because amplifications of the 3q26 and 8q24 regions are likely independent events (171), demonstrated by the poor overlap of tumors with PIK3CA and MYC amplification in HNSCC (TCGA,
Moreover, MYC amplification is equally as common in PIK3CA-point mutant samples and therefore it would be assumed to drive resistance in these models as well (TCGA, www.cbioportal.org).

Multiple studies have suggested that it is not PIK3CA amplification, but SOX2 amplification that is the driver event in 3q amplification (85, 173). This is reinforced by our findings that two PIK3CA-amplified cells (FaDu and PCI6A) were resistant to siRNA knockdown of PIK3CA (Figure 5), which would imply that PIK3CA is not the sole oncogenic driver in these cell lines. We also conducted a preliminary analysis of PIK3CA-SOX2 co-amplification in HNSCC cell lines and found that most cell lines harbored both CNV events with nearly 100% correlation (Figure 14). Additionally, SOX2 expression, irrespective of amplification status, appears to be connected to oncogenic processes, as one group confirmed high expression levels of SOX2 independent of gene amplification in smoking-induced malignancies of the esophagus and lung (85). SOX2 encodes a transcription factor that maintains pluripotency in undifferentiated stem cells, which is suggestive of an important role in EMT in early metastatic events (85,87,174). This is supported by the fact that patients with 3q amplification have a worse prognosis than patients with normal diploid number of the 3q arm (83,84). Furthermore, SOX2 has been implicated as a lineage-survival oncogene in lung and esophageal cancers, meaning that upon dysregulation, its function in lineage development affords the cell tumorogenic potential (174).

SOX2 amplification alone however cannot cause cells to transform, suggesting that multiple cooperating genes are required (85). PIK3CA, on the other hand, can sufficiently facilitate and sustain malignant transformation (22-25,68). In lung SCC, co-amplification of SOX2 and the FGFR2 gene, which functions upstream of PI3Kα signaling, can drive the induction of malignancy (85). This demonstrates a potential reliance of SOX2 on PI3K/AKT signaling for oncogenesis. Multiple studies have shown that SOX2-binding to the mTOR promoter can impact pluripotency (175), but more importantly, global mTOR function (176). Specifically, Corominas-Faja and colleagues demonstrated that the overexpression of SOX2 in MCF-7 cells favorably impacted mTOR and mTOR-associated gene expression. Three notable mTOR-inhibitor genes
experienced a negative 3-fold change in expression: PRKAA1, which encodes AMPK, DEPTOR, which encodes the mTORC1 inhibitor Deptor, and DDIT4, which is a stress response gene (176). Moreover, the insulin receptor, upstream of PI3K/AKT signaling, was upregulated by 3-fold, while the expression level of S6K1 was also increased with overexpression of SOX2 (176). This provides a rational explanation for potent mTORC1 activity after PI3Kα inhibition in cells with PIK3CA and SOX2 co-amplification. Moreover, one study showed that the only way to suppress the effects of overexpressed SOX2 in esophageal cancer cell lines was the complete inhibition of the PI3K/AKT signaling axis (90), as was the case with our study. Based on our results, and the known evidence for SOX2-PI3K/AKT pathway interplay, further studies should analyze the role of SOX2 on the PI3K/AKT pathway in terms of gene expression and pathway activation. Moreover, it would also be valuable to study the potential, oncogenic additive effects SOX2 overexpression affords to non-cancerous cell lines. Finally, a retrospective study of the patient data looking at treatment failure, staging, and progression to metastasis in relation to SOX2 status in these tumors would provide valuable inference as to the effects of SOX2 on HNSCC cancer progression.

Future directions should seek to explain what secondary factors maintain mTORC1 activity during BYL719 treatment in PIK3CA-amplified cell lines. A simple explanation may be that higher concentrations of BYL719 are needed to sufficiently inhibit all active PI3Kα, however this model seems simplistic. The evidence for SGK3 activation is appealing, although future studies are needed to clearly understand the interplay, if any, between phospho-SGK3 and hVps34, and their combined effects on mTOR-nutrient sensing. As the intracellular interactome continues to grow, additional PI3K/AKT pathway relationships will be elucidated, and should merit individual attention due to high degrees of redundancy in signaling. Unfortunately from studies in our lab, resistance was not mediated through the RAS/RAF/MEK/ERK pathway despite significant interplay between the two axes, yet this will probably not be absolute for all pathways and all small molecule inhibitors. Finally, it is our primary hypothesis that co-amplification of SOX2 is a key mediator of BYL719 resistance. Future studies should seek to directly understand the effects of SOX2 on mTOR and mTOR-associated genes in HNSCC. Moreover, our lab is currently studying the effects of SOX2 overexpression on
BYL719 resistance by using RNAi-knockdown of SOX2 in combination with BYL719 (Nichols et al., unpublished). If a mutual interdependency between SOX2 and PI3K/AKT signaling in HNSCC can be established, then future studies can aim towards firmly teasing the precise mechanism.

As is the case with any study that uses cell lines, results are inferred from model systems that are divergent from the actual tumor biology. Cell lines undergo in vitro growth selection introducing changes in ploidy number, chromosomal rearrangements, point mutations and amplifications, while requiring an immortalization step in order to propagate and persist for many passages. Additionally, tissue culture systems are devoid of the tumor microenvironment as well as an active immune system, both of which significantly impact tumor activity in patients. Moreover, all HPV-positive HNSCC cell lines used in this study were uncharacteristic of regular HPV-positive patients (5,13,14): all were from tobacco smokers and/or habitual drinkers, most presented with advanced (Grade 3) disease, and/or were immortalized from recurrent tumors (177-180).

In order to circumvent the limitations with in vitro work, we generated cell line and patient-dervied xenografts in NSG mice. Despite affording the study an understanding of drug response with an intact tissue microenvironment and whether BYL719 can be effectively delivered to impact tumor growth, the use of immunocompromised mice does not allow us to study the effects with an intact immune system. Due to the difficulty in establishing patient tumors in immunocompromised mice, at the time of writing, we have not had the chance to compare the effects of BYL719 in a NSG mouse population harboring PIK3CA-WT, or PIK3CA-amplified patient tumors. The advantage of PDX over cell line-derived xenografts is that they are representative of actual patient malignancies.

Finally, when analyzing real-time PCR amplification data, a clear definition of what constituted an oncogenic amplification in the PIK3CA gene was difficult to establish. The TCGA classifies all cell lines with one extra copy of PIK3CA as being amplified; therefore we decided that all cell lines or patient samples with a ΔΔCt<1 would be called amplified as well. The FaDu and Cal27 cell lines were used as positive
and negative controls, however a more appropriate control for normalization in cell line studies would have been any diploid cell line rather than the germline, patient controls we used. Future studies should seek to understand the significance of one to multiple extra gene copies on oncogenic transformation

The advent of NGS has revolutionized the way we look at cancer biology, and cancer treatment. Specifically in head and neck cancer, it has elucidated the key underlying differences between HPV-positive and HPV-negative disease states, while providing a detailed summary of which mutations are present at high percentages, and which ones are amenable to targeting. As has been previously discussed, \textit{PIK3CA} is the only directly, drug targetable oncogene with much greater frequencies in HPV-positive disease. As such, this study aimed to first identify a target for small molecule inhibition, followed by the elucidation of biomarkers and mechanisms of resistance and sensitivity. As has been outlined in this discussion, cell lines with \textit{PIK3CA} H1047R mutations are exquisitely sensitive to BYL719. Conversely however, cell lines with \textit{PIK3CA} amplification or co-mutation and amplification are resistant. This was further demonstrated in cell line-derived, and patient-derived HNSCC xenografts, confirming the effectiveness of BYL719. Through this study, I was able to demonstrate that all resistant cell lines had some degree of mTORC1 activity when treated with BYL719. Additionally, most HPV-positive cell lines maintained AKT phosphorylation at Serine 473 during BYL719 treatment as well. More importantly however, and irrespective of HPV-status, combination therapy specific for the oncogenic \textit{PIK3CA}, and the effector of resistance mTORC1, managed to reverse this resistance and reduce cell viability. The use of combination therapy probably has a greater effect over dual-PI3K-mTOR inhibitor because each drug is able to inhibit one specific component thereby ensuring maximal downregulation of target activity. The significance of this research should translate directly into clinical practice, where I predict that only patients with \textit{PIK3CA} mutant, and not \textit{PIK3CA} amplified tumors, should receive BYL719 treatment. Moreover, a combination therapy of BYL719 and an inhibitor of mTORC1 (mTORC2 impact merits further attention as well) would instead be predicted to be most effective for those patients with \textit{PIK3CA} amplifications. The need for pre-clinical research studies of drug effectiveness is vital, with the ultimate goal to predict how tumors will respond.
However, when cancer cells prove to be inexplicably irresponsive, such as I found in my studies, or become refractory to drug treatment this provides researchers an opportunity to understand the underlying mechanisms of resistance. It is these studies that may lead to appropriate clinically useful counter measures based on combination therapy.
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  Podium Presentation

  Abstract: *Targeting PIK3CA in head and neck cancer with BYL719, an alpha specific PI3K inhibitor*
  Poster Presentation

  Abstract: *Targeting PIK3CA in head and neck cancer*
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  Abstract: *Targeting PIK3CA in head and neck cancer*
  Poster Presentation

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