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Short running title

PI3K/Akt and EGFR in head and neck cancer

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Abstract

Resistance to therapies targeting the epidermal growth factor receptor (EGFR), such as cetuximab, remains a major roadblock in the search for effective therapeutic strategies in head and neck squamous cell carcinoma (HNSCC). Due to its close interaction with the EGFR pathway, redundant or compensatory activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been proposed as a major driver of resistance to EGFR inhibitors. Understanding the role of each of the main proteins involved in this pathway is utterly important in order to develop rational combination strategies able to circumvent resistance. Therefore, the current work reviewed the role of PI3K/Akt pathway proteins, including Ras, PI3K, tumor suppressor phosphatase and tensing homolog, Akt and mammalian target of rapamycin in resistance to anti-EGFR treatment in HNSCC. In addition, we summarize PI3K/Akt pathway inhibitors that are currently under (pre)clinical investigation with focus on overcoming resistance to EGFR inhibitors. In conclusion, genomic alterations in and/or overexpression of one or more of these proteins are common in both human papillomavirus (HPV)-positive and HPV-negative

HNSCC tumors. Therefore, downstream effectors of the PI3K/Akt pathway serve as promising drug targets in the search for novel therapeutic strategies that are able to overcome resistance to anti-EGFR treatment. Co-targeting EGFR and the PI3K/Akt pathway can lead to synergistic drug interactions, possibly restoring sensitivity to EGFR inhibitors and hereby improving clinical efficacy. Better understanding of the predictive value of PI3K/Akt pathway alterations is needed to allow the identification of patient populations that might benefit most from these combination strategies.

Keywords

HNSCC, therapeutic resistance, targeted therapy, cetuximab, PI3K/Akt pathway inhibitors, combination therapy

1. Introduction

Head and neck cancer is the collective term for a heterogenous group of epithelial malignancies primarily originating in the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx.[1] The vast majority (>90%) of these head and neck cancers originate from the uncontrolled growth of cells with squamous histology and can therefore be referred to as head and neck squamous cell carcinomas (HNSCC).[2] Worldwide, HNSCC is the sixth most common cancer type with roughly over 800,000 new individuals diagnosed each year, and this number is expected to rise even more over the next decade.[3] Tobacco smoking and alcohol consumption have been identified as major causative factors, as substantial exposure can lead to the accumulation of multiple independent genetic alterations, drastically increasing the risk to develop HNSCC.[4, 5] Historically, about 70-75% of HNSCC cases have been attributed to smoking and alcohol.[6, 7] However, the human papillomavirus (HPV) has been acknowledged as another major risk factor of an increasing proportion of HNSCC (overall prevalence of 36%).[8, 9] In this regard, oral and oropharyngeal HPV infections have been shown to promote oropharyngeal HNSCC.[10] Over the years, it has become clear that HPV-positive malignancies represent a biologically distinct entity with a significant different pathogenesis and better prognosis compared to HPV-negative malignancies.[10-13]

Despite this increasing knowledge on the molecular characteristics of HNSCC, the 5-year survival remains relatively low, especially in the HPV-negative cohort (48% in HPV-negative and 80% in HPV-positive HNSCC).[14-16] This is due to the limited response rates (RRs) with the current treatment options, which are often associated with serious side effects.[17-19] Therefore, it is becoming more important to further unravel the molecular carcinogenesis of HNSCC. This can elucidate the genetic and biological heterogeneity of the disease as well as the importance of inter-individual variation in the human

genome for therapy selection.[20] Eventually, this will lead to the development of novel innovative and personalized therapeutic strategies.

In this context, the epidermal growth factor receptor (EGFR) has been shown to become overexpressed in approximately 90-95% of all HNSCCs[21], which is associated with advanced disease and reduced survival.[22, 23] This observation led to particular interest in the EGFR as a therapeutic target in both the laboratory and clinical settings. As such, monoclonal antibodies (e.g. cetuximab and panitumumab) as well as small-molecule tyrosine kinase inhibitors (e.g. erlotinib and gefitinib) targeting EGFR have been studied thoroughly over the past decades.[24-27] This resulted in the clinical implementation of one of the first successful targeted therapies, i.e. the EGFR-specific antibody cetuximab – either as monotherapy or in combination with conventional therapies, such as radiotherapy or chemotherapy.[17, 18] However, despite the enhanced EGFR expression in the majority of HNSCC tumors and initial promising results, therapeutic resistance remains a major roadblock in the search to effective HNSCC therapies. Indeed, only a small subset of HNSCC patients benefit from cetuximab as a single agent (<15% in patients who failed platinum-based therapies)[28] or combined with chemotherapy in the first-line recurrent/metastatic disease setting (36%)[17], as patients are often intrinsically resistant or become resistant (acquired resistance) after prolonged treatment.[29] Currently, treatment options are limited, especially for HNSCC patients who exhibit resistance to anti-EGFR therapies. Therefore, it is of great importance to unravel the molecular mechanisms underlying resistance to EGFR inhibitors, as this may lead to the establishment of new innovative therapeutic strategies that are able to overcome resistance and/or provide new biomarkers that can be used to predict the therapeutic response to EGFR blockade.[30] In this regard, increasing evidence suggests that aberrant signaling of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in resistance to EGFR-targeted therapies. In the following paragraphs, we will provide an overview of the PI3K/Akt pathway as a compensatory mechanism for resistance to EGFR-targeting agents and present preclinical and clinical findings of PI3K/Akt pathway inhibition, with focus on overcoming resistance to EGFR inhibitors. The majority of the reviews discussing the use of PI3K/Akt pathway inhibitors for the treatment of HNSCC are focusing on monotherapies or combinations of these agents with radiotherapy or chemotherapy.[31-33] The current review is the first to focus entirely on (i) the interaction of EGFR and PI3K/Akt; and (ii) overcoming resistance to EGFR-inhibitors by combining EGFR and PI3K/Akt inhibitors.

2. EGFR in relation to the PI3K/Akt pathway

EGFR (HER1 or ErbB1) is a ubiquitously expressed transmembrane protein and the prototype member of the HER or ErbB tyrosine kinase family. The receptor can be activated by binding of different polypeptide ligands, such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF) and epiregulin, to the extracellular

domain.[34] Ligand binding to EGFR leads to receptor homo- or hetero-dimerization, which triggers intrinsic tyrosine kinase activity in the C-terminal domain. This eventually leads to a downstream phosphorylation and activation cascade, resulting in a wide range of cellular responses, such as proliferation, invasion, adhesion, angiogenesis and survival.[35-39] Downstream effector molecules of these signaling pathways are potentially involved in the development of resistance to drugs targeting EGFR signaling.[40, 41] One of the pathways is the kirsten rat sarcoma viral oncogene homolog (Ras)/Raf/mitogen-activated protein kinase (MAPK) pathway, which is EGFR's best-characterized downstream pathway and an essential route in the regulation of cell survival and proliferation. Ras activation by EGFR leads to the recruitment and activation of the protein kinase Raf that, through intermediate steps, phosphorylates MAPK-1 and -2.[42, 43] Activated MAPKs exert their effect in the nucleus where they phosphorylate and regulate specific transcription factors, such as Elk1 and c-Myc, leading to altered gene expression.[44-47]

However, physiological or oncogenic activation of Ras does not only stimulate the Raf/MAPK pathway, it can also directly activate the PI3K/Akt pathway. The latter is involved in various biological processes essential for normal cellular functionality, including survival, proliferation, differentiation, angiogenesis, protein synthesis and glucose metabolism[48]. Besides these physiological functions, the PI3K/Akt pathway is also associated with a number of oncogenic processes and is one of the most frequently dysregulated pathways in cancer, including HNSCC.[49, 50] As such, aberrant signaling can lead to the stimulation of cell growth, inhibition of cell death and the promotion of invasion and migration[51-53], which is all favoring cancer cells.

PI3K can be activated by Ras and is composed of a regulatory p85 and a catalytic p110 subunit.[54] The regulatory p85 subunit binds and integrates signals from a wide range of transmembrane and intracellular proteins, leading to a conformational modification that activates the p110 subunit.[55, 56] Additionally, the p110 subunit can also be directly activated by activated Ras[57], highlighting the close interaction between EGFR stimulation and PI3K downstream signaling. Upon activation, PI3K catalyzes the phosphorylation of phosphatidylinositol (4,5)-biphosphate (PIP_2) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP_3). Successively, PIP_3 acts as a docking site for the pleckstrin-homology domain of Akt, leading to a non-activating conformational change, and thereby exposing two phosphorylation sites. These specific sites must be phosphorylated as well by their activators, e.g. phosphoinositide-dependent kinase (PDK) 1 and 2, in order to completely activate Akt.[58-60]

There are three isoforms of Akt that are closely related to each other, i.e. Akt1, Akt2 and Akt3.[61] Activation of Akt leads to the phosphorylation of a variety of (isoform-specific and/or -non-specific) downstream substrates, such as mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3), that affect cell growth, cell cycle distribution and survival.[62-64] More specifically, Akt inhibits tuberous sclerosis complexes 1 and 2 (TSC1/2) through phosphorylation[65], which releases the

inhibition on Ras homolog enriched in brain (RHEB). Activated RHEB subsequently activates mTOR.[66] mTOR is a highly conserved serine-threonine kinase that is able to form two different types of multiprotein complexes, i.e. mTOR complexes 1 and 2 (mTORC1/2).[67] Both complexes are composed of mTOR with disheveled, Egl-10, and pleckstrin (DEP) domain-containing mTOR-interacting protein (DEPTOR)[68] and mammalian lethal with SEC13 protein 8 (mLST8)[69]. However, mTORC1 is defined by the interaction of mTOR with regulatory-associated protein of mTOR (raptor)[70] and proline-rich Akt substrate 40 kDa (PRAS40)[71], whereas rapamycin-insensitive companion of mTOR (rictor)[72], protein observed with rictor (protor)[73] and mammalian stress-activated protein kinase interacting protein 1 (mSin1)[74] are the key components of mTORC2. mTORC1 phosphorylates ribosomal S6 kinase 1 (p70S6K1) that, in turn, activates ribosomal protein S6.[75] In addition, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) is another downstream primary effector of mTORC1. Inhibition of 4EBP1 results in the release of eIF4E.[76, 77] The mTORC1-mediated phosphorylation of these downstream substrates ultimately leads to the stimulation of protein synthesis and cell growth.[78] On the other hand, the best-known function of mTORC2 is the phosphorylation of Akt[79], hereby contributing to cell survival and proliferation. Besides, it is also involved in cytoskeleton organization and cellular and tissue homeostasis.[78]

PI3K-dependent signaling is regulated by the cytoplasmic tumor suppressor phosphatase and tensing homolog (PTEN) that is able to dephosphorylate PIP_3 back to PIP_2, which terminates the signaling cascade by bringing the cell to its resting state again.[80] Interestingly, PTEN is also able to translocate to the nucleus (often referred to as nuclear PTEN) through various mechanisms. Over the past years, it has become clear that nuclear PTEN has specific functions that differ from cytoplasmic PTEN. More specifically, PTEN localized in the nucleus plays a significant role in chromosome and cellular stability, DNA repair and cell cycle arrest.[81] The above described crosstalk between the EGFR and PI3K signaling pathways is schematically presented in figure 1.

As such, it is clear that the Ras/Raf/MAPK pathway and PI3K/Akt pathway are highly interconnected and that both pathways are stimulated by EGFR through activated Ras.

3. Role of the PI3K/Akt pathway in resistance to anti-EGFR treatment in HNSCC

Over the past years, it became clear that intrinsic and acquired resistance mechanisms substantially limit the therapeutic benefit of cetuximab treatment in HNSCC. Therefore, there has been an increasing interest in unravelling the mechanisms that drive resistance to EGFR-targeted therapies in order to (i) maximize clinical RRs by biomarker-driven patient selection; and (ii) develop new therapeutic strategies to overcome resistance.[82] Mutations in genes resulting in overexpression of ligands and/or constitutive activation of key signaling mediators downstream of EGFR might be involved in the development of resistance. In this context, various resistance-mediating molecular

alterations and pathways have been proposed, including the PI3K/Akt signaling pathway (Figure 2A). Moreover, increasing evidence indicates that the PI3K/Akt pathway frequently remains activated despite anti-EGFR treatment and therefore plays an important role in resistance to EGFR-targeting therapies.[83-86]

3.1 RAS/RAF alterations

RAS proteins are proto-oncogenes encoded by three ubiquitously expressed genes, i.e. *HRAS, NRAS* and *KRAS*. RAF proteins, on the other hand, are encoded by *ARAF, BRAF* and *CRAF* and defined as essential effectors of the RAS signaling cascade. The RAS pathway is one of the most frequently mutated pathways in various types of cancer. Aberrant RAS signaling is associated with hyper-proliferation and increased cell survival.[87, 88] In the context of resistance, it has been demonstrated in colorectal cancer that activating *KRAS* and *BRAF* mutations are associated with therapeutic resistance to cetuximab.[89-91] As 58% of metastatic colorectal cancer patients bear mutations in one of these two genes, genomic testing is nowadays standard of care to predict the efficacy of anti-EGFR therapies in metastatic colorectal cancer.[92] In contrast, *KRAS* and *BRAF* mutations are relatively rare events in HNSCC, suggesting an insignificant role in predicting therapeutic response of HNSCC patients.[93-96] Nevertheless, a comprehensive analysis of the mutational landscape of HNSCC revealed that *KRAS* mutations are more frequent than originally thought (but still rare) in HPV-positive tumors (6%) compared to HPV-negative tumors (1%).[97] Furthermore, Rampias et al. demonstrated that cetuximab sensitivity could be restored by silencing *HRAS* in *HRAS* mutant HNSCC cell lines, suggesting a potential role of *RAS* mutations in cetuximab resistance.[98] In the clinical setting, there are some indications towards this hypothesis too. As such, it was recently demonstrated that *KRAS/HRAS* mutations are associated with poor progression-free survival (PFS) in HNSCC patients treated with cetuximab in the first-line recurrent/metastatic (R/M) setting, but not in patients treated with cetuximab and radiotherapy.[99] These results suggest that *HRAS/KRAS* mutations might influence cetuximab sensitivity in HNSCC patients receiving cetuximab with or without chemotherapy. However, more research is necessary to define the precise role of these mutations in patients receiving radiotherapy. Additionally, Braig et al. confirmed by next generation sequencing that activating *RAS* mutations are not very common in tumors from cetuximab-naive HNSCC patients.[100] Moreover, they also compared these data with liquid biopsies acquired during and after cetuximab/platinum/5-fluorouracil treatment (EXTREME regimen). They concluded that following cetuximab treatment, about one-third of the patients had acquired *KRAS*, *NRAS* or *HRAS* mutations. Interestingly, *RAS* mutations could not be detected in the non-progressive subset of patients, while acquired *RAS* mutations were found in nearly half of the patients showing on-treatment disease progression. These findings suggest that acquisition of activating *RAS* mutations is correlated with clinical resistance to the EGFR inhibitor cetuximab.[100]

3.2 PI3K mutational changes and its contribution to resistance

In contrast to intrinsic *KRAS* mutations, genomic alterations in one of the major components of the PI3K/Akt pathway (e.g. *PIK3CA, AKT1/2/3* and *PTEN)* are relatively common and can be found in approximately 66% of HNSCC patients.[101, 102] Moreover, a study analyzed the whole-exome sequencing data of 151 HNSCC tumors and elucidated that PI3K is the most frequently mutated mitogenic pathway downstream of EGFR. Furthermore, they found that the presence of multiple changes in the PI3K signaling pathway is associated with a more advanced disease.[103] In this regard, the PI3K/Akt signaling pathway is upregulated in more than 90% of HPV-positive and -negative HNSCC.[104] In case of HPV-positive tumors, not only mutations, but also HPV infection itself can contribute to the activation of the PI3K/Akt pathway. More specifically, it has been described that the HPV E6 and E7 oncoproteins, which are persistently expressed in HPV-positive tumors, are able to activate mTORC1[105] and upregulate Akt activity[106], respectively.

Global gene expression and pathway analysis between cetuximab-resistant and -sensitive tumors using a patient tumor transplant model showed that molecules of the PI3K/Akt pathway were upregulated in cetuximab-resistant compared to -sensitive tumors.[107] In addition, activation of the PI3K/Akt pathway was shown to be associated with inferior PFS and overall survival (OS) and was also suggested to predict resistance to EGFR-targeted therapy in the E2303 phase II trial.[108] Overall, this indicates compensatory activation of the PI3K/Akt pathway (by mutational changes and/or, in case of HPV-positive HNSCC, expression of HPV oncoproteins) as a main mechanism of resistance to EGFR blockade in HNSCC.

Previous research on the characterization of the mutational landscape of HNSCC reported mutations in *PIK3CA*, which encodes for the catalytic p110 subunit of PI3K, in 8% of investigated HNSCC samples.[109] However, more recent TCGA data described the *PIK3CA* gene as one of the most frequently mutated genes in both HPV-positive and -negative HNSCC patients, with mutations in the PIK3CA gene in 21% of the HNSCC samples. Out of all *PIK3CA* mutations found, 73% were located at Glu542Lys and Glu545Lys in the helical domain, and His1047Arg/Leu in the kinase domain, all three hotspots that promote activation of PI3K. In approximately a quarter of the cases, *PIK3CA* mutation was accompanied by amplification of the gene.[102] Interestingly, depending on the HPV status of the patient, *PIK3CA* mutations seem to be more common and localized at different regions of the gene. As such, HPV-positive HNSCC samples have a higher incidence of *PIK3CA* mutations and/or amplifications (56%), which are often located in the helical domain of *PIK3CA*. In contrast, in HPV-negative HNSCC, mutations and/or amplifications are less frequent (34%) and more scattered.[102, 110-112] Besides mutations in *PIK3CA*, recurrent focal amplifications for 3q26/28 are frequently present in both HPV-positive and -

negative tumors. This 3q26/28 region includes squamous lineage transcription factors *TP63* and *SOX2* as well as the oncogene *PIK3CA*.[102] In addition, PI3K overexpression and subsequent upregulated activity was observed in 27.2% of HNSCC samples (Figure 2A).[113]

Nevertheless, to the best of our knowledge, no data provides definite evidence of *PIK3CA* mutations as one of the responsible factors for the limited efficacy of EGFR-targeted therapies. In this regard, a recent study performed a hotspot *PIK3CA* mutational and PI3K p110 expression analysis but failed to confirm PI3K as a predictive biomarker for cetuximab resistance. However, it is worth mentioning that sample sizes were limited and not all *PIK3CA* abnormalities were included in the analysis.[114] To the contrary, CAL27 HNSCC cells that were genetically engineered to express activating *PIK3CA* and *KRAS* mutations, did not demonstrate a sustained response to cetuximab, even though an initial short-lasting beneficial effect was observed.[115] Also, the CAL33 HNSCC cell line used in the study of Rebucci et al. harbored a *PIK3CA* activating mutation and was identified as intrinsically resistant to cetuximab, suggesting a potential role of the mutation in the sensitivity to cetuximab.[116] Furthermore, in the recent study of Leblanc et al., activating *PIK3CA* mutations were associated with poor PFS in HNSCC patients receiving cetuximab in the first-line R/M disease setting.[99] In light of the reported prevalence of *PIK3CA* mutations, amplifications and recent findings, further examination of the *PIK3CA* mutational status as a potential biomarker to predict cetuximab resistance might provide novel, more conclusive insights.

3.3 PTEN loss as a potential resistance signature

The loss of PTEN is a frequently occurring event in various malignancies, including HNSCC.[102, 117-120] As mentioned previously, PTEN is responsible for inhibiting the PI3K/Akt pathway by dephosphorylating PIP_3 back to PIP_2. Therefore, PTEN inactivation or deletion can lead to the same effect as activating mutations and epigenetic alterations in the PI3K/Akt pathway and is often associated with more aggressive tumors, poor PFS and OS.[121, 122] Even partial loss of PTEN function is sufficient to (further) initiate tumor development of some cancer types and a decrease in PTEN levels below 50% accelerates tumor progression.[123] As such, loss of PTEN may play an important role in resistance to EGFR blockade. In HNSCC, *PTEN* loss of function mutations have been reported throughout literature at various frequencies (2% to 24%), demonstrating the extremely high heterogeneity in the HNSCC mutational landscape.[109, 124] Similar to mutations in the *PIK3CA* gene, *PTEN* genomic alterations are more frequently observed in HPV-associated HNSCCs. For example, Sangale et al. reported *PTEN* loss (assessed by FISH) in over 30% of HPV-associated oropharyngeal cancers.[125] In another study, next-generation sequencing of DNA samples from 252 formalin-fixed paraffin-embedded HNSCC tumor samples revealed *PTEN* mutations or loss in 15% of HPV-positive compared to 5% of HPV-negative tumors.[111]

However, when looking at the expression levels of PTEN, it seems that the genomic alterations seen in HPV-positive tumors are not necessarily inactivating ones. For example, analysis of 65 tonsillar tumors using immunohistochemistry revealed that both nuclear and cytoplasmic PTEN expression was preserved more frequently in HPV-positive (73%) compared to HPV-negative (43%) tumors[126], despite the finding that *PTEN* mutations more often occur in HPV-positive HNSCC. Without HPV stratification, low or absent PTEN expression can be observed in approximately 10-30% of HNSCCs[102, 103, 126-128] and this often leads to aggressive tumors with worse prognosis in locoregional disease.[129, 130] Moreover, in the study of Bian et al., the PTEN protein level was found to be decreased or even undetectable in 80% (16/20) of the HNSCC samples (HPV status not specified) as compared to six mucosa control samples, suggesting that loss of the expression of PTEN is a common event in HNSCC.[131]

Various mechanisms have already been described that may explain the loss of PTEN expression, including reduced protein synthesis, augmented protein degradation, or other posttranslational modifications.[128] On the genomic level, loss of PTEN expression may also be caused by epigenetic silencing of the gene[132, 133], as inactivation of different tumor suppressor genes by hypermethylation has already been reported in HNSCC.[134, 135]

Over the years, it has been hypothesized that PTEN loss might be part of a signature characteristic for resistance to anti-EGFR therapy, as this may lead to compensatory activation of the PI3K/Akt pathway (Figure 2A). Indeed, PTEN loss has already been associated with cetuximab and erlotinib resistance in colorectal[136] and lung cancer[137], respectively. Moreover, in a cetuximab-resistant NSCLC cell line, generated from NCI-HCC827 NSCLC cells, it was shown that increased proteasomal degradation of PTEN, resulting in constitutive activation of Akt, is involved in acquired cetuximab resistance. As such, cetuximab-resistant NCI-HCC827 clones were characterized by Akt hyperactivation and considerably decreased protein levels of PTEN.[138] In addition, it was reported that various cell lines, including PTEN-deficient epidermoid carcinoma cells, were resistant to EGFR-inhibiting agents.[139] This finding suggests a potential role of PTEN loss in resistance to EGFR inhibitors in HNSCC. Moreover, the study of Da Costa et al. was able to confirm PTEN expression as a prognostic factor in metastatic HNSCC, although it could not be identified as a predictive biomarker with statistically significant evidence.[140] Nevertheless, their findings do suggest a possible role for the loss of PTEN in predicting cetuximab resistance and require further investigation in a larger cohort of patients.[140] Another recent study analyzed PTEN expression in samples from patients included in two clinical trials of cetuximab-based therapy for R/M HNSCC, i.e. a randomized trial of cisplatin plus placebo versus cisplatin plus cetuximab (E5397) and a randomized trial of cetuximab + sorafenib versus cetuximab monotherapy (NCI-8070). Their results also suggested that loss of PTEN protein expression may be associated with cetuximab resistance. However, it is again worth mentioning that sample size used in this study was limited and further

validation of PTEN as predictive biomarker for resistance is merited.[114] Similar findings regarding PTEN and anti-EGFR therapy resistance were reported by Cohen et al.[141] Results from their phase III randomized clinical trial for metastatic HNSCC suggested that PTEN expression was a predictive biomarker for resistance to afatinib, a second-generation tyrosine kinase inhibitor targeting EGFR, ErbB2 and ErbB4.[141] Furthermore, loss of PTEN protein expression was recently shown to have a negative predictive value in HNSCC patients treated with cetuximab in combination with radiotherapy.[99]

Taken together, loss of PTEN protein may diminish the effect of multiple EGFR inhibitors in HNSCC and could be considered as a potential predictive biomarker for EGFR-targeted therapy response (Figure 2A).

3.4 Altered Akt expression frequently occurs during cetuximab resistance

Regarding the other downstream effector molecules of the PI3K/Akt pathway, mutations in genes encoding for Akt and mTOR are almost non-existing, while overexpression of these proteins occurs more often (Figure 2A).[113] Akt is a key regulator of various processes driving aberrant cell growth. Constitutive activation of Akt is a frequent abnormality observed in several types of cancers, including HNSCC.[142] Moreover, the active state of the Akt protein is detected in 50% of preneoplastic lesions.[143] Previous research has indicated that the expression and activation of Akt is also associated with accelerated tumor progression, as shown in immortalized murine keratinocyte cell lines as a model for squamous malignancies.[144] In addition, immunohistochemical analysis with antibodies against phosphorylated Akt confirmed the presence of the active form of Akt in mouse skin SCC. Furthermore, the activation status of Akt was examined in HNSCC-derived cell lines and clinical samples from HNSCC patients, which led to two observations: (i) Akt is frequently activated in human HNSCC, as shown by the elevated kinase activity; and (ii) the pattern of expression and localization of Akt is correlated with the progression of the disease.[142] In another study, active Akt could be detected in 60% of HPV-positive and 80% of HPV-negative HNSCC samples.[145] As mentioned previously, upregulated Akt activity in HPV-positive HNSCC might (partially) be induced by the expression of the viral oncoprotein E7.[106]

As increased Akt signaling seems to play an important role in carcinogenesis, it might also be related to resistance to cetuximab and/or other EGFR-targeting therapies. Indeed, it has already been suggested that persistent Akt activation may be an underlying mechanism of resistance to cetuximab in both HNSCC[108, 116, 146, 147] and colorectal cancer.[147] Rebucci et al. studied the cellular response to cetuximab treatment in cetuximab-resistant and -sensitive cell lines by Western blot analysis and found significant differences in phosphorylation of Akt.[116] More specifically, in the cetuximab-sensitive A431 epidermoid carcinoma cell line, cetuximab treatment significantly inhibited Akt phosphorylation, whereas phosphorylated Akt levels remained unmodified following cetuximab therapy in resistant

HNSCC cell lines. Cetuximab-resistant CAL33 cells harbored a mutation in exon 20 of the *PIK3CA* gene, which was found to be causal for the persistence of Akt activation. These results imply that cell lines acquiring mutations that lead to constitutive activation of the PI3K/Akt pathway, become minimally dependent on canonical EGFR ligand-induced signaling for cellular growth and thus are more resistant to cetuximab treatment.[116] In colorectal cancer, similar results have been reported.[148] However, CAL27 HNSCC tumors retro-engineered to express *PIK3CA* and *RAS* oncogenes were initially sensitive to treatment with cetuximab, although they relapsed within one month.[115] Nevertheless, these studies provide some evidence that persistent Akt activation, seen in *PIK3CA* mutated cells, might be an important player underlying cetuximab resistance.[116]

In accordance with previously discussed results, tumor kinase profiling of cetuximab-sensitive and acquired resistant HNSCC cell lines also showed that increased Akt1/2/3 phosphorylation after cetuximab treatment is characteristic for acquired cetuximab-resistant HNSCC cell lines.[149] Similarly, activation of Akt by phosphorylation has been correlated with sensitivity to the EGFR-targeted agent gefitinib in both HNSCC cell lines and tumor specimens.[150] Therefore, over the past years, phospho-Akt has been suggested as a potentially useful predictive biomarker. In this context, analysis of a cohort of 50 oral squamous cell carcinoma patients who were treated with cetuximab-based induction chemotherapy, showed that diminished expression of phosphorylated Akt was associated with better disease-free survival in these patients.[151] This finding suggests that efficient response to cetuximab therapy can be predicted by the phospho-Akt levels in the patient.

3.5 mTOR and its potential to mediate resistance

Similar to phospho-Akt, elevated mTOR activity has gained interest in the field of EGFR-targeted therapy resistance. During cancer, aberrant activation of mTOR is known to induce metabolic changes, such as dysregulation of glucose, fatty acid, amino acid and lipid metabolism.[152] Furthermore, inhibition of mTOR could prevent the proliferation of cancer cells.[153-155] Notably, increased mTOR activity is a frequent event in both HPV-positive and -negative HNSCC[156] and is suggested to play a central role in HNSCC tumorigenesis and tumor progression.[157, 158] The phosphorylated active form of p70S6K1, which is a translation regulator and a downstream effector of mTOR, is often accumulated in HNSCC patients samples and HNSCC-derived cell lines.[159, 160] In the study of Wang et al., cetuximab-resistant CAL27 cells, harboring activating *PIK3CA* and *RAS* mutations, were characterized by increased expression of phosphorylated S6K1, indicative for elevated mTOR activity.[115] This suggested that cetuximab-resistant cells may have an increased ability to activate mTOR in a more efficient manner compared to cetuximab-sensitive cells. The underlying mechanism of this selective increase in mTOR activity remains to be elucidated and requires more investigation.[115] In addition, the precise role of

mTOR in the development and maintenance of resistance to EGFR-targeted therapies is still largely unclear.[161]

4. Preclinical studies on targets of the PI3K/Akt pathway in combination with EGFR-targeted agents in HNSCC

4.1 PI3K inhibitors in combination with EGFR inhibition

Due to its central position in the PI3K/Akt pathway and its high incidence of molecular alterations, PI3K has been suggested as a compelling drug target for cetuximab-resistant HNSCC. Over the past years, a wide range of PI3K inhibitors have been developed, going from pan-PI3K inhibitors, targeting all four isoforms of class I PI3K, to isoform-selective inhibitors.[162] A number of them were preclinically investigated by pharmaceutical companies and academic institutions to test their potential in overcoming resistance to EGFR inhibitors (Table 1, Figure 2B). In this regard, the combination of cetuximab with the PI3Kα-selective inhibitor alpelisib (BYL719, Figure 3A) was shown to exert synergistic activity in HNSCC cell lines with different molecular status and also demonstrated a clear anti-tumor effect in a *PIK3CA*-mutant mouse HNSCC xenograft model.[162, 163] Similarly, the addition of alpelisib to cetuximab had an additive anti-tumor effect in the cetuximab-sensitive KYSE180 xenograft model. Moreover, in the KYSE180_CR model (acquired cetuximab resistant model), the combination treatment restored cetuximab sensitivity to a level similar to that of cetuximab monotherapy in the cetuximab-sensitive model.[164] Furthermore, PX-866 (a wortmannin analogue and an oral, irreversible pan-PI3K inhibitor, Figure 3B) combined with cetuximab was shown to be more effective in a patient-derived HNSCC xenograft mouse model compared to cetuximab alone.[165] Lattanzio et al. evaluated the anti-proliferative effect of the oral pan-PI3K inhibitor buparlisib (Figure 3C) in combination with cetuximab with/without radiotherapy in cetuximab-resistant HNSCC cell lines with or without *PIK3CA* mutations.[166] Treatment of cetuximab followed by buparlisib showed synergistic activity in inhibiting cell proliferation in both *PIK3CA* mutated and wildtype HNSCC cell lines. When radiotherapy was added to the treatment schedule, the anti-proliferative effect of this triple combination therapy was enhanced only in the *PIK3CA* wild type cell line. Activation of mTORC2 complex and caspase proteins in the *PIK3CA* wild type cell line were suggested as potential mechanisms underlying the synergistic combination of cetuximab plus buparlisib. In the *PIK3CA* mutated cell line, increased sensitivity of these mutated cells to PI3K inhibition was suggested as an explanation for the observed synergism. In addition, EGFR-ERK signaling induced by radiation and an increase in DNA repair protein levels in a MAPK-dependent manner, which results in radioresistance, might explain the similar anti-proliferative effects observed in the *PIK3CA* mutated cell line between the treatment schedule with and without radiotherapy.[166] Similarly, in an *in vivo* study using an orthotopic mouse xenograft HNSCC model, it

was demonstrated that the combination of cetuximab and buparlisib with/without irradiation both produced the highest anti-tumor activity compared to control, leading to almost complete tumor growth arrest. Interestingly, only the triple combination was synergistic in this HNSCC xenograft model.[167] Furthermore, the efficacy of copanlisib (Figure 3D), another pan-PI3K inhibitor with preferential activity against PI3Kα and PI3Kδ isoforms of PI3K, has been preclinically investigated in combination with cetuximab using patient-derived xenograft (PDX) models. Adding copanlisib to treatment with cetuximab resulted in an increased tumor response in 21 out of 33 PDX models tested, with 14 out of 16 cetuximab-resistant tumors showing response to combined treatment.[168] Similarly, Rebucci et al. investigated whether LY294002 (Figure 3E), a synthetic non-selective PI3K inhibitor, in combination with cetuximab is able to restore the sensitivity of resistant CAL33 cells to cetuximab treatment.[116] Interestingly, CAL33 harbor a *PIK3CA* mutation and are characterized by unmodified Akt phosphorylation levels following cetuximab monotherapy. Treatment with LY294002 plus cetuximab was shown to decrease Akt phosphorylation and induced significant growth inhibition in cetuximab-resistant CAL33 cells compared to cetuximab as a single agent.[116] Furthermore, the PI3Kα/δ-selective inhibitor, pictilisib (GDC-0941, Figure 3F) combined with the EGFR inhibitor erlotinib demonstrated synergistic effects in different HNSCC cell lines compared to pictilisib alone.[169] Taken together, these preclinical results support the hypothesis that inhibition of PI3K in combination with EGFR blocking antibodies might be able to restore sensitivity to EGFR inhibitors in resistant HNSCC patients.

4.2 Akt inhibitors in combination with EGFR inhibition

Targeting Akt is considered as a highly attractive anti-cancer strategy. Similar to PI3K, Akt represents a central component of the PI3K/Akt signaling pathway, which is commonly disrupted in HNSCC. As such, multiple Akt inhibitors have been developed and investigated as a single agent for their ability to inhibit cell proliferation, induce cell death and prevent metastasis in HNSCC.[169-171] Preclinical studies focusing on the combination of an Akt-inhibitor with anti-EGFR targeted therapy to restore the sensitivity and thus overcome resistance to EGFR-targeted therapies are very scarce throughout literature. To the best of our knowledge, we have reported on the only study that investigated the combination of the allosteric Akt inhibitor MK2206 (Figure 4) with cetuximab in a panel of cetuximab-sensitive and -resistant HNSCC cell lines (Table 1, Figure 2B). We reported an additive to synergistic interaction between MK2206 and cetuximab in different treatment schedules, suggesting that this combination might be a promising therapeutic strategy to overcome acquired cetuximab resistance in HNSCC.[172] Thus, for some unknown reason, inhibition of the regulators and targets of Akt (e.g. PI3K and mTOR), instead of inhibition of Akt itself, seems to be more attractive to combine with EGFR targeting. A potential reason for this could be that there might be an immunological interaction between PI3KCA inhibition and cetuximab. In this regard, it was recently suggested that the PI3K inhibitor buparlisib is

able to alleviate tumor immune suppression by promoting IFNγ secretion.[173] However, further research regarding this topic is still necessary.

4.3 mTOR inhibitors in combination with EGFR inhibition

mTOR is one of the most widely studied substrates of the PI3K/Akt pathway in terms of the (pre)clinical development of targeted therapies (Table 1, Figure 2B). This could be explained by the fact that it mediates many of Akt's functions, thus killing two birds with one stone.[174] The best-known mTOR inhibitor is rapamycin (Figure 5A), also known as sirolimus. Rapamycin was originally used as an immunosuppressant.[175, 176] Following the discovery of the anti-tumoral activity of rapamycin in different tumor types, rapamycin analogues, also known as rapalogs, were developed and represent the first generation of anti-tumor mTOR inhibitors (e.g. temsirolimus and everolimus).[177-179] These rapalogs bind primarily to a domain adjacent to the kinase active site of mTORC1, together with the immunophilin termed FKBP12. Hereby, first generation mTOR inhibitors inhibit only some of the functions of mTORC1. The second generation mTOR inhibitors (e.g. OSI-027 and AZD8055) are considered more potent as they block mTOR kinase in a direct manner, inhibiting both mTORC1 and mTORC2.[179, 180] Inhibition of mTOR in HNSCC seems to be promising and in-depth analysis of the molecular basis of therapeutic resistance in HNSCC suggests that mTOR co-targeting strategies might provide an effective option in bypassing this resistance.[181, 182]

Already in 2007, it was shown that co-targeting mTOR and EGFR by respectively, temsirolimus (Figure 5B) and erlotinib, resulted in additive anti-tumor effects in a HNSCC xenograft mouse model established with the Detroit 562 cell line that has intermediate susceptibility to EGFR inhibitors. However, the combined treatment failed to be superior in comparison with the best single agent (i.e. temsirolimus) in the HEP2 cell line, which is known to be resistant to EGFR inhibitors.[183] These findings suggest that the combination of temsirolimus plus erlotinib is only partially capable of overcoming anti-EGFR drug resistance in HNSCC. Furthermore, Bozec et al. investigated the addition of temsirolimus to a previously established triple combination therapy, consisting of radiotherapy, cetuximab and bevacizumab in nude mice engrafted with the cetuximab-resistant CAL33 cell line.[184] Administration of this triple combination together with temsirolimus had an additive effect and resulted in a significantly greater growth inhibition, decreased tumor proliferation, delayed tumor regrowth and decreased expression of anti-apoptotic markers as compared to both the triple combination alone and temsirolimus alone, without any significant toxicities during treatment.[184] The study of Wang et al. demonstrated that concomitant administration of the mTOR inhibitors rapamycin or everolimus (Figure 5C) plus cetuximab resulted in a remarkably increased anti-tumor response in HNSCC tumor xenografts, with almost no residual tumor masses at the end of the combination treatment.[115] Importantly, the combination of mTOR and EGFR inhibition also prevented tumor growth

in HNSCC cells that were resistant to cetuximab as a single agent, indicating its potential as a novel combination strategy to overcome cetuximab resistance. Decreased cell proliferation, inhibition of lymphangiogenesis and increased autophagy were suggested as responsible mechanisms underlying the effect of the combination therapy. As cetuximab is known to induce antibody-dependent cellular cytotoxicity, the authors also highlighted the hypothesis that cetuximab treatment may lead to a cytotoxic immune response against EGFR-overexpressing HNSCC cells, which might synergize with mTOR growth-signaling inhibition.[115] An *in vivo* study investigating the anti-tumor efficacy of temsirolimus combined with cetuximab, cisplatin and 5-fluorouracil (Cet-C/5-FU) in an orthotopic xenograft model of HNSCC showed that, although the addition of temsirolimus to the Cet-C/5-FU combination led to a significant decrease of tumor proliferation compared to Cet-C/5-FU alone, the highest tumor inhibition and almost complete tumor growth arrest was seen when temsirolimus was combined with cetuximab alone. This dual combination also demonstrated the highest inhibitory effects on MAPK and PI3K/Akt signaling pathways and consequently also on cell proliferation.[158] Similarly, Lattanzio et al. demonstrated that temsirolimus plus cetuximab exerted a synergistic effect *in vitro* in the CAL33 HNSCC cell line.[185] As the CAL33 cell line was previously described as intrinsically resistant to cetuximab[116], the latter suggests that the temsirolimus-cetuximab combination might be an efficient option for the treatment of cetuximab-resistant tumors. This is in accordance with the study of Niehr et al., which reported that the combination of temsirolimus with cetuximab was able to restore cetuximab sensitivity in a HNSCC cell line with acquired resistance to cetuximab.[186]

Not only rapalogs, but also second-generation mTOR inhibitors have been preclinically investigated over the past years. In this regard, the combination of OSI-027 (also known as A7486, Figure 5D), an oral second generation mTORC1/2 inhibitor, with erlotinib demonstrated a synergistic growth-inhibiting effect in different HNSCC cell lines compared to either drug alone. Using an HNSCC xenograft model, OSI-027 in combination with cetuximab was shown to significantly improve anti-tumor efficacy compared to cetuximab alone. Thus, the addition of OSI-027 enhanced the sensitivity of the tumor to cetuximab. These findings suggest that the second-generation mTOR inhibitor OSI-027 in combination with EGFR inhibitors may be able to improve treatment responses in HNSCC patients.[187] More recently, it has been shown that the second generation mTOR inhibitor AZD8055 (Figure 5E) in combination with cetuximab produced effective inactivation of downstream members of the PI3K/Akt pathway. However, this combination exerted only little to no additional antiproliferative effect compared to single agent treatment in three out of five HNSCC cell lines tested. Nevertheless, when investigating this specific combination therapy in PDX models selected on the basis of well-described *PIK3CA*-activating mutations or for high intrinsic resistance to cetuximab, a significant growth delay in all five PDX models could be observed, whereas either agent administered alone was almost ineffective at reducing tumor growth. These results suggest that the combination therapy of cetuximab plus

AZD8055 had at least an additive anti-tumor effect in different *in vivo* tumor models, including intrinsically cetuximab-resistant PDX models.[188]

4.4 Dual PI3K/mTOR inhibitors in combination with EGFR inhibition

Dual inhibitors of PI3K and mTOR simultaneously target the active sites of both enzymes and have therefore a possible advantage over anti-cancer agents targeting only one component of the pathway. Indeed, dual PI3K/mTOR inhibitors block the pathway both upstream and downstream of Akt. Consequently, Akt activation as a result of the disruption of the mTORC1-S6K-IRS1 negative feedback loop, which is reported to occur with rapalogs, is avoided.[189, 190] Furthermore, preclinical studies have also suggested that dual PI3K/mTOR inhibitors have a broader efficacy across more genotypes than agents targeting PI3K or mTOR alone.[189, 191] Importantly, dual PI3K/mTOR inhibitors have proven their efficacy in preclinical HNSCC models (Table 1, Figure 2B). For example, the combined treatment of the dual PI3K/mTOR inhibitor PKI-587 (Figure 6A) and cetuximab was able to enhance sensitivity to cetuximab, even in HNSCC cell lines characterized as cetuximab-resistant. Moreover, *in vivo* evaluation in nude mice xenografted with EGFR-resistant KYSE30 cells showed that the combination treatment significantly reduced tumor growth and prolonged mice survival.[161] This suggests that PKI-587 might be able to overcome cetuximab resistance in HNSCC. However, dual PI3K/mTOR inhibitors do not always seem successful in preclinical HNSCC studies. For example, Swick et al. reported that the combination of dual PI3K/mTOR inhibitor NVP-BEZ-235 (Figure 6B) with cetuximab had little to no additional antiproliferative effect in a panel of HNSCC cell lines.[188] Further research on combination strategies with dual PI3K/mTOR inhibitors in the context of anti-EGFR resistance might be interesting to get insight in novel promising therapeutic options in HNSCC.

In conclusion, blocking activity upstream of Akt is more efficacious then blocking Akt itself or blocking downstream of Akt. This suggests there are more pathways involved between PI3K/PTEN and Akt.

5. Clinical studies evaluating combinations of PI3K/Akt pathway and EGFR inhibition in HNSCC patients

As discussed above, much preclinical effort has been made to investigate the potential of combination strategies regarding anti-EGFR targeted therapies and agents targeting the PI3K/Akt signaling pathway in HNSCC. The vast majority of the preclinical results provide a strong indication that these PI3K/Akt-targeted agents are promising new cancer therapeutics that are effective in overcoming resistance to EGFR-targeted therapies. Therefore, several clinical trials have been conducted over the past years to evaluate the efficacy and safety of PI3K/Akt pathway inhibitors with additional anti-EGFR therapy.

5.1 PI3K inhibitors in combination with EGFR inhibition

After promising *in vitro* and *in vivo* findings, various clinical studies were set up to further investigate the combination of PI3K and EGFR inhibition in HNSCC patients. Only a few of them have been completed, whereas the majority of the studies are still ongoing (Table 2).

5.1.1 Alpelisib

In a phase Ib dose-escalation study investigating the combination of alpelisib and cetuximab in platinum-resistant R/M HNSCC patients (NCT01602315), the most common side effects (any grade) included hyperglycemia, rash, stomatitis, dry skin, hypomagnesemia, decreased appetite, diarrhea, fatigue and paronychia. Based on the observed dose-limiting toxicities, 300 mg alpelisib was considered as the recommended phase II dose (RP2D) in combination with standard weekly doses of cetuximab. In addition, this combination showed promising signs of anti-tumor activity in 10 evaluable patients receiving a dose of 300 mg being one partial response (PR), three unconfirmed PRs, five stable disease (SD) and one case in whom the response was unknown.[192] More recently, the phase Ib trial of Dunn et al. evaluated the addition of alpelisib to cetuximab and radiation in locally advanced HNSCC patients (NCT02282371).[193] The rationale behind this combination is based on studies demonstrating that (i) cetuximab and alpelisib are potent radiosensitizing agents[18, 194]; and (ii) both agents show synergism in a preclinical model for HNSCC.[163] Based on dose-limiting toxicities, the RP2D was determined to be 250 mg alpelisib daily combined with cetuximab and radiation. Alpelisib likely enhanced common toxicities associated with radiotherapy and cetuximab, but overall, the combination was considered to be safe. Interestingly, all 11 evaluable patients showed complete response following combination therapy and 10 remained disease free for a median follow-up period of 23.5 months. Further development of this combination might be interesting for patients in whom (platinum-based) chemotherapy is contraindicated or for patients with an activating alteration in the PI3K/Akt pathway.[193]

5.1.2 PX-866

A phase I dose-finding study assessed the safety and maximum tolerated dose (MTD)/RP2D of the oral pan-PI3K inhibitor PX-866 in combination with cetuximab in patients with incurable HNSCC or colorectal cancer (NCT01252628). Similar to the MTD of single agent PX-866, the RPD2 for this specific combination was 8 mg/day PX-866.[195] Furthermore, PX-866 combined with cetuximab also showed to be well-tolerated in HNSCC patients.[196] The most common all-grade and grade 3/4 adverse events in 11 evaluable patients were manageable and included anticipated gastrointestinal toxicities (diarrhea (90.1%, 18.2%), nausea (54.5%, 0%), vomiting (72.2%, 0%), hypomagnesemia (72.2%, 0%), fatigue

(54.5%, 0%), rash (45.5%, 0%) and peripheral edema (40%, 0%), which are all known side effects of either PX-866, other PI3K inhibitors or cetuximab.[195-197] No formal dose-limiting toxicities could be observed. These results suggest that combining PX-866 and cetuximab at the MTD of each single agent is feasible. This finding is encouraging, since combination therapies are generally most effective when all agents are given at their MTD. Furthermore, the combination showed promising signs of anti-cancer activity in nine evaluable patients. PR was observed in four patients and PR or SD was present in eight patients after cycle two. Interestingly, the partial RR of the combination (66% for cetuximab-naïve and 33% for cetuximab pre-treated patients) was higher than the expected single agent RR for cetuximab in HNSCC (i.e. 13%). Furthermore, both cetuximab-naïve and cetuximab pre-treated patients showed clinical responses, suggesting that PX-866 may be able to overcome cetuximab resistance in addition to enhancing the activity of cetuximab. However, the study's small sample size is a limiting factor, making it difficult to draw any definite conclusions about PX-866's efficacy and the possibility to combine PX-866 with cetuximab at full doses for multiple cycles.[196]

This combination was further investigated in a randomized, phase II clinical study, which enrolled 83 patients with advanced, platinum-refractory HNSCC who had received at least one but no more than two prior systemic treatment regimens (NCT01252628). Despite the encouraging (pre)clinical results discussed above, the combination treatment failed to be superior over cetuximab monotherapy in terms of PFS (80 days versus 80 days), OS (211 days versus 256 days) and RR (10% versus 7%). Whereas the majority of the patients enrolled in this study were HPV-positive patients (56%), neither HPV-positive nor HPV-negative patients obtained clinical benefit for the combination of the PI3K inhibitor PX-866 and cetuximab. This lack of clinical benefit might be explained by the fact that patients were enrolled without any molecular preselection. In fact, sensitivity to PI3K inhibitors might be dependent on the presence of genetic alterations in the PI3K/Akt pathway, such as *PIK3CA* mutations and PTEN loss. These alterations were underrepresented in the 46 tumors analyzed in this study. However, none of the eight patients (17%) whose tumors did harbor a *PIK3CA* mutation, showed any response to the combination therapy, making it difficult to explain this lack of clinical benefit. Although the addition of PX-866 to cetuximab was generally well-tolerated, overall toxicity was higher in the combination arm. Especially, the incidence of nausea (53% versus 23%), vomiting (45% versus 15%) and diarrhea (40% versus 21%), causing electrolyte imbalances, was increased. While severe adverse events (grade 3 or higher) were infrequent, they were more common in the combination arm.[198]

5.1.3 Buparlisib

Recently, clinical studies have been investigating the efficacy of the pan-PI3K inhibitor buparlisib in HNSCC patients. Treatment with buparlisib in combination with paclitaxel already demonstrated a significant survival improvement in R/M HNSCC patients (median OS of 10.4 months vs. 6.5 months

with paclitaxel alone).[199] As EGFR and PI3K co-targeting approaches have demonstrated promising anti-tumor activity in preclinical models[166, 167], the pilot, dose-escalation study of Brisson et al. tried to determine the MTD of buparlisib administered concomitant with cetuximab in R/M HNSCC (NCT01816984).[200] However, the highest dose of buparlisib tested (100 mg) was reached without patients presenting any dose-limiting toxicities. Therefore, this dose of buparlisib in combination with cetuximab was recommended to be tested in an expansion cohort to further evaluate safety, tolerability and preliminary efficacy. The most common all-grade side effects of the combined therapy in 12 patients were hyperglycemia (91.6%), hypomagnesemia (83.3%), anorexia (66.7%), fatigue (66.7%), pain (66.7%), hypoalbuminemia (58.3%) and rash (58.3%). The simultaneous treatment with buparlisib and cetuximab demonstrated good tolerability and an attractive toxicity profile in R/M HNSCC patients. Interestingly, the combination showed beneficial effects in these patients, including those who had previously received cetuximab. In this regard, out of 12 evaluable patients, one cetuximab pre-treated patient achieved PR (8.3%) and four patients (three cetuximab pre-treated and one cetuximab-naïve patient) achieved SD (33.3%). This suggests that the combination of buparlisib and cetuximab is able to overcome cetuximab resistance in HNSCC patients. Therefore, further study of this combination is warranted, especially in cetuximab-resistant HNSCC patients, given the favorable toxicity profile and preliminary beneficial results demonstrated in this pilot study.[200]

5.2 mTOR inhibitors in combination with EGFR inhibition

As mentioned earlier, extensive preclinical data suggests that using mTOR inhibitors in combination with EGFR-blocking antibodies might be a promising strategy to circumvent therapeutic resistance to EGFR-targeted therapy. As a result, numerous phase I/II clinical trials have been carried out over the past decade in order to evaluate whether these combination therapies would be appropriate strategies in the treatment of HNSCC (Table 2).

5.2.1 Temsirolimus

As multiple preclinical studies demonstrated synergism between EGFR-inhibiting agents and temsirolimus[183-185], various clinical trials have evaluated this combination in patients with HNSCC. In a phase I clinical trial of temsirolimus plus cetuximab in patients with advanced solid tumors, including HNSCC, dosages escalated from 15 to 25 mg and 150 to 250 mg/m^2 for temsirolimus and cetuximab, respectively (NCT02215720). Dose-limiting toxicities occurred, such as pulmonary embolism, stomatitis and acneiform rash in three out of 39 patients enrolled in this study. Based on the results, the weekly dosage of 25 mg temsirolimus in combination with 250 mg/m^2 cetuximab was selected as the MTD for this combination. In addition, the study reported that 46.2% of the patients exhibited SD, while the overall RR was low, with a disappointing 5% in 37 evaluable patients. Several patients

terminated their treatment due to progressive disease (77%), adverse events (10%), patient's decision (5%) or doctor's decision (8%). Unfortunately, only 74% of patients were molecularly screened for aberrations in the EGFR and/or PI3K/Akt pathways, limiting the observations on the possible association between molecular alterations and anti-tumor activity. Overall, the authors did not recommend further clinical evaluation of this combination due to limited activity and its significant toxicity profile.[201]

In another phase I trial, the triple combination of the vascular endothelial growth factor (VEGF)-targeted antibody bevacizumab, cetuximab and temsirolimus was investigated in 21 patients with advanced malignancies, including nine patients with HNSCC (NCT01552434).[202] EGFR and VEGF(R) inhibitors have been reported to work synergistically, which can be attributed to the fact that their targets share common downstream signaling pathways.[203-205] On the other hand, temsirolimus is known to inhibit the PI3K/Akt pathway and attenuate hypoxia-inducible factor 1α (HIF-1α) levels. PI3K/Akt pathway hyperactivation and elevated HIF-1α levels are both suggested as mechanisms of resistance for cetuximab[107] and bevacizumab[206], respectively. Therefore, this combination strategy has a strong rationale and might be a promising strategy to avoid the emergence of therapeutic resistance. Out of eight evaluable patients with HNSCC, two patients showed PR and one patient had SD for more than 6 months following the combination regimen. However, 14% (3/21) of the patients were withdrawn from the study due to toxicities. The most common non-hematologic toxicities (any grade) included dermatitis, fatigue, hypercholesterolemia, hyperglycemia, hypertriglyceridemia, mucositis and proteinuria.[202] All of these adverse events have previously been reported as common side effects following therapy with temsirolimus, cetuximab or bevacizumab as a single agent.[207-212] Interestingly, PTEN loss was reported in one HNSCC patient. This patient had a hopeful 23% decrease of tumor lesions but progressed after three cycles of treatment. Again, molecular analysis was limited to those patients of whom tissue was available, making it impossible to identify any molecular biomarkers. Taken together, although the combination showed clinical efficacy in HNSCC, careful management of the reported toxicities will be required for future clinical development.[202]

Before it was reported that the combination of temsirolimus and EGFR inhibition had an unfavorable safety profile, its clinical efficacy had already been investigated in a couple of phase II clinical trials in HNSCC patients (Table 2). For example, clinical activity with primary endpoint PFS was investigated for temsirolimus in combination with erlotinib in patients with platinum-refractory R/M HNSCC (NCT01009203). A total of 12 patients were enrolled, but six had to withdraw early due to severe toxicities and treatment-unrelated death, prompting early study termination.[213] The RP2D used in this study was based upon a phase I study in patients with recurrent glioblastoma multiforme[214], which highlights the fact that promising regimens in one cancer type cannot always be translated to another

cancer type, because, among others, differences in patients characteristics may impact on that. As a matter of fact, HNSCC patients are characterized by unique disease- and treatment-related co-morbidities, which may have increased the toxicity profile that was not observed in glioblastoma patients. Due to the early withdrawals in this study, the evaluation of the clinical efficacy of the combination regimen was inadequate. However, preliminary results suggest that *PIK3CA* mutational and PTEN expression status could be used as biomarker candidates for future studies in the setting of mTOR blockade.[213] Although the latter study indicated that dual mTOR-EGFR blockade is unsafe in R/M HNSCC, the randomized phase II MAESTRO trial, investigating temsirolimus with or without cetuximab, was able to successfully enroll patients and complete the study without prohibitive toxicity (NCT01256385). The combination of temsirolimus with cetuximab demonstrated potential clinical activity, while temsirolimus as a single agent did not show any activity in HNSCC patients. However, combining temsirolimus with cetuximab did not improve the median PFS in this patient population compared to temsirolimus alone.[215, 216] Taken together, the combination of temsirolimus and EGFR inhibition has a severe toxicity profile that may often not be tolerable for HNSCC patients. In addition, these combination therapies demonstrated only limited clinical efficacy in R/M HNSCC patients. Therefore, further clinical development is not recommended.

5.2.2 Everolimus

Although combining the mTOR inhibitor everolimus with cetuximab was effective in preclinical *in vivo* studies[115], clinical trials were often not as encouraging. The phase I dose-escalation study evaluating everolimus in combination with cetuximab enrolled a total of 29 patients with advanced cancer, including HNSCC. Everolimus was tested at three dose levels in combination with cetuximab: 30 mg, 50 mg and 70 mg. At none of these dose levels, dose-limiting toxicities were observed in one-third or more of the patients tested, leading the investigators to conclude that 70 mg weekly was the MTD. The most common grade ≥2 side-effects of the combination treatment were rash (34%), fatigue (24%), elevated alkaline phosphatase (21%), hypoalbuminemia (21%), anemia, vomiting, hypomagnesemia and hypersensitivity (each 17%).[217] The reported adverse events were consistent with previous results from trials evaluating cetuximab[218, 219] or everolimus[220, 221] as a single agent. Regarding clinical efficacy of the combination, 16 patients were evaluable for response, with five patients (including one HNSCC patient) maintaining SD for 4 to 19 months. In summary, the combination of everolimus and cetuximab had a manageable toxicity profile and resulted in prolonged disease control in a subset of patients.[217] However, the latter study was one of the few successful studies investigating combinations with everolimus and EGFR inhibition in HNSCC. Similar to the phase II study of Bauman et al.[213], a phase I trial evaluating the triple combination of cisplatin, cetuximab and everolimus as a potential strategy to overcome cetuximab resistance in patients with R/M HNSCC was terminated prematurely due to

toxicities (NCT01009346).[222] In the phase Ib dose-escalation study of Saba et al., the triple combination of carboplatin, cetuximab and everolimus demonstrated a manageable toxicity profile when everolimus was administered at the lowest dose level (i.e. 2.5 mg/day) in patients with R/M HNSCC (NCT01283334).[223] However, increasing the dose of everolimus beyond 2.5 mg/day was not feasible due to the emergence of grade ≥3 gastrointestinal toxicities and hyponatremia.[223] This is in accordance with previously reported studies, which were unable to proceed with the desired dose escalations of everolimus due to severe toxicities.[222, 224, 225] Notably, the MTD of 2.5 mg everolimus every other day determined in this study is rather low compared to the RP2D of 70 mg/week everolimus in combination with standard cetuximab reported in the phase I clinical trial of Ciunci et al.[217] Common grade ≥3 side-effects of the combination therapy included leukopenia (5.3%), neutropenia (9.0%) and hyperglycemia (6.6%). Interestingly, preliminary results on the efficacy of the combination treatment in 13 evaluable patients showed an encouraging RR of 61.5% (8/13, all PRs) and PFS of 8.15 months with two patients even maintaining a response for more than 12 and 37 months, respectively. The performed biomarker analysis in this study showed a significant correlation between phosphorylated mTOR and OS, whereas various biomarkers had a significant predictive discrimination power of best response, with phosphorylated p44/42 staining being the most predictive.[223]

Overall, despite the preclinical evidence that mTOR is a promising therapeutic target, the triple combination of platinum-based chemotherapy, cetuximab and everolimus demonstrated poor tolerability with unexpected toxicities even at low dose levels. Although the reasons for this increased incidence of toxicities remain unclear, it is possible that cis/carboplatin could have exacerbated the toxicities of the targeted agents.[222, 223]

Nevertheless, based on the promising results of a phase I/II clinical study in advanced NSCLC patients[224], the dual combination of everolimus with the EGFR inhibitor erlotinib was investigated in a phase II clinical trial in R/M HNSCC patients. This study hypothesized that inhibition of the PI3K/Akt pathway via mTOR may also enhance the effectiveness of erlotinib in R/M HNSCC and prevent or delay the emergence of resistance (NCT00942734). The most frequent grade ≥3 side-effects included mucositis (17%), fatigue (14%), diarrhea, rash, infections and head and neck edema (each 8%). Regarding the efficacy in 35 evaluable patients, three patients (8%) showed PR at 4 weeks, one of which was confirmed at 12 weeks. Disappointingly, the overall RR at 12 weeks was only 2.8%, with the median duration of response (from first response to progressive disease) being 1.9 months. In addition, SD was observed in 27 patients (77%) at 4 weeks, with 11 (31%) confirmed at 12 weeks. Median PFS and OS was 11.9 weeks and 10.25 months, respectively.[226] In comparison with the results of a previously published phase II clinical trial evaluating erlotinib as single agent in R/M HNSCC[227], the

767 combination of everolimus with erlotinib failed to improve the clinical efficacy of erlotinib in R/M
768 HNSCC patients.
769
770 **6. Conclusions and future perspectives**
771 Therapeutic resistance remains a major problem in the field of HNSCC and limits the efficacy of
772 available treatment regimens with EGFR-targeted therapies. The two main pathways downstream of
773 EGFR i.e. Ras/Raf/MAPK pathway and PI3K/Akt pathway are highly interconnected and can both be
774 stimulated by activated Ras following EGFR stimulation. Due to its close interaction with the EGFR
775 pathway, redundant or compensatory activation of the PI3K/Akt pathway has been proposed as one
776 of the major drivers of resistance to EGFR inhibitors. Therefore, the current work thoroughly reviewed
777 the role of Ras, PI3K, PTEN, Akt and mTOR in resistance to anti-EGFR treatment in HNSCC. Genomic
778 alterations in and/or overexpression of one or more of these proteins are common in both HPV-
779 positive and HPV-negative HNSCC tumors. Although no definitive predictive biomarkers have been
780 identified so far, a large set of genomic and proteomic studies indicate that alterations in the PI3K/Akt
781 pathway are important players underlying resistance to EGFR-targeted therapies. As the role of *RAS*
782 mutations and increased mTOR activity in the prediction of resistance is not unambiguously, we
783 believe future studies should focus on the validation of *PIK3CA* mutations, loss of PTEN expression and
784 elevated phospho-Akt levels as predictive biomarkers in larger cetuximab-based clinical trials. This
785 would support optimal patient selection, ultimately resulting in increased response rates to cetuximab-
786 based therapies.
787 Besides proper patient selection, co-targeting EGFR and the PI3K/Akt pathway is the most promising
788 therapeutic strategy to overcome EGFR-targeted therapy resistance in the treatment of head and neck
789 cancer. Various preclinical studies have provided encouraging results, showing that the combination
790 of EGFR and PI3K/Akt pathway inhibitors often leads to synergistic anti-tumor effects. However, this
791 could not always be translated to the patient, as certain combinations resulted in substantial toxicity
792 and/or limited clinical efficacy in clinical studies. In general, combinations with PI3K inhibitors have
793 shown more favorable results in terms of toxicity and preliminary clinical efficacy compared to mTOR
794 inhibitors. Moreover, based upon the study of Brisson et al.[200], buparlisib is regarded as the most
795 promising PI3K inhibitor to combine with EGFR inhibition for the treatment of HNSCC patients. In order
796 to improve preclinical to clinical translation in the future, we recommend the use of three-dimensional
797 patient-derived HNSCC organoids for the further development of novel combination regimens with
798 PI3K inhibitors. Patient-derived HNSCC organoids have recently emerged as a novel preclinical model
799 in cancer research and offer the possibility to accurately predict drug response of individual HNSCC
800 patients in the clinic.[228-230] Additionally, these models are faster, easier and less expensive to generate
801 than patient-derived xenograft mouse models.

We believe that future (pre)clinical studies should focus on combinations with PI3K inhibitors (more specifically buparlisib) rather than on mTOR inhibitors, due to the significant toxicity profile of the latter seen in combination with EGFR-targeted therapies. Further evaluation of other therapeutic strategies involving the PI3K/Akt pathway besides targeting PI3K, Akt and mTOR in combination with EGFR-targeted therapies might also lead to effective circumvention of resistance to EGFR inhibition. For example, future studies could explore, although challenging, novel methods such as protein delivery, miRNA targeting and gene editing to restore the loss of PTEN protein expression in HNSCC tumors.

In future clinical studies, it is important to stratify patients based on their HPV status, as two phase III trials (RTOG 1016[231] and De-ESCALaTE[232]) recently showed that HPV-positive HNSCC patients are not very responsive to cetuximab treatment, indicating the need for different treatment approaches in this subset of patients. Activating mutations in *PIK3CA* and *PTEN* loss are more frequently occurring events in HPV-positive HNSCC, whereas EGFR overexpression and amplification are mostly seen in HPV-negative HNSCC. Together with the fact that the expression of HPV viral oncoproteins can contribute to the activation of the PI3K/Akt pathway, this indicates that tumor growth in HPV-positive HNSCC is mostly driven by PI3K/Akt pathway signaling rather than by signaling through EGFR. Therefore, monotherapeutic approaches with PI3K/Akt pathway inhibitors should be considered as a promising strategy for future clinical trials in HPV-positive HNSCC patients. On the other hand, HPV-negative HNSCC patients might be the population that could mostly profit from the described co-targeting approaches in this review. In light of the recent success of the anti-programmed cell death 1 immune checkpoint inhibitor pembrolizumab[233] and the potential immunomodulating effects of PI3K inhibition[173], it might be interesting to investigate a triple combination strategy consisting of an EGFR inhibitor, a PI3K inhibitor and an immune checkpoint inhibitor in future studies. However, more research on the potential impacts of PI3K inhibitors on the immune system is still needed to provide a strong rationale for the proposed triple combination therapy.

In conclusion, we can state that, based upon the information summarized in this review, inhibition of the PI3K/Akt pathway will play an important role in improving the therapeutic response in HNSCC.

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Biosketches

Hannah Zaryouh

Hannah Zaryouh graduated summa cum laude in 2019 from the Faculty of Pharmaceutical, Biomedical and Veterinary Sciences at the University of Antwerp (Belgium), with a M.Sc. in Biomedical Sciences – Molecular and Cellular Biomedical Sciences. In the same year, she started her Ph.D. project focusing on novel therapeutic strategies for head and neck cancer at the Center for Oncological Research (CORE) at the University of Antwerp thanks to the starting grant "Emmanuel van der Schueren 2019" ("Kom op tegen Kanker"/Stand Up To Cancer).

Ines De Pauw

Ines De Pauw graduated in Molecular and Cellular Biomedical Sciences with high distinction at the University of Antwerp. In September 2014, she initiated her Ph.D. study at the Center for Oncological Research Antwerp (University of Antwerp) thanks to the starting grant "Emmanuel van der Schueren 2014" ("Kom op tegen Kanker"/Stand Up To Cancer). In January 2015, she received a grant of the University Research Fund (UA BOF DOCPRO) to continue her Ph.D. thesis. Her Ph.D. concentrated on the identification of new predictive biomarkers for the use of EGFR-targeted therapies as well as testing novel combination therapies in order to overcome intrinsic and acquired resistance to these EGFR targeting drugs. She obtained her Ph.D. degree (Medical Sciences) at the Faculty of Medicine, University of Antwerp, in 2019. She is now working as a postdoctoral researcher at the Center for Oncological Research Antwerp (University of Antwerp) with the financial support of "Kom op tegen Kanker" (Stand Up To Cancer). Her research interest focuses on targeted therapies and immune therapy in head and neck squamous cell carcinoma.

Hasan Baysal

Hasan Baysal earned his M.Sc. degree majoring in Molecular and Cellular sciences with great distinction in 2017 from the university of Antwerp based on his undergraduate thesis, titled "In vitro study on the effectiveness of afatinib to overcome cetuximab resistance in colorectal cancer." with dr. Ines De Pauw. Based on his contributions to this work, he was awarded a co-authorship publication as well as the opportunity to start a graduate fellowship under supervision of prof. dr. An Wouters, in the research group for "Targeted and Combination Therapy Team" at the Center for Oncological Research Antwerp (CORE, University of Antwerp). His current research focuses on investigating drug combinations that target both the epidermal growth factor receptor and the innate immune system as a novel therapeutic approach for the treatment of head and neck cancer. The early results and collaborations with fellow researchers have translated in one first-author publication in the 'British Journal of Cancer' and several additional co-authorship publications. In addition, he is also working on the characterization of the NK cell population in head and neck cancer patients and the identification of novel biomarkers and targetable molecules as a novel therapeutic approach for the treatment of head and neck cancer. Besides his scientific research output, he has mentored two master dissertations, both of which have inspired aspiring early researchers to pursue doctoral fellowships.

Marc Peeters

Marc Peeters is Professor of oncology at the Antwerp University (Belgium). He is head of the oncology department at the Antwerp University Hospital and coordinator of the Multidisciplinary Oncology Center Antwerp. He is also chairman of the College of Oncology. Previously, he was Coordinator of the Digestive Oncology Unit at The University Hospital in Ghent (Belgium). He completed his medical studies at the Catholic University in Leuven (Belgium). He finished his training in Internal Medicine at the UZ Gasthuisberg in Leuven and underwent additional training in Oncology and Digestive Oncology at the UZ Gasthuisberg, the Institut Gustave Roussy in Villejuif, the University of Pennsylvania Hospital in Philadelphia, the Royal Marsden Hospital in London, and the Memorial Sloan-Kettering Cancer Center in New York. Dr. Peeters is Secretary of the Flemish Society of Gastroenterology. He is treasurer of the Belgian Group of Digestive Oncology and member of the Belgian Society of Medical Oncology, The European Society of Medical Oncology, The American Society for Clinical Oncology, and the gastrointestinal group of the European Organization for Research and Treatment of Cancer. His research expertise includes the identification of molecular markers and therapy modulation in

digestive tumors. He has been involved in many clinical studies on therapeutic agents for gastrointestinal tumors.

Jan B. Vermorken

Jan B. Vermorken graduated in 1970 (University of Amsterdam, Netherlands), became board-certified specialist in internal medicine in 1975, received his Ph.D. in Medical Sciences in 1986 and was officially registered as a Medical Oncologist in 1992. From May 1997 until October 1, 2009, he was Professor of Oncology at the University of Antwerp, and Head of the Department of Medical Oncology at the University Hospital Antwerp (UZA), in Edegem, Belgium. After his retirement he remained connected to both University and UZA (consultant). His main fields of interest are gynecologic oncology and head and neck oncology. He was founding chair of the Gynecologic Cancer InterGroup (1997–2003), and strongly involved in establishing the Head and Neck Cancer International Group (HNCIG) in 2015 and chaired both the EORTC Gynecologic Cancer Group (1983–1989) and the EORTC Head and Neck Cancer Group (2006–2009)). He devotes a large part of this time to teaching, professional training, and continuing medical education. Professor Vermorken is member of multiple scientific societies and editorial boards of International journals, reviewer of many cancer journals, and (co)author of more than 700 publications. He was Editor-in-Chief of Annals of Oncology (2009-2014), and is chief editor of the head and neck cancer section of The Oncologist (since 2003), and the head and neck section of Frontiers in Oncology (since 2015). He received the ESMO award in 2007 and on March 1, 2013 he received the title of Commander in the Order of Leopold for his contributions to oncology.

Filip Lardon

Filip Lardon studied biology/physiology at the University of Hasselt (bachelor's degree, 1985–1987) and the University of Antwerp (master's degree, 1987–1989). In 1995, he obtained his Ph.D. degree in Medical Sciences at the Faculty of Medicine and Health Sciences, University of Antwerp (doctoral thesis "Cell cycle kinetics of human bone marrow progenitors: in vitro effects of hematopoietic growth factors and growth inhibitors"). In 1998, he was appointed as associate professor at the department of Oncology at the University of Antwerp, and in 2012, he became full professor and head of the Center for Oncological Research. He is (co)author of more than 150 international peer reviewed scientific publications, (co)promotor of more than 50 different research projects and author of 7 books. Since 2016, he is also vice-rector of the University of Antwerp.

An Wouters

An Wouters obtained her master's degree in Biomedical Sciences in 2004 at the University of Antwerp (UAntwerp, Belgium, summa cum laude). She performed her Ph.D.-research in the field of cancer research, focusing on combination therapies under normoxia and hypoxia, at the Center for Oncological Research (CORE, UAntwerp, prof. Dr. Filip Lardon, prof. Dr. Marc Peeters) and obtained her Doctor in Medical Science degree in 2010. As postdoctoral researcher, she oriented her research interests towards targeted cancer therapy and the role of the hypoxic microenvironment. Currently, she is professor in Experimental Oncology and coordinator of the 'Targeted and Combination Therapy Team' at CORE (UAntwerp). She is (co-)author of more than 55 international peer-reviewed scientific publications, with a H-index of 15.

Tables

Table 1 Preclinical trials evaluating PI3K/Akt pathway inhibitors in combination with EGFR-targeted therapy in patients with HNSCC.

Treatment	Experimental setting	Treatment schedule	Effect	Possible mechanism involved	Reference
PI3K inhibitors in combination with EGFR inhibition					
Cetuximab + alpelisib	*In vitro* *In vivo:* xenograft model (type NA)	Simultaneously Duration NA	Synergism	Combined inhibition of EGFR and PI3Kα	[163]
	In vivo: CLX-model	Simultaneously Duration NA	Additive effects	Combined inhibition of EGFR and PI3Kα	[164]
Cetuximab + PX-866	*In vivo:* PDX-model	Simultaneously 25-29 days	Additive to synergistic effects	Combined inhibition of EGFR and PI3K	[165]
Cetuximab + buparlisib +/- RT	*In vitro*	Cytotoxicity assay and western blot Sequentially: cetuximab (+/- RT) → buparlisib Each drug 2 days with/without RT on day 1 Sequentially: buparlisib (+/- RT) → cetuximab Each drug 2 days with/without RT on day 1 Simultaneously: Buparlisib + cetuximab +/- RT 4 days with/without RT on day 1	cetuximab → buparlisib: synergism cetuximab + RT → buparlisib: synergism in cetuximab-sensitive cell line buparlisib → cetuximab: antagonism buparlisib + RT → cetuximab: NA buparlisib + cetuximab: antagonism buparlisib + cetuximab + RT: NA	Cetuximab-sensitive cell line Synergism: activation of mTORC2 complex and caspase proteins Cetuximab-resistant cell line Synergism: higher sensitivity of mutated cells to PI3K inhibition No synergistic effect with RT: EGFR-ERK signaling induced by radiation and an increase in DNA repair protein levels in a MAPK-dependent manner, resulting in radioresistance Antagonism: activation of alternative pathways	[166]
	In vivo: CLX-model	Simultaneously *In vivo* growth experiments: 10 days Buparlisib: 5 days a week Cetuximab: once a week RT: 3 days a week	buparlisib + cetuximab: additive effects buparlisib + cetuximab + RT: synergism	Buparlisib + cetuximab: combined inhibition of MAPK and PI3K pathway, resulting in antiproliferative effects Buparlisib + cetuximab + RT:	[167]

				induction of apoptotic cell death	
Cetuximab + copanlisib	*In vivo:* PDX-model	Simultaneously *In vivo* growth experiments: 21 days	Tumor control and improved tumor response	Combined inhibition of EGFR and PI3K	[168]
Cetuximab + LY294002	*In vitro*	Simultaneously Growth inhibition assay and cell cycle analysis: 3 days	Growth inhibition and restored cetuximab sensitivity of resistant cells	Reduction in Akt phosphorylation and cell cycle arrest in G_0/G_1	[116]
Erlotinib + pictilisib	*In vitro*	Simultaneously Cytotoxicity assay: 3 days	Synergism	Combined inhibition of EGFR and PI3K	[169]
Akt inhibitors in combination with EGFR inhibition					
Cetuximab + MK2206	*In vitro*	Simultaneously Cytotoxicity assay: cetuximab 7 days and MK2206 last 3 days	Additive to synergistic effects	Inhibition of Akt phosphorylation	[172]
mTOR inhibitors in combination with EGFR inhibition					
Erlotinib + temsirolimus	*In vivo:* CLX-model	Simultaneously *In vivo* growth inhibition assay: 28 days FNA biopsies: 7 days	Additive effect in erlotinib-sensitive cell line No synergistic effect in erlotinib-resistant cell line	Inhibition of Akt activity, MAPK and p70 phosphorylation	[183]
RT + cetuximab + bevacizumab + temsirolimus	*In vivo:* CLX-model	Simultaneously *In vivo* growth experiments: 14 days Each drug: 5 days a week RT: 3 days a week	Additive effects	Inhibition of Akt phosphorylation and reduction of cell proliferation	[184]
Cetuximab + rapamycin/everolimus	*In vivo:* CLX-model	Simultaneously *In vivo* growth experiments: 22 days – 50 days Western blot: 4 days and 20 days	Improved anti-tumor response (rapid tumor collapse)	Decreased cell proliferation, inhibition of lymphangiogenesis and increased autophagy ADCC effect of cetuximab might synergize with mTOR signaling inhibition	[115]
Cetuximab + cisplatin + 5-FU + temsirolimus	*In vivo:* CLX-model	Simultaneously *In vivo* growth experiments: 10 days Temsirolimus: 5 days a week Cetuximab: once a week Cisplatin + 5-FU: once a week	Cetuximab + temsirolimus: synergism Full combination: no synergistic effects	Combined inhibition of EGFR/MAPK and mTOR pathway Inhibition of tumor vessel formation	[158]
Cetuximab + temsirolimus	*in vitro*	Cytotoxicity assay and western blot Simultaneously	Synergism	Downregulation of pEGFR, pAkt, p-p70S6K1 and p4E-BP1	[185]

		4 days Sequentially: Cetuximab → temsirolimus Each drug 2 days Sequentially: Temsirolimus → cetuximab Each drug 2 days	Antagonism Additive effects	Upregulation of pEGFR, p-p70S6K1 and p4E-BP1 and downregulation of pAkt Upregulation of pEGFR, p-p70S6K1 and p4E-BP1	
	In vitro	Simultaneously Cytotoxicity assay: short term 3 days or long term 7 days	Increased growth-inhibitory effects Restored cetuximab sensitivity of resistant cells	Combined inhibition of EGFR and mTOR	[186]
Erlotinib + OSI-027 (*in vitro*)	*In vitro*	Simultaneously *In vitro* Cytotoxicity assay: 3-5 days Western blot: 24h Clonogenic assay: 5 days	*In vitro:* synergism	Enhanced inhibition of mTORC1/2 activity and downstream effectors	[187]
Cetuximab + OSI-027	*In vivo:* CLX-model	Simultaneously 14 days	Reduced tumor volume	Enhanced inhibition of mTORC1/2 activity and downstream effectors	[187]
Cetuximab + AZD8055	*In vitro* *In vivo:* PDX-model	Simultaneously *In vitro* Growth inhibition assay: 3-4 days Clonogenic assay: 7-21 days *In vivo:* 14 days	No synergistic effects in three cell lines Additive effects in two other cell lines At least additive effects *in vivo*	Cetuximab: reduction of pEGFR and pMAPK1 AZD8055: reduction of pAkt and pS6	[188]
Dual PI3K/mTOR inhibitors in combination with EGFR inhibition					
Cetuximab + PKI-587	*In vitro* *In vivo:* CLX-model	Simultaneously *In vitro* Cell density assay: NA Western blot and ELISA analysis: 24h *In vivo*: 21 days	Synergism	Dephosphorylation/inactivation of Akt, p70S6K and pERK1/2 Cetuximab-resistant cell lines: induction of autophagy cell death	[161]

				Cetuximab-sensitive cell lines: induction of apoptotic cell death	
Cetuximab + NVP-BEZ-235	*In vitro*	Simultaneously Growth inhibition assay: 3-4 days Clonogenic assay: 7-21 days	No synergistic effects in three cell lines Additive effects in two other cell lines	Combined inhibition of EGFR, PI3K and mTOR	[188]

Abbreviations: NA, not available; 5-FU, 5-fluorouracil; CLX-model, cell line xenograft model; PDX-model, patient-derived xenograft model; RT, radiotherapy; ADCC, antibody-dependent cellular cytotoxicity; FNA, fine needle aspiration.

Table 2 Clinical trials evaluating PI3K/Akt pathway inhibitors in combination with EGFR-targeted therapy in patients with HNSCC.

Clinical trial identifier	Phase	Initiation of the study	Treatment schedule	Tumor type	Outcome	Status	Reference
PI3K inhibitors in combination with EGFR-targeted therapy							
Alpelisib							
NCT01602315	Ib/II	2012	Arm A: alpelisib 300 or 400 mg/day (tablets) with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks) Arm B: alpelisib 300 mg/day (drinkable suspension) with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks)	R/M HNSCC	MTD: alpelisib 300 mg/day (tablets) Arm A: at 300 mg/day: 1/10 PR 3/10 unconfirmed PR 5/10 SD at 400 mg/day: 1/2 PR 1/2 PD Arm B: no responses	Terminated due to slow recruitment	[192]
NCT02282371	I	2014	Alpelisib 200-300 mg/day with cetuximab 400 mg/m^2 prior to IMRT and then at 250 mg/m^2/week during IMRT (1 fraction/day up to a total of +/- 70 Gy)	Locoregionally advanced HNSCC	MTD: alpelisib 250 mg/day 11/11 CR 10/11 disease free (median follow-up period 23.5 months)	Active, not recruiting	[193]
NCT02298595	I/II	2014	Alpelisib 200-350 mg/day with cisplatin 75 mg/m^2/week and cetuximab 400 mg/m^2 and then at 250 mg/m^2/week	HPV-associated oropharyngeal HNSCC	NA	Withdrawn	https://clinicaltrials.gov
PX-866							

NCT01252628	I/II	2010	Phase I: PX-866 6 or 8 mg/day with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week IV (cycle of 3 weeks) Phase II: Arm A: PX-866 8 mg/day with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 3 weeks) Arm B: PX-866 8 mg/day (cycle of 3 weeks)	R/M HNSCC, metastatic colorectal carcinoma	Phase I: MTD: PX-866 8 mg/day 4/9 PR 4/9 SD 1/9 PD Phase II: Arm A: median PFS: 80 days Median OS: 211 days 4/42 PR 19/42 SD 14/42 PD Arm B: median PFS: 80 days Median OS: 256 days 3/41 PR 20/41 SD 11/41 PD	Completed	[196, 198]
Buparlisib							
NCT01816984	I/II	2013	Buparlisib 100 mg/day for a 7-day run-in period followed by buparlisib 80-100 mg/day with cetuximab 500 mg/m^2 every 14 days	R/M HNSCC	MTD: buparlisib 100 mg/day 1/12 PR 4/12 SD 5/12 PD Cetuximab pre-treated patients: 1/11 PR 3/11 SD	Active, not recruiting	[200]
Copanlisib							
NCT02822482	Ib/II	2016	Copanlisib with cetuximab every week (cycle of 4 weeks), dosing regimens NA	R/M HNSCC with PI3KCA mutation/amplification and/or PTEN loss	NA	Active, not recruiting	https://clinicaltrials.gov
mTOR inhibitors in combination with EGFR-targeted therapy							
Temsirolimus							
NCT01015664	I/II	2009	Cisplatin 75 mg/m^2 on day 1 with temsirolimus 10-25 mg/week and cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks)	R/M HNSCC	NA	Terminated	https://clinicaltrials.gov

NCT01552434	I	2012	Temsirolimus 5 or 12.5 mg/week with bevacizumab 2.5-10 mg/kg (day 1 and 15) and cetuximab 100 mg/m^2 and then at 75 mg/m^2/week (cycle of 4 weeks)	Advanced/metastatic tumors, including HNSCC	MTD: temsirolimus 5 mg/week with bevacizumab 10 mg/kg biweekly and cetuximab 100/75 mg/m^2/week 2/18 PR 4/18 SD HNSCC patients: 2/8 PR 1/8 SD	Recruiting	202
NCT02215720	I	2014	Cetuximab 400 mg/m^2 loading dose and then 7 days later cetuximab 150-250 mg/m^2/week with temsirolimus 15-25 mg/week	Advanced/metastatic solid tumors, including HNSCC	MTD: cetuximab 250 mg/m^2/week with temsirolimus 25 mg/week Median PFS: 2.0 months Median OS: 7.5 months 2/39 PR 18/39 SD	Unknown	201
NCT02215720	I	2014	Temsirolimus 15 mg with cetuximab 400 mg/m^2, more detailed regimen NA	Advanced/metastatic solid tumors	NA	Unknown	https://clinicaltrials.gov
NCT01009203	II	2009	Temsirolimus 15 mg/week and erlotinib 150 mg/day (cycle of 4 weeks)	R/M HNSCC	Median PFS: 1.9 months Median OS: 4.0 months 1/9 PR (patient withdrawn due to toxicity)	Terminated due to high patient withdrawal rate	213
NCT01256385	II	2010	Arm A: temsirolimus 25 mg/week with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks) Arm B: temsirolimus 25 mg/week (cycle of 4 weeks)	R/M HNSCC	Arm A: median PFS: 89.0 days Median OS: 205 days 1/40 CR 4/40 PR Arm B: median PFS: 93.5 days Median OS: 181 days 1/40 PR	Completed	215, 216
Everolimus							
NCT01009346	I/II	2009	Everolimus 2.5-10 mg/day with cetuximab 250 mg/m^2/week and cisplatin 40 mg/m^2 (day 1 and 8) or carboplatin (day 1 and 8) using the Calvert formula (cycle of 4 weeks)	R/M HNSCC	Median PFS: 2.8 months Combination was poorly tolerated even at the lowest dose level of everolimus 2.5 mg/day	Terminated due to toxicity	222

NCT01332279	I	2011	Erlotinib in combination with everolimus and radiotherapy, dosing regimens NA	R/M HNSCC	NA	Withdrawn (sponsor withdrawal)	https://clinicaltrials.gov
NCT01283334	I/II	2011	Everolimus 2.5-10 mg/day with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week and carboplatin at doses sufficient to produce an area under the curve of 2 mg/ml/min on days 1, 8, and 15 (cycle of 4 weeks)	R/M HNSCC	MTD: everolimus 2.5 mg every other day Objective RR: 61% Median PFS: 8.15 months 8/13 PR	Completed	[223]
NCT01637194	I	2012	Everolimus daily with cetuximab weekly, dosing regimens NA	R/M HNSCC or colon cancer	NA	Completed	https://clinicaltrials.gov
NA	I	NA	Arm A: everolimus 30-70 mg/week for 3 weeks followed by everolimus 30-70 mg/week with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks) Arm B: cetuximab 400 mg/m^2 and then at 250 mg/m^2/week for 3 weeks followed by everolimus 30-70 mg/week with cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 4 weeks)	Advanced malignancies	MTD: everolimus 70 mg/week 5/16 SD	Completed	[217]
NCT00942734	II	2009	Everolimus 5 mg/day with erlotinib 150 mg/day (cycle of 4 weeks)	R/M HNSCC	Median PFS: 11.9 weeks Median OS: 10.25 months At 4 weeks: 3/35 PR 27/35 SD At 12 weeks: 1/35 PR 11/35 SD	Completed	[226]
NCT01133678	II	2010	Arm A: everolimus 5 mg/day with cisplatin 75 mg/m^2 (day 1), paclitaxel 175 mg/m^2 (day 1) and cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 3 weeks)	LA HNSCC	NA	Unknown	https://clinicaltrials.gov

			Arm B: placebo daily with cisplatin 75 mg/m^2 (day 1), paclitaxel 175 mg/m^2 (day 1) and cetuximab 400 mg/m^2 and then at 250 mg/m^2/week (cycle of 3 weeks)				
Sirolimus							
NCT00940381	I	2009	Sirolimus 3 mg and then at 1mg/day with cetuximab 100 mg/m^2 and then at 65 mg/m^2/week (cycle of 4 weeks)	Advanced malignancies	NA	Completed	https://clinicaltrials.gov
Ridaforolimus							
NCT01212627	I	2010	Ridaforolimus 20 mg/day with cetuximab, dosing regimen NA (cycle of 4 weeks)	Advanced HNSCC, lung and colon cancer	NA	Terminated (Determination to stop enrollment made due to funding)	https://clinicaltrials.gov

Abbreviations: NA, not available; R/M HNSCC, recurrent/metastatic head and neck squamous cell carcinoma; MTD, maximum tolerated dose; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival; IMRT, intensity-modulated radiation therapy.

Figures

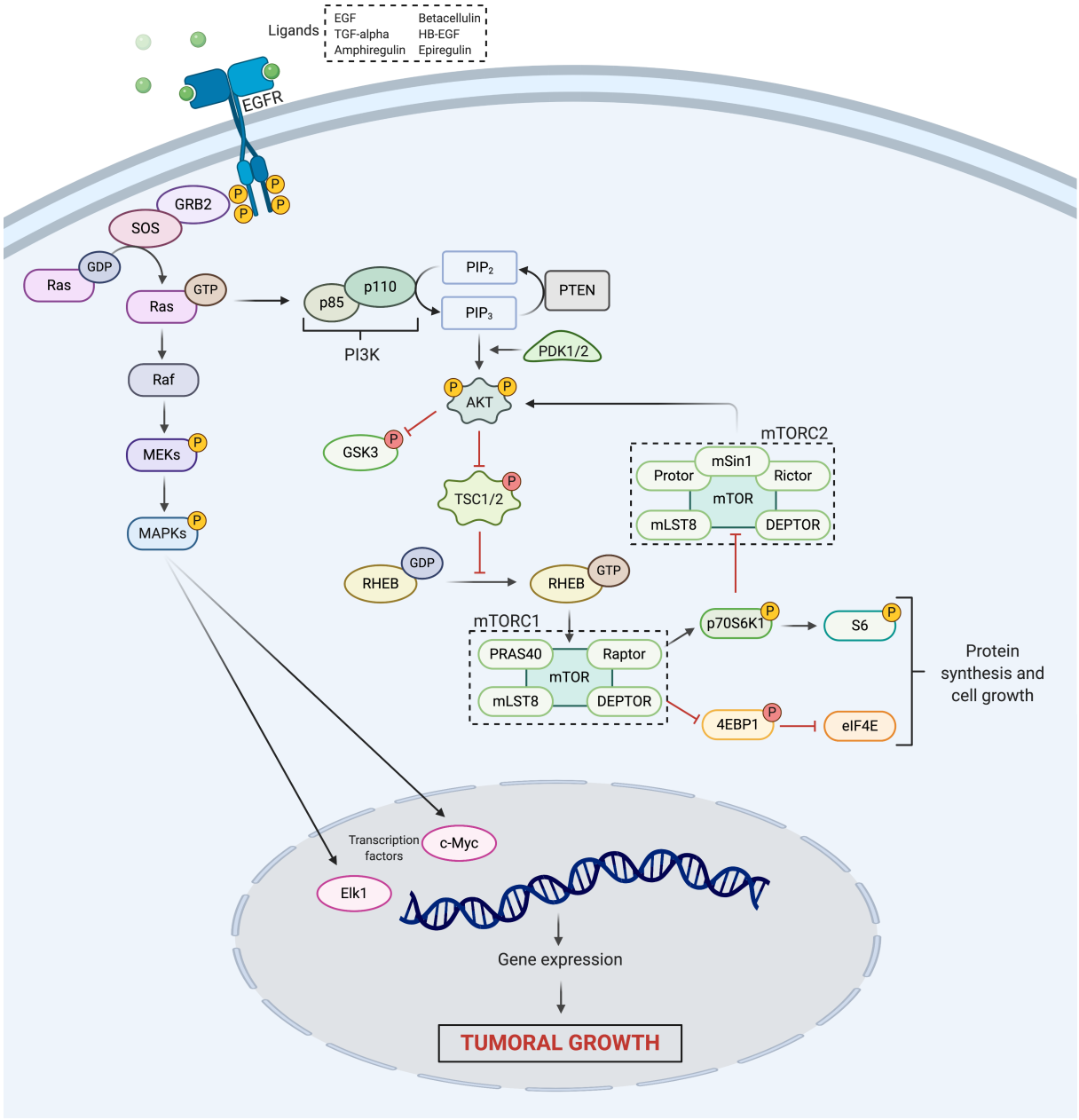

Figure 1 Schematic overview of the crosstalk between EGFR stimulation and the PI3K/Akt signaling pathway through activated Ras. Physiological or oncogenic activation of Ras leads to the stimulation of the Raf/MAPK signaling pathway. The activation signal can also be transferred to the PI3K/Akt pathway by binding of activated Ras to the p110 catalytic subunit of PI3K, showing that these pathways are highly interconnected. Activated PI3K phosphorylates PIP_2 to PIP_3, which activates Akt through phosphorylation by PDK1/2 and mTORC2. Activation of Akt leads directly or indirectly to the phosphorylation of a variety of downstream effectors, such as mTOR and GSK3, that affect cell growth, cell cycle entry and survival. Other pathways following EGFR activation are not shown. 'P' in a yellow circle indicates phosphorylation with activating effects. 'P' in a red circle indicates phosphorylation

with inhibitory effects. This figure was adapted from “PI3K/Akt, RAS/MAPK, JAK/STAT Signaling”, by BioRender.com (2021) and retrieved from https://app.biorender.com/biorender-templates.

Abbreviations: EGF, epidermal growth factor; TGF-alpha, transforming growth factor alpha; HB-EGF, heparin-binding epidermal growth factor; EGFR, epidermal growth factor receptor; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless adaptor protein; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Ras, kirsten rat sarcoma viral oncogene homolog; MEKs, mitogen-activated protein kinase kinases; MAPKs, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol (4,5)-biphosphate; PIP_3, phosphatidylinositol (3,4,5)-triphosphate, PTEN, phosphatase and tensin homolog; PDK1/2, phosphoinositide-dependent kinase-1/2; GSK3, glycogen synthase kinase 3; TSC1/2, tuberous sclerosis complexes 1 and 2; RHEB, Ras homolog enriched in brain; mTORC1, mammalian target of rapamycin complex 1; PRAS40, proline-rich Akt substrate 40 kDa; DEPTOR, disheveled, Egl-10, and pleckstrin domain-containing mTOR-interacting protein; mLST8, mammalian lethal with SEC13 protein 8; Raptor, regulatory-associated protein of mTOR; mTORC2, mammalian target of rapamycin complex 2; Rictor, rapamycin-insensitive companion of mTOR; Protor, protein observed with rictor; mSin1, mammalian stress-activated protein kinase interacting protein 1; p70S6K1, ribosomal p70S6 kinase 1; S6, ribosomal protein S6; 4EBP1, eukaryotic initiation factor 4E binding protein 1; eIF4E, eukaryotic initiation factor 4E.

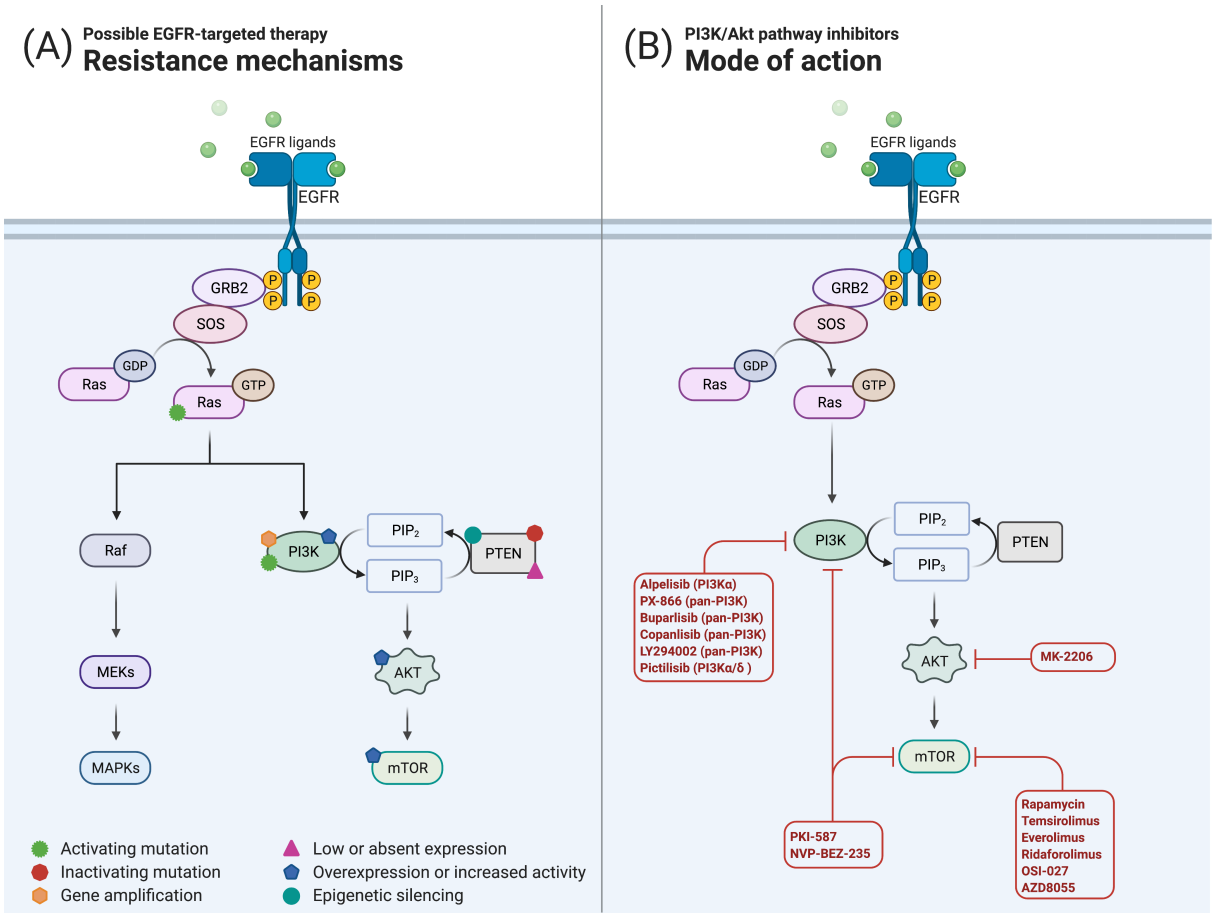

Figure 2 Schematic representation of possible resistance mechanisms and PI3K/AKT pathway inhibitors described in this review. (A) Possible resistance mechanisms to EGFR-targeted therapies in HNSCC focusing on the PI3K/AKT pathway that could explain aberrant activation of this pathway during EGFR blockade. Alterations of the pathway components are indicated with different symbols. (B) Overview of PI3K, Akt, mTOR and dual PI3K/mTOR inhibitors indicating their mode of action in the PI3K/Akt signaling pathway. PI3K isoform selectivity is shown between brackets for the PI3K inhibitors. This figure was created with BioRender.com.

Abbreviations: EGFR, epidermal growth factor receptor; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless adaptor protein; GDP, guanosine diphosphate; GTP, guanosine triphosphate; MEKs, mitogen-activated protein kinase kinases; MAPKs, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol (4,5)-biphosphate; PIP_3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog; mTOR, mammalian target of rapamycin.

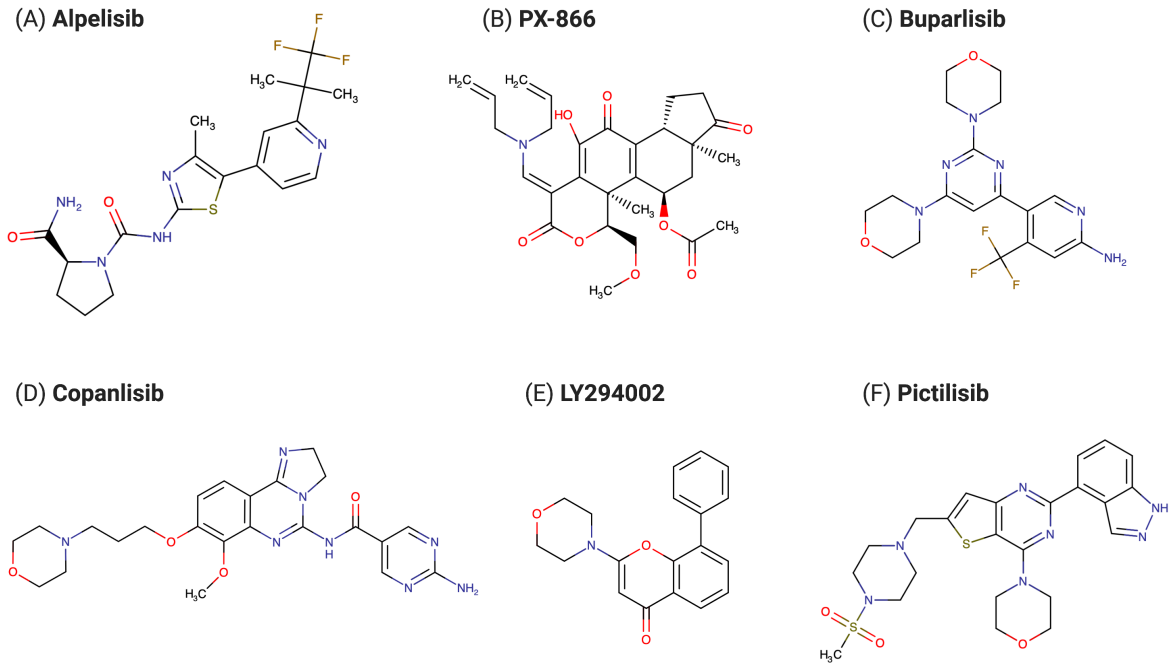

Figure 3 Structure of phosphatidylinositol 3-kinase inhibitors. (A) Alpelisib, a PI3Kα-selective inhibitor. (B) PX-866, a pan-PI3K inhibitor. (C) Buparlisib, a pan-PI3K inhibitor. (D) Copanlisib, a pan-PI3K inhibitor with preferential activity against PI3Kα and PI3Kδ. (E) LY294002, a pan-PI3K inhibitor and (F) pictilisib, a PI3Kα/δ-selective inhibitor.[234]

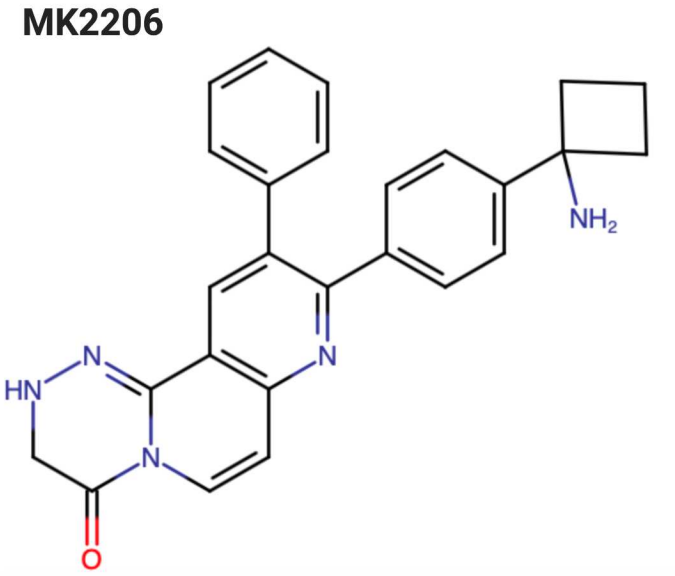

Figure 4 Structure of Akt inhibitor MK2206.[234]

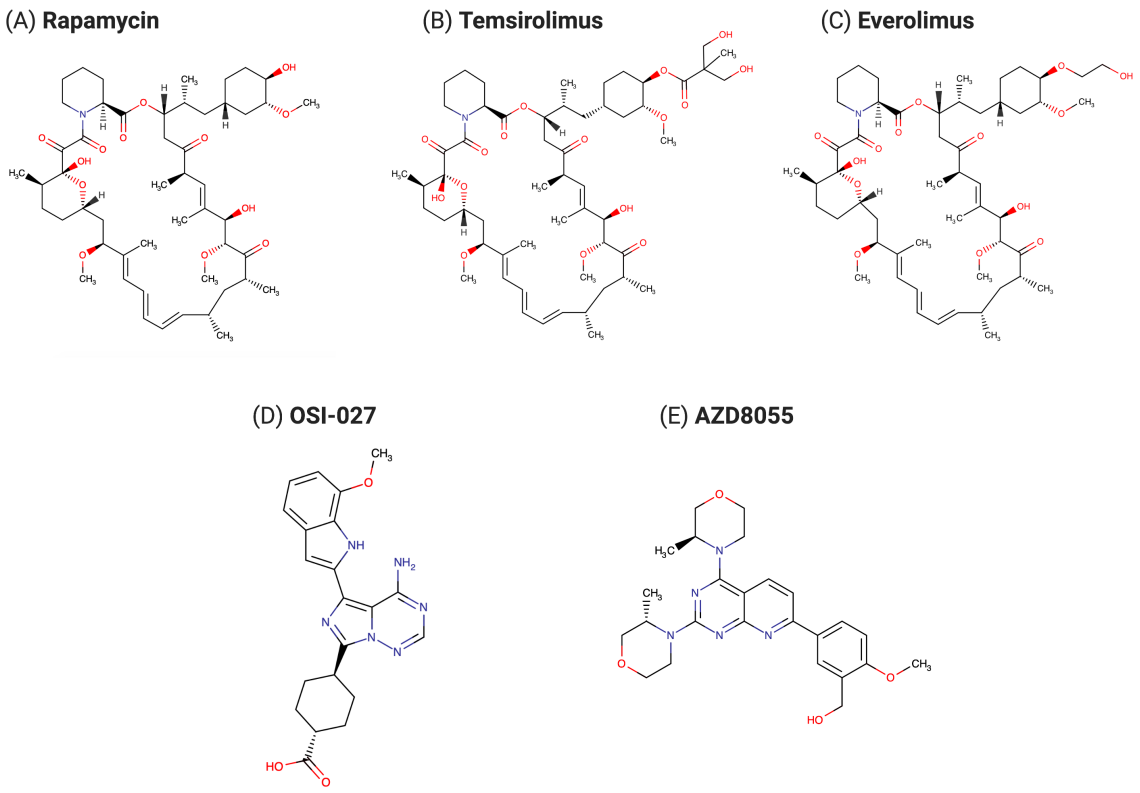

Figure 5 Structure of mammalian target of rapamycin inhibitors. First generation mTOR inhibitors (A) rapamycin, (B) temsirolimus and (C) everolimus, inhibiting only mTORC1. Second generation mTOR inhibitors (D) OSI-027 and (E) AZD9055, inhibiting both mTORC1 and mTORC2.

Abbreviations: mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2.[234]

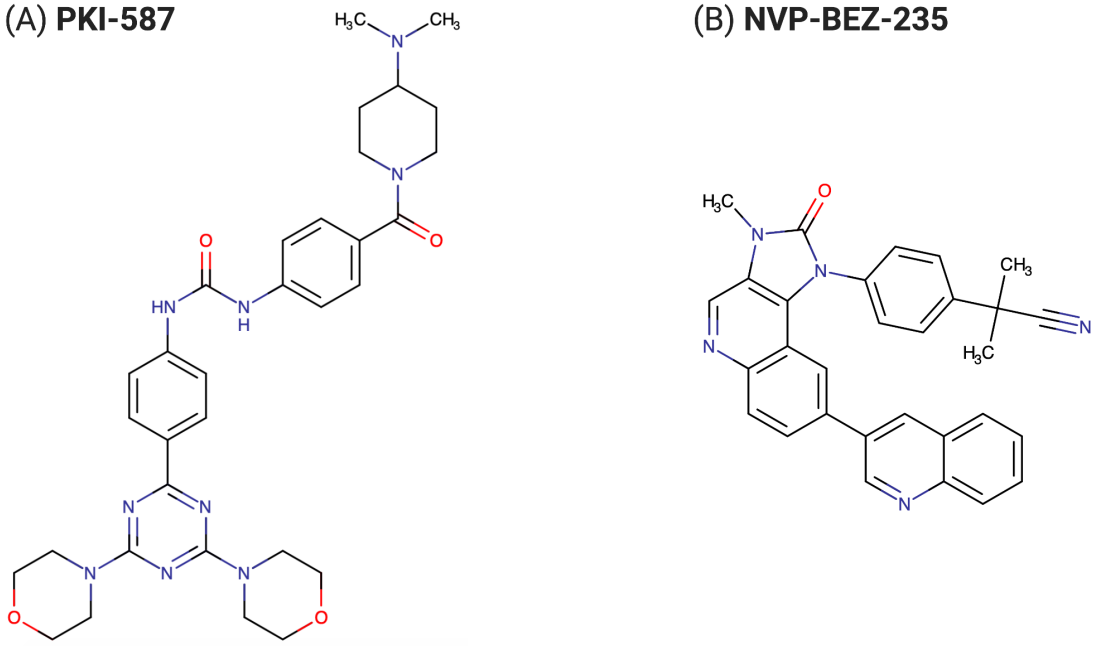

Figure 6 Structure of dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitors. (A) PKI-587 and (B) NVP-BEZ-235.[234]