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# Evaluation of Co-Inhibition of ErbB Family and PI3K Kinases for HPV-Negative Head and Neck Squamous Cell Carcinoma

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Keywords: HNSCC; PI3K inhibitors; ErbB inhibitor; targeted therapy

*Article*

# Evaluation of Co-Inhibition of ErbB Family and PI3K Kinases for HPV-Negative Head and Neck Squamous Cell Carcinoma

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**Abstract:** The ErbB/HER family of protein-tyrosine kinases (ErbB) and the phosphatidylinositol 3-kinase (PI3K) represent crucial targets in the treatment of head and neck squamous cell carcinoma (HNSCC). We previously reported that a combination therapy using Afatinib (ErbB inhibitor) and Copanlisib (PI3K inhibitor), both FDA-approved kinase inhibitors, suppressed the growth of HPV-positive HNSCC. In our current study, we further evaluated the efficacy and clinical potential of this combination therapy for treating HPV-negative HNSCC in vitro and in animal model. We found that the combination treatment of Afatinib and Copanlisib dramatically enhanced inhibition of cell proliferation and reduced cell survival when compared to treatment with either inhibitor in two HPV negative HNSCC cell lines. Notably, this combination led to significant inhibition of xenograft tumor growth in mice, without any apparent effects on body weight. Copanlisib alone effectively blocked PI3K/Akt signaling, but caused up-regulation of HER2 and HER3 phosphorylation as previously reported in other types of cancer. However, the combination treatment with Copanlisib and Afatinib completely blocked phosphorylation of the ErbB family (including HER3) and Akt, while also remarkedly increasing apoptosis. These results suggest that co-targeting the ErbB family and PI3K kinases by a combination treatment of Afatinib and Copanlisib can have clinical potential for patients.

**Keywords:** HNSCC; PI3K inhibitors; ErbB inhibitor; targeted therapy

## 1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer globally, with 890,000 new cases and 450,000 deaths each year [1-3]. Based on etiological factors, HNSCC is classified into two types of disease: either HPV-positive or HPV-negative. The occurrence of HPV-negative HNSCC is associated with the use of tobacco and excessive consumption of alcohol, while HPV-positive HNSCC is related to human papillomavirus (HPV) infection [4-6]. Reports indict that patients with HPV-positive HNSCC have a higher 5-year survival rate (~80%) than HPV-negative patients (~50%) [7]. However, since HPV-positive HNSCC occurs mainly in oral and oropharyngeal tissues rather than other regions of head and neck, and may also progress to recurrent/metastatic disease in a significant portion of patients [8], there is an urgent need for new therapeutics to treat both HPV-positive and HPV-negative HNSCC [9, 10].

Classical therapies for HNSCC patients without distant metastasis typically include surgical resection, radiation therapy, chemotherapy, or a combination of these regimens. The specific therapeutic approach depends on various factors such as pre-existing clinical conditions, location of the cancer, and the TNM stages of the tumor. A combination of these treatments could reduce the rate of recurrence and distant metastasis for patients with local-regional disease. However,

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chemotherapy remains the primary option for patients with recurrent and distant metastatic HNSCC [11]. Cisplatin has been the most commonly used anticancer drug for treatment of advanced HNSCC, but, while many newly diagnosed patients with advanced HNSCC initially respond well to cisplatin-based chemotherapies, most patients either have intrinsic resistance or will eventually develop acquired resistance to cisplatin, leading to death within one year [12]. Immunotherapy has been recently introduced for refractory HNSCC, but its impact has been limited [12, 13]. Therefore, it remains of the utmost importance to find new therapeutic alternatives

The epidermal growth factor receptor (EGFR), a member of the ErbB kinases family, is notably overexpressed in 90-95% of HNSCC, and plays a crucial role in the cancer's pathogenesis and clinical course [14-16]. EGFR controls the activation of several essential pathways such as PI3K/Akt/mTOR and RAS-RAF-MAPK (MEK)-ERK, which regulate cell proliferation, survival, and migration [17, 18]. In 2006, the monoclonal EGFR antibody cetuximab was approved by FDA for treatment of HNSCC in combination with the standard therapy [19-21]. However, the use of cetuximab resulted in very limited improvement in survival rates for patients undergoing cisplatin-based therapy [22]. In addition, small molecule kinase inhibitors like Gefitinib and Erlotinib, while effective in targeted therapies for Non-Small Cell Lung Cancer (NSCLC), have not demonstrated any benefits for HNSCC patients [23, 24].

Increasing evidence demonstrates the importance of the ErbB family, which contains EGFR, HER2, HER3, and HER4, in the carcinogenesis of HNSCC and its response to therapies. HER2 and HER3 form heterodimers with EGFR and play a role in PI3K/Akt activation. In addition, HER2 and HER3 are also associated with resistance to EGFR and PI3K inhibitors in cancer [25, 26]. These results indicate that targeting the ErbB family kinases could more effectively suppress HNSCC compared to solely using EGFR inhibitors [27, 28]. In fact, FDA-approved ErbB family inhibitor, Afatinib, has shown positive results in HNSCC clinical trials and is now listed on the National Comprehensive Cancer Network (NCCN) guidelines as a third-line single agent for HNSCC treatment [28-32]. Understanding the mechanisms behind resistance to Afatinib and exploring methods to avoid that resistance would be beneficial.

Phosphatidylinositol 3-Kinase (PI3K) is one of the most important downstream effectors of the EGFR/ErbB receptor family. The genes *PIK3CA, PIK3CB,* and *PIK3CD* encode three highly homologous catalytic isoforms of class IA PI3K, p110α, p110β, and p110δ, respectively. These isoforms associate with any of five regulatory isoforms: p85α, its splicing variants p55α and p50α, p85β, and p55γ [33]. The most important PI3K-p85 complex is PI3Kα/p85α. Recent studies demonstrate that mutations of PIK3CA, which codes for PI3Kα, are one of the most frequent mutations in HNSCC. In addition, PIK3CA/PI3Kα amplification or overexpression were also identified in HNSCC. Furthermore, PI3K/Akt signaling is activated in 34% of HPV-negative HNSCC tumors and 56% of HPV-positive tumors, which describe the prognosis of HNSCC [34-37]. PI3K activation in turn activates Akt, which then phosphorylates its substrates, such as TSC2, PRAS40, GSK3β, and FOXO, to regulate multiple cellular functions that consequently control cell proliferation, survival, and response to therapies. The tumor suppressor gene *PTEN* encodes the PTEN protein that dephosphorylates PIP3 to inhibit the PI3K pathway. Mutations that resulted in PTEN loss or deceased PTEN expression were frequently observed in HPV-positive and negative HNSCC [38, 39]. These alterations further result in the activation of PI3K/Akt [40]. Therefore, PI3Ks is one of the most attractive targets for the treatment of HNSCC [41-43].

We previously reported that co-targeting the ErbB family and PI3K, through a combination of Afatnib and Copanlisib, suppressed growth of HPV-positive HNSCC [44]. In this study, we further explored whether this combination is also effective at suppressing the growth of HPV-negative HNSCC. We found that the combination of Afatinib and Copanlisib led to significant inhibition of cell proliferation and induction of apoptosis in HPV-negative HNSCC. Furthermore, this combination suppressed tumor growth in xenograft models, while having no obvious effect on body weight loss in mice. These results highlight the feasibility of this combination for the treatment of HPV-negative HNSCC.

## 2. Materials and Methods

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*2.1. Cell Culture*

HNSCC cell lines, Cal27, and FaDu were purchased from ATCC and were authenticated by short tandem repeat analysis (STR) and tested for mycoplasma contamination in the Translational Core Facility of the University of Maryland Marlene and Stewart Greenebaum Cancer Center. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/mL penicillin and streptomycin (Gibco).

*2.2. Antibodies and Inhibitors*

The following antibodies were purchased from Cell Signaling: phospho-Akt-S473 (CST-4508), phospho-Akt-T308 (CST-9275), Akt (CST-2938), phospho-S6K-T389 (CST-9205), S6K (CST-9202), phospho-HER2-Y1248 (CST-2247), HER2 (CST-4290), phospho-HER3-Y1289 (CST-2842), HER3 (CST-12708), C-caspase-3 (CST-xxx), and β-actin (CST-4967). Gefitinib, Erlotinib and Afatinib and all PI3K inhibitors were purchased from Selleck Chemicals.

*2.3. Cell Lysis and Western Blot Analysis*

Cell lysis and Western blot analysis were performed as previously described [44, 45].

*2.4. Analyzing apoptosis by Annexin V/propidium iodide staining*

Apoptosis analysis by Annexin V/propidium iodide staining was performed as previously described [44, 45].

*2.5. Cell Viability Assay*

Cell viability was assessed by sulforhodamine B (SRB) staining as described previously [46]. Each experiment was performed in triplicate. To determine synergy of drug combination, the combination index values were determined according to the Chou–Talalay method [47] using CalcuSyn software.

*2.6. Tumor Xenograft Formation in Mice*

FaDu cells were subcutaneously injected on the right flank Nu/nu mice (Envigo, Frederick MD) at a density of 0.5 x$10^6$ cells/ml in the presence of 33% Matrigel™ (Fisher Scientific). When tumors reached approximately 200 $mm^3$, mice were randomized to one of four treatment groups (7 mice/group): vehicle control, Copanlisib (6 mg/kg, IP), Afatinib (6 mg/kg, PO), or a combination of Copanlisib and Afatinib. Tumor volume was measured twice per week using electronic calipers and animals were weighed 5 days per week. Tumor volume was calculated as (L x $W^2$)/2, where W is the smaller dimension and L is tumor length. Mice were euthanized on Day 32 of the treatment, and the tumors were excised, weighed, fixed, and frozen.

*2.7. Statistical Analysis*

All *in vitro* data are shown as mean ± SD and animal data was shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad Software Inc.).

**3. Results**

*3.1. HPV-Negative HNSCC Cell Lines are Sensitive to Afatinib.*

We determine the $EC_{50}$ values to EGFR/ErbB family inhibitors, Gefitinib, Erlotinib, and Afatinib in two HPV-negative HNSCC cell lines, Cal27 and FaDu. Both cell lines are relatively resistant to Erlotinib and sensitive to Gefitinib. However, they are very sensitive to Afatinib (**Figure 1A** and **B**). These results suggest that Afatinib is the more effective small molecule inhibitor for treatment of HPV negative HNSCC in comparison to EGFR inhibitors.

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Figure 1

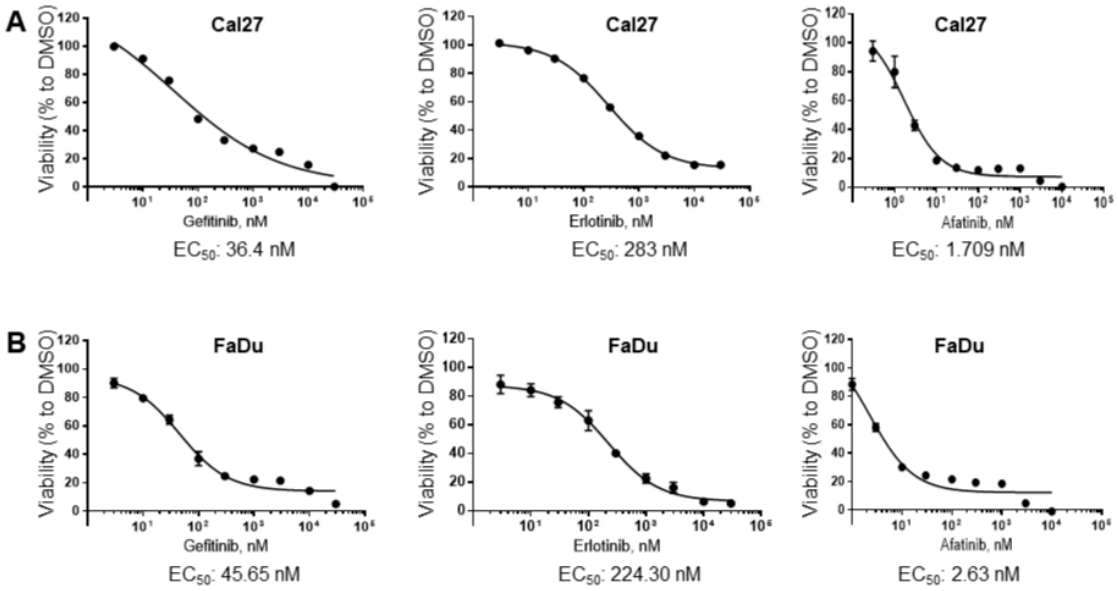


**Figure 1.** Afatinib more effectively inhibited cell proliferation in HPV-negative HNSCC compared to Gefitinib and Erlotinib. A and B. Cal27 (A) and FaDu (B) cells were treated with DMSO or increasing concentrations of Gefitinib, Erlotinib, and Afatinib for 96 hours and cell proliferation was measured by SRB assay and $EC_{50}$ were determined by GraphPad Prism version 7.04.

*3.2. Copanlisib is the Most Effective PI3K Inhibitor to Suppress HPV-Negative HNSCC Proliferation.*

To identify more effective PI3K inhibitors, we determined the $EC_{50}$ values of six PI3K inhibitors, which included three pan-PI3K inhibitors, Copanlisib, BKM120, and GDC09410; the PI3Kα inhibitor BYL719; the PI3Kβ inhibitor AZD8186; and the PI3Kδ inhibitor Cal-101 (**Figure 2A** and **B**). Cal27 cells were strongly resistant to PI3Kδ, and relatively resistant to PI3Kα and PI3Kβ inhibitors. However, they were much more sensitive to Pan PI3K inhibitors, Copanlisib, GDC0941 and BKM120. Moreover, they showed much lower $EC_{50}$ to Copanlisib in comparison to BKM120 and GDC0941. Similar results were found in FaDu cells (**Supplementary Figure 1A** and **Figure 2B**). These results suggest that Copanlisib is the most effective small molecule PI3K inhibitor for treatment of HPV-negative HNSCC.

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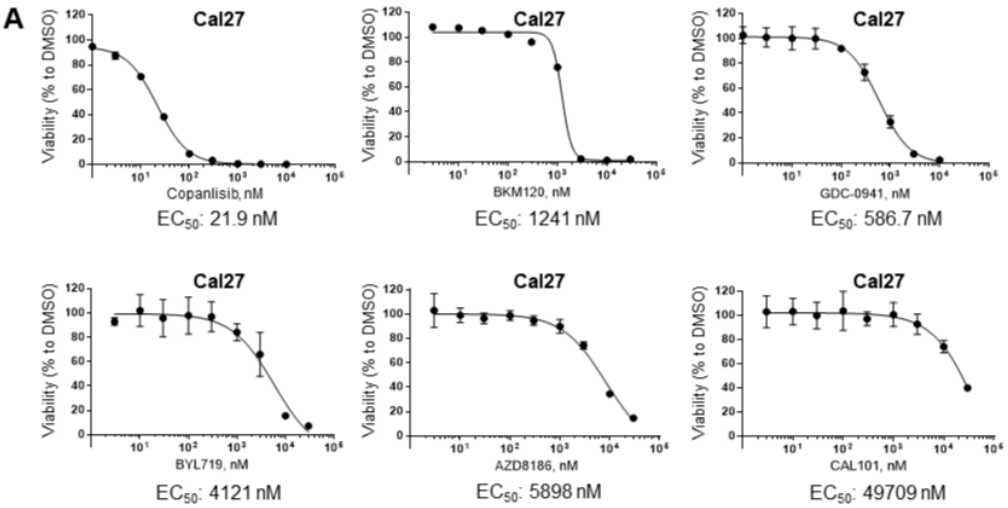
Figure 2




B

| | Pan-PI3K inhibitors | | | PI3Kα inhibitor | PI3Kβ inhibitor | PI3Kδ inhibitor |
|---|---|---|---|---|---|---|
| | Copanlisib | BKM120 | GDC-0941 | Byl719 | AZD8186 | Cal-101 |
| Cal27 cells | 21.9 nM | 1241 nM | 586.7 nM | 4121 nM | 5898 nM | 49709 nM |
| FaDu cells | 25.96 nM | 1759 nM | 310.4 nM | 3022 nM | 2923 nM | 20692 nM |

**Figure 2.** Copanlisib more effectively inhibited cell proliferation compared to other PI3K inhibitors in Cal27 cells. A. Cal27 cells were treated with DMSO or increasing concentrations of Copanlisib and other PI3K inhibitors for 96 hours and cell proliferation was measured by SRB assay. The growth curves are shown. The experiments were performed in triplicate. The associated $EC_{50}$ to the 6 inhibitors were determined by GraphPad Prism version 7.04. B. $EC_{50}$ to six PI3k inhibitors in Cal27 and FaDu cells were listed.

*3.3. Synergistic Inhibition of Cell Proliferation by a Combination of Afatinib and Copanlisib.*

Our goal was to test whether simultaneous inhibition of ErbB family and PI3K pathways could more effectively inhibit HPV negative HNSCC proliferation. Based on the data described above, we selected Afatinib and Copanlisib for the combination therapy. Similar to the results in **Figures 1 and 2**, Afatinib or Copanlisib alone inhibited cell proliferation, however, the combination caused enhanced inhibition of cell proliferation in both Cal27 (**Figure 3A**) and FaDu cells (**Figure 3C**). Furthermore, the related combination index (CI) value for each combination was calculated according to the Chou–Talalay method method [47] using CalcuSyn software. The CI values for all combinations were less than 1.0, which indicated a synergistic effect in the combination of Afatinib and Copanlisib (**Figure 3B and D**). These data indicate that Afatinib and Copanlisib synergistically inhibit HPV-negative HNSCC cell proliferation.

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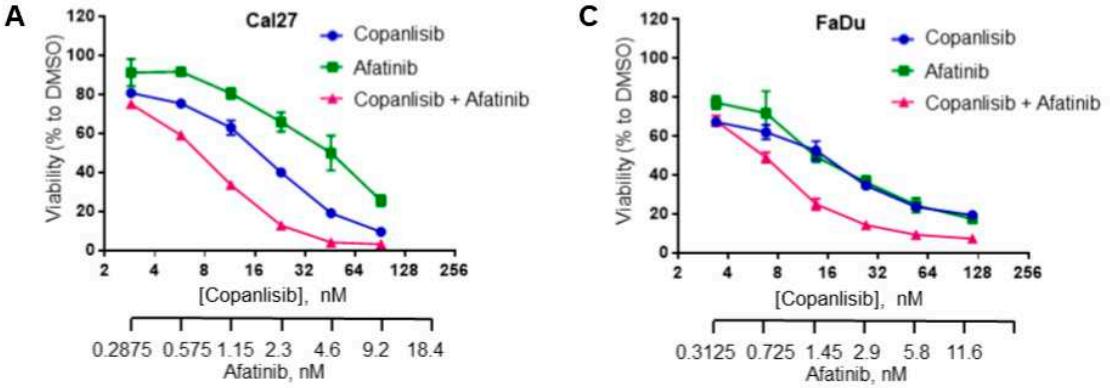
Figure 3




B

| Copanlisib (nM) | Afatinib (nM) | CI |
|---|---|---|
| 2.875 | 0.2875 | 0.756 |
| 5.75 | 0.575 | 0.774 |
| 11.5 | 1.15 | 0.592 |
| 23 | 2.3 | 0.384 |
| 46 | 4.6 | 0.245 |
| 92 | 9.2 | 0.383 |

D

| Copanlisib (nM) | Afatinib (nM) | CI |
|---|---|---|
| 3.375 | 0.3125 | 0.742 |
| 6.75 | 0.725 | 0.692 |
| 13.5 | 1.45 | 0.588 |
| 27 | 2.9 | 0.493 |
| 54 | 5.8 | 0.476 |
| 118 | 11.6 | 0.74 |

**Figure 3.** Synergistic inhibition of cell proliferation by combination of Afatinib and Copanlisib *in vitro*. A and C. Cal27 (A) and FaDu (C) cells were treated with different concentrations of Afatinib, Copanlisib, or their combinations for 96 hours and cell proliferation was measured by SRB assay. The experiments were performed in triplicate. B and D. The combination index values (CI values) for different combinations were determined using CalcuSyn, Version 2.0 (C and D).

*3.4. Synergistic Inhibition of Xenograft Tumor Growth by the Combination of Afatinib and Copanlisib in Mice.*

It was important to evaluate the anti-tumor activity of the Afatinib and Copanlisib combination *in vivo* by using a mouse xenograft model. FaDu cells were inoculated into the mice, and when the tumors reached approximately 200 $mm^3$, mice were randomized into four groups for treatment with vehicle control, Copanlisib, Afatinib, or the combination of both. We originally intended to treat the mice for more than 6-8 weeks, but we had to terminate the experiment on day 32 due to tumor necrosis in the majority of the control mice. The average tumor volume in groups treated with either Copanlisib or Afatinib was lower than that of the control group, but there were no significant differences in tumor volume among these three groups, which might be due to tumor necrosis in the control group (**Figure 4A**). However, tumor volumes in the combination treatment group were significantly lower than the control group by the end of the study (**Figure 4A**).

Similarly, after the tumors were excised and weighed at the end of the study, there was no significant difference in tumor weight (mg) between the groups treated with either Copanlisib or Afatinib (**Figure 4B**). However, tumor weight in the combination treatment group was significantly lower compared with single reagent treated groups ($P<0.05$, **Figure 4B**) and notably lower compared with control group ($P<0.01$, **Figure 4B**). In summary, while Afatinib or Copanlisib alone had modest inhibitory effects on tumor growth, it did not reach statistical significance, whereas the combination of the two drugs significantly inhibited tumor growth (**Figure 4A and B**).

More importantly, all doses of Copanlisib and Afatinib in single and combination treatment were well-tolerated, because there was no significant weight loss observed during the study (**Figure 4C**). These results demonstrate the feasibility of the Afatinib and Copanlisib combination in suppressing HPV-negative HNSCC.

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Figure 4

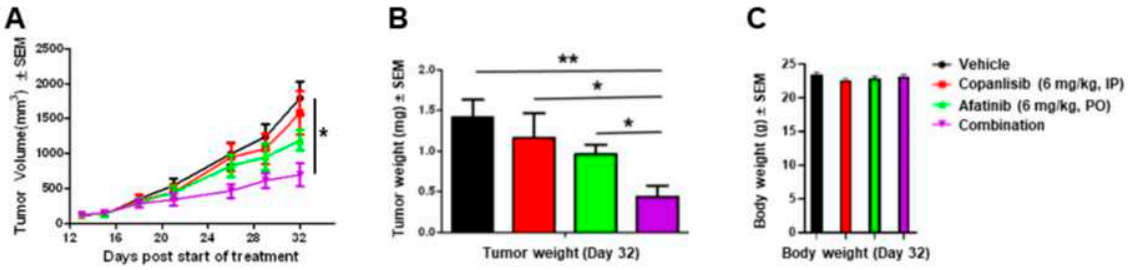


**Figure 4.** Inhibition of HNSCC growth by combination of Afatinib and Copanlisib *in vivo*. A, B, and C. FaDu cells were inoculated into mice. When tumors reached approximately 200 $mm^3$, mice were randomized to one of four treatment groups (7 mice/group): vehicle control, Copanlisib (6 mg/kg, IP), Afatinib (6 mg/kg, PO), or a combination of Copanlisib and Afatinib. The treatments were performed for 32 days. The xenograft tumor volumes (A), final average weights of tumors (B), and average body weights of mice (C) in each group were compared. (*P<0.05, **P<0.01).

*3.5. A combination of Afatinib and Copanlisib Induces Apoptosis.*

We tested whether a combination of Afatinib and Copanlisib could cause more apoptosis compared to either single treatment. Cal27 cells were treated with Afatinib (0.5 μM), Copanlisib (30 nM), or their combination for 48 hours before apoptosis assay was performed. Afatinib and Copanlisib alone induced cell apoptosis, whereas their combination significant increased cell apoptosis (**Figure 5A** and **Supplementary Figure 2**). Since FaDu cells showed higher $IC_{50}$ to Afatinib and Copanlisib in comparison to Cal27 cells, we chose higher concentrations of Afatinib and Copanlisib for the treatments. Treatment with Copanlisib (125 nM) caused significant apoptosis, whereas treatment with Afatinib (1.0 μM) did not cause significantly increased apoptosis. However, a combination of Copanlisib (125 nM) and Afatinib (1.0 μM) led to significantly increased apoptosis compared to either of the single treatment (**Figure 5B** and **Supplementary Figure 3**). These data demonstrate that Afatinib and Copanlisib cooperate to induce apoptosis in HPV-negative HNSCC.

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Figure 5

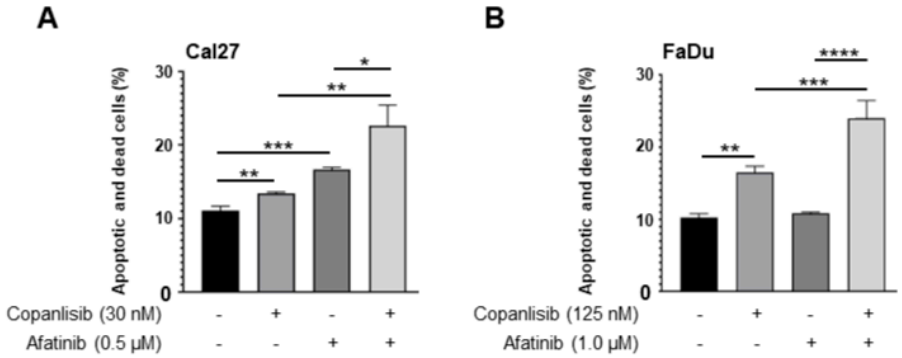


**Figure 5.** A combination of Afatinib and Copanlisib increased cell apoptosis compared to Afatinib or Copanlisib treatment alone. A and B. Cal27CP (A) and FaDu (B) cells were treated with vehicle control, Copanlisib, Afatinib, or a combination for 48 hours, and cell apoptosis was analyzed by Annexin V/propidium iodide staining. The experiments were performed in triplicate, early and late-stage apoptotic, and dead cells were counted, and statistical analysis was performed. $P$ values < 0.05 were considered to be statistically significant. Note: in A, (*P<0.05, **P<0.01, ***P<0.0001). .

*3.6. Combination of Afatinib and Copanlisib Completely Inhibits ErbB and PI3K Pathways Resulting in Induction of Caspase Cleavage.*

Previous studies demonstrated that PI3K inhibitors induced HER2 and HER3 phosphorylation, which thus conferred resistance to PI3K inhibitor [48, 49]. We recently showed that Copanlisib induced an increase of P-HER2 Y1248 and P-HER3 Y1289 in HPV+ HNSCC cells, while a combination of Afatinib and Copanlisib blocked phosphorylation of HER2 and HER3 [44]. We further tested whether Copanlisib induce P-HER2 Y1248 and P-HER3 Y1289 in HPV-negative Cal27 (**Figure 6A**) and FaDu cells (**Figure 6B**). Similarly, Copanlisib significantly inhibited phosphorylation of Akt and induced phosphorylation of P-HER2 Y1248 and P-HER3 Y1289, whereas the combination of Copanlisib and Afatinib completely blocked both phosphorylation of Akt, HER2 and HER3. In addition, increased caspase-3 cleavage was induced by the combination compared to the single treatment (**Figure 6A** and **B**).

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Figure 6

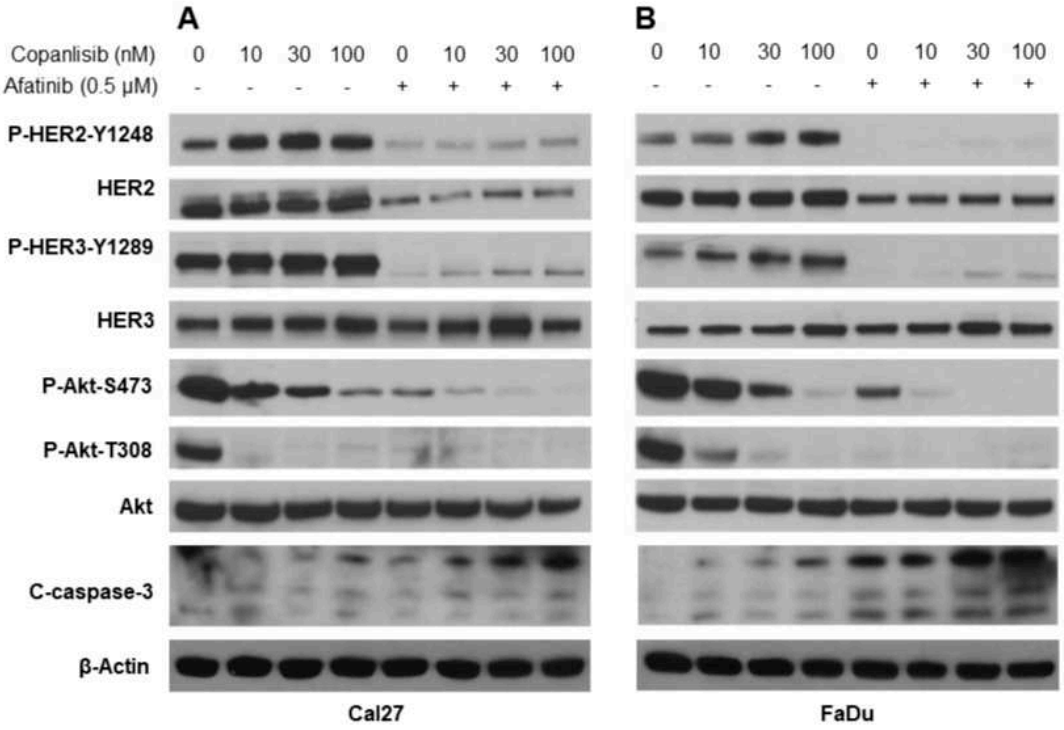


**Figure 6.** Inhibition of both ErbB and PI3K/Akt pathways and induced caspase cleavage by combination of Afatinib and Copanlisib. A-B. Combination of Copanlisb and Afatinib effectively blocks the phosphorylation of HER2, HER3 and Akt and induces more caspase 3 cleavage. Cal27 (A), and FaDu (B) cells were treated with increasing concentrations of Copanlisib, Afatinib (0.5 μM), or their combination for 24 hours before lysed. The indicated proteins were detected by Western blot analysis.

## 4. Discussion

In this study, we tested the efficacy of co-inhibiting the ErbB family and PI3K through the combination of Afatinib and Copanlisib to inhibit HPV-**negative** HNSCC. Our results showed that the combination of Afatinib and Copanlisib caused dramatic inhibition of cell proliferation and suppressed cell survival *in vitro* in comparison to treatment with either Afatinib or Copanlisib alone. Notably, the combination led to significant inhibition of xenograft tumor growth without affecting the body weight of the mice. These results suggest that the combination of Afatinib and Copanlisib may have clinical potential for the treatment of HPV-negative HNSCC.

The EGFR/ErbB and PI3K/Akt/mTOR pathways have been the most attractive pathways to target for treatment of HNSCC due to over-expression or activating mutation of PIK3CA and loss function mutations of PTEN [6, 50-53]. It has been reported that constitutive activation of PI3K/Akt/mTOR pathway due to the alterations in PIK3CA, PTEN, Akt or mTOR is associated with resistance to EGFR inhibitor [6]. Our data showed that treatment with Afatinib alone cannot completely block the phosphorylation of Akt (Figure 6). Furthermore, it has been reported that PI3K inhibition led to increased phosphorylation and total levels of HER3, which confer resistance to PI3K inhibitors [48, 49, 54-56]. Our data also showed that Copanlisib increased phospho-HER3 (Y1289), which was counteracted by the addition of Afatinib (Figure 6). Notably, the combination of Copanlisib and Afatinib induced significant caspase-3 cleavage in addition to the complete inhibition of ErbB and PI3K/Akt pathways (Figure 6). These results provide a rationale for the co-inhibition of ErbB and PI3K as a method to treat HNSCC.

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We recently reported that the combination of Afatinib and Copanlisib effectively suppressed HPV-positive HNSCC. The combination therapy blocked both ErbB and PI3K/Akt pathways, which was accompanied by deceased E6 and E7, and the induction of Apoptosis, indicating increased efficacy of this combination in HPV+ HNSCC [44]. A publication by Milewska et al, reported that cell lines from multiple cancers, including HNSCC with PIK3CA mutations, are sensitive to the combination of Afatinib and Copanlisib [57]. As the basal level of PI3K/Akt is also high in HPV-negative HNSCC and plays essential roles in the regulation of growth, metastasis, and sensitivity to chemo- and targeted therapies [6, 42, 58, 59], it would be reasonable to predict that this combination would also be beneficial in HPV-negative HNSCC with upregulated PI3K/Akt signaling. Afatinib has shown positive results in HNSCC clinical trials and is now listed on the National Comprehensive Cancer Network (NCCN) guidelines as a third-line single agent for HNSCC treatment[28-32]. Our results indicate that the combination of Afatinib with Copanlisib would more effectively suppress HNSCC in patients with refractory disease.

Immunotherapy, including immune checkpoint blockade (ICB) targeting PD-L1/PD-1 using PD-1 inhibitor, Nivolumab or Pembrolizumab, was another important advancement in the treatment of advanced HNSCC. Afatinib modulates PD-L1 expression in multiple cancers, including gastric cancer [60]. In addition, it has been reported that PI3K inhibitors such as BKM120 deceased the expression of PD-L1 in HNSCC cells [61]. It would be interesting to determine the effects of Afatinib, Copanlisib, and their combination on the expression of PD-L1 in HNSCC and immune cells, such as T-cells, and the impact of the combination of Afatinib and Copanlisib on immunotherapy.

## 5. Conclusion

Our results suggest that co-targeting the ErbB family and PI3K kinases by a combination treatment of Afatinib and Copanlisib can have clinical potential for HNSCC patients.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Copanlisib more effectively inhibited cell proliferation compared to other PI3K inhibitors in FaDu cells, Figure S2: Combination of Copanlisib and Afatinib induced more apoptosis compared to either single treatment in Cal27 cells, Figure S3: A combination of Copanlisib and Afatinib induced more apoptosis compared to either single treatment in FaDu cells.

**Author Contributions:** Xinyan Geng: Data curation, Formal analysis, Writing – original draft. Shirin Azarbarzin: Data curation, Formal analysis. Zejia Yang: Data curation, Formal analysis. Rena G. Lapidus: Data curation, Formal analysis. Xiaoxuan Fan: Data curation, Formal analysis. Yong Teng: Formal analysis, Writing – review & editing. Ranee Mehra: Formal analysis, Writing – review & editing. Kevin J. Cullen: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing, Funding acquisition. Hancai Dan: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing, Funding acquisition.

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