

**Rationale for using irreversible EGFR Inhibitors in combination with PI3K inhibitors for  
advanced Head and Neck Squamous Cell Carcinoma**

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**Abbreviations:**

EGFR:                epidermal growth factor receptor

DMSO:               dimethyl sulfoxide

HNSCC:              head and neck squamous cell carcinoma

HPV:                human papilloma virus

HSA:                highest single agent

IC<sub>50</sub>:               half-maximal inhibitory concentration

IGFR:               insulin-like growth factor receptor

PI3K:               phosphatidylinositol 3-kinase

## Abstract

Head and neck squamous cell carcinoma (HNSCC) is a common and debilitating form of cancer characterized by poor patient outcomes and low survival rates. In HNSCC, genetic aberrations in PI3K and EGFR pathway genes are common, and small molecules targeting these pathways have shown modest effects as monotherapies in patients. While emerging preclinical data support the combined use of PI3K and EGFR inhibitors in HNSCC, in-human studies have displayed limited clinical success so far. Here, we examined the responses of a large panel of patient-derived HNSCC cell lines to various combinations of PI3K and EGFR inhibitors, including EGFR agents with varying specificity and mechanistic characteristics. We confirmed the efficacy of PI3K and EGFR combination therapies, observing synergy with alpha isoform selective PI3K inhibitor HS-173 and irreversible EGFR/ErbB2 dual inhibitor afatinib in the majority of models tested. Surprisingly, however, our results demonstrated only modest improvement in response to HS-173 with reversible EGFR inhibitor gefitinib. This difference in efficacy was not explained by differences in ErbB target selectivity between afatinib and gefitinib; despite effectively disrupting ErbB2 phosphorylation, the addition of ErbB2 inhibitor CP-724714 failed to enhance the effect of HS-173 gefitinib dual-therapy. Accordingly, while irreversible ErbB inhibitors showed strong synergistic activity with HS-173 in our models, we observed that none of the reversible ErbB inhibitors were synergistic. Therefore, our results suggest that the ErbB inhibitor mechanism of action may be critical for enhanced synergy with PI3K inhibitors in HNSCC patients and motivate further preclinical studies for ErbB and PI3K combination therapies.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common form of cancer by incidence worldwide and is often associated with either high alcohol and tobacco use or infection with high-risk human papilloma virus (HPV) (Howlader N, April 2016; Kamangar et al., 2006). The disease has 5-year survival rates of less than 50% for HPV negative tumors and around 80% for HPV positive tumors, and we believe that overall survival for patients will be improved by advancing novel therapeutic approaches that target aberrations common to different subsets of HNSCC tumors (Giefing et al., 2016; Ludwig et al., 2016). Furthermore, the development of effective rational combination therapies may be critical for overcoming common resistance mechanisms that emerge following targeted monotherapy. We believe this approach may have utility for both adapting clinical paradigms with adjuvant/neoadjuvant agents as well as advancing personalized medicine approaches through the development of rational combination therapies for the most prominent molecular alterations in HNSCC.

Of the potential targetable molecular alterations common to HNSCC, the phosphoinositide-3 kinase (PI3K) pathway is disrupted through genomic amplifications or activating point mutations in >30% of tumors (Gillison et al., 2015; Lui et al., 2013; Michmerhuizen et al., 2016; Murugan et al., 2013) and the epidermal growth factor receptor (EGFR) is overexpressed in >90% of tumors (Gillison et al., 2015; Lui et al., 2013; Ozanne et al., 1986). Inhibitors to each of these pathways have already been advanced individually in HNSCC. For example, in a recent phase II trial, pan-PI3K inhibitor BKM120 (buparlisib) with paclitaxel improved survival as compared to paclitaxel and placebo in recurrent and metastatic HNSCC patients (Soulieres et al., 2017), and EGFR antibody cetuximab is currently in clinical use after demonstrating improved outcomes in combination with radiotherapy or cisplatin (Bonner et al., 2006; Vermorken et al., 2008). Thus, while PI3K and EGFR targeting therapies have been used with some clinical success, response rates are still relatively low and innate or

acquired resistance mechanisms appear to be widespread (Boeckx et al., 2013; Bonner et al., 2006; Michmerhuizen et al., 2016; Rodon et al., 2014; Soulieres et al., 2017; Vermorken et al., 2008).

Preclinical data indicate that dual-therapies directed against both PI3K and EGFR pathways might improve responses in HNSCC (Anisuzzaman et al., 2017; D'Amato et al., 2014; Lattanzio et al., 2015; Michmerhuizen et al., 2016; Rebucci et al., 2011; Silva-Oliveira et al., 2017; Young et al., 2013). Given these promising data, several clinical trials assessing the combination have been opened in HNSCC, most of which use the EGFR targeting antibody cetuximab in combination with various inhibitors of PI3K (e.g. NCT01816984, NCT2282371, NCT02822482). Unfortunately, however, one recently completed study showed no significant improvement in patient survival with the addition of pan-PI3K inhibitor PX-866 to cetuximab (Jimeno et al., 2015). These surprising data suggested that a deeper understanding of the molecular mechanisms of action that drive response to PI3K and EGFR therapies is necessary to fully interpret the results of these trials.

Here, due to the early reported disparity between *in vitro* and clinical trial results, we conducted further studies characterizing the responses to various classes of PI3K and EGFR dual therapies in HNSCC. We used a panel of genetically diverse HNSCC cell lines to examine responses to combinations of PI3K and EGFR inhibitors; in doing so, we sought to assess patterns of response that might translate to future clinical trial design and/or serve as a guide for future precision medicine protocols in HNSCC.

## **Materials and Methods**

### *Cell Culture*

Cells were cultured in a humidified incubator at 37°C with 5% (vol/vol) CO<sub>2</sub>. UM-SCC cells (University of Michigan) and Cal-33 cells (a kind gift from Dr. Anthony Nichols) were previously derived from HNSCC patient tumor samples and cultured in DMEM with 10% FBS,

1X Pen/Strep, 1X NEAA (Brenner et al., 2010). HSC-2, HSC-4 (both from Japanese Collection of Research Bioresources through Sekisui XenoTech, Kansas City, KS) and Detroit 562 (American Type Culture Collection, Manassas, VA) cells were cultured in EMEM with 10% FBS, 1X Pen/Strep. All cell lines were genotyped to confirm authenticity and were mycoplasma negative.

Details of DNA copy number analysis are published elsewhere (Ludwig et al., 2018). All UM-SCC cell lines were confirmed to contain *PIK3CA* as previously reported from Nimblegen V2 exome capture based experiments (Liu et al., 2013). Cal-33, HSC-2 and HSC-4 copy number data were obtained from the publicly available canSAR database (Bulusu et al., 2014; Halling-Brown et al., 2012). *EGFR* mutation status and/or copy number was similarly assessed using data from Nimblegen V2 exome capture based experiments (Liu et al., 2013) for UM-SCC cell lines, the canSAR database for HSC-2, HSC-4, and Cal-33 (Bulusu et al., 2014; Halling-Brown et al., 2012), and previously published work for Detroit 562 (Young et al., 2013).

### *Genomic DNA Purification*

Cells from models with *PIK3CA* mutations (Cal-33, HSC-2, HSC-4, Detroit 562, UM-SCC-43, UM-SCC-19, UM-SCC-85) were harvested and washed in PBS, then frozen at -20°C. The thawed cell pellet was re-suspended in 700  $\mu$ L of Nuclei Lysis Solution (Promega, Madison, WI) for one hour at 55°C. Then, 200  $\mu$ L of Protein Precipitation Solution (Promega) was added to the sample, which was mixed and placed on ice for at least five minutes before being centrifuged at 13,000 RPM and 4°C for five minutes. The supernatant was transferred to a tube containing 600  $\mu$ L of isopropanol and centrifuged at 13,000 RPM for one minute. After aspirating the resulting supernatant, the DNA pellet was washed in 200  $\mu$ L of 70% ethanol, dried, and re-suspended in 30-50  $\mu$ L of nuclease-free water.

### *Sanger Sequencing*

Genomic DNA was amplified using PCR with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and the primers with sequences listed in **Figure S1**. After being inserted into the pCR8 vector system or processed using the Qiagen QIAquick PCR purification kit, PCR products were submitted for Sanger sequencing at the University of Michigan DNA Sequencing Core on the 3730XL DNA Sequencer (Applied Biosystems, Foster City, CA) as described elsewhere (Birkeland et al., 2017). Sequences were aligned using the DNASTAR Lasergene software suite.

### *Chemicals*

All compounds (BYL719, HS-173, BKM120, afatinib, gefitinib, erlotinib, BMS-566924, AEE788, TAK-285, CUDC-101, and dacomitinib) were purchased from Selleck Chemicals (Houston, TX). Each inhibitor was initially dissolved in 100% sterile dimethyl sulfoxide (DMSO) to 10 mM and then diluted in media to the indicated concentrations for studies *in vitro*.

### *Resazurin Cell Viability Assay*

Resazurin cell viability assays were performed as described previously (Birkeland et al., 2016b; Michmerhuizen et al., 2016; Shum et al., 2008). To study relative cell viability, 2,000 cells per well (for all cell lines except HSC-2, for which the cell density was reduced to 1,000 cells per well due to large cell size and rapid growth rate) were seeded (in 50  $\mu$ L volume) in 384-well microplates using a Biotek (Winooski, VT) Multiflo liquid handling dispensing system. Cells were allowed to adhere overnight prior to treatment. Inhibitors were prepared by hand from 10 mM stocks at 200X concentration in a 96 well plate, then diluted 10X concentration in media in a second 96-well plate using the Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform and VWorks Automation Control Software. The intermediate plate with inhibitors in

media was used to treat the cells with the desired compound concentration, again using liquid handling robotics, such that cells were treated with complete media containing 0.5% inhibitor or DMSO in a 10-point two-fold dilution series. Each treatment was administered in quadruplicate. Cells were stained with 10  $\mu$ L of 440  $\mu$ M resazurin (Sigma, St Louis, MO) dissolved in serum-free media for 12-24 hours before fluorescent signal intensity was quantified. Quantification occurred after 72 hour treatment using the Biotek Cytation3 fluorescence plate reader at excitation and emission wavelengths of 540 and 612 nm, respectively. Data were plotted using Prism 7 and fit with concentration response curves using the log(inhibitor) vs. response -- Variable slope model with four parameters ( $IC_{50}$ , top, bottom, and Hill slope) allowed to vary.

#### *Annexin V Apoptosis Assay*

To study Annexin V presentation, 115,000 Detroit 562 cells or 100,000 UM-SCC-59 cells per well were seeded in six-well plates. After 24 hours, media was aspirated and replaced with 3 mL of fresh, complete media. 1 mL of media containing DMSO or inhibitor(s) was added to each well. Cells were cultured for 72 hours, at which time, media was collected from each well. Each well was then washed in PBS, which was also collected. Finally, cells were trypsinized and added to the suspension. Samples were then centrifuged, washed once with PBS, and counted using the Countess Automated Cell Counter (Invitrogen). 100,000 cells per sample were stained with Annexin V FITC and PI using the Dead Cell Apoptosis Kit (ThermoFisher, Waltman, MA) according to manufacturer's instructions. 5  $\mu$ L of Annexin V FITC and 5  $\mu$ L of PI were added to each sample. Samples were incubated at room temperature in the dark for 15 minutes and analyzed using the Bio-Rad ZE5 or MoFlo Astrios EQ Cell Sorter at the University of Michigan Flow Cytometry Core.

#### *Western Blotting*

Cells at 70-80% confluency were treated with DMSO or inhibitor prior to harvesting and lysing in radioimmunoprecipitation assay buffer (catalog No. 89900; ThermoFisher) containing 1% NP-40 and 0.1% SDS. 8-20 micrograms of each cell harvest were used, and standard Western blot protocols were followed as previously described (Birkeland et al., 2016a). Primary antibodies (described in detail in **Table S1**) were incubated overnight at 4°C or for at least one hour at room temperature, followed by a goat anti-rabbit horseradish peroxidase (catalog No. 111-035-045; Jackson ImmunoResearch, West Grove, PA) secondary antibody at room temperature for one hour as described elsewhere (Tillman et al., 2016). The blots were then visualized with chemiluminescence and imaged. 300dpi or greater images were digitally retained from all Westerns and representative blots are shown. ImageJ software was used to quantify protein expression and compare treatment responses.

### *Synergy Analysis*

The effects of combination treatments were analyzed with Combenefit software (Di Veroli et al., 2016) using the highest single agent (HSA) model (Berenbaum, 1981; Bliss, 1939; Borisy et al., 2003; Greco et al., 1995; Mathews Griner et al., 2014). For each cell line and pair of inhibitors, the number of concentration combinations with scores greater than 20 were counted. These counts were averaged across at least two (and as many as five) independent replicates for each experiment. Experiments having more than eight concentration combinations with scores greater than 20 were considered additive or synergistic. We compared the number of concentration combinations with scores above 20 for HS-173 and afatinib (afatinib combination score) as well as HS-173 and gefitinib (gefitinib combination score). Cell lines were considered more responsive to the afatinib combination if the afatinib combination score exceeded the gefitinib combination score by eight or more.

### *Statistical Analysis*

To determine if statistically significant differences occurred with combination treatments, a two-way ANOVA was performed in R to compare the natural logarithm of the percentage of living cells following vehicle, HS-173, gefitinib or afatinib, or combination treatment. The interaction between HS-173 and gefitinib or afatinib treatment indication was tested by F-test for the synergy effect of drug combination. In total, four separate tests on drug combination (HS-173 combined with gefitinib or afatinib for UM-SCC-59 and Detroit 562 cell lines) were performed simultaneously, so Bonferroni correction was used to adjust p-values.

## Results

### *Subsets of HNSCCs Respond to PI3K + EGFR Inhibitor Combination Therapies*

To first probe the co-dependence of HNSCC cell lines to PI3K and EGFR pathway inhibitors, we compared the response of a small panel of models to the PI3K $\alpha$  inhibitor HS-173 (Lee et al., 2013; Rumman et al., 2016) and irreversible pan-EGFR/ErbB2 inhibitor afatinib (Li et al., 2008) as monotherapies and in combination. We selected HS-173 as the PI3K inhibitor as it was the most effective and isoform selective small molecule in our panel of cell lines. Afatinib was used as the ErbB inhibitor; this drug was approved by the FDA in 2016 as a first-line treatment for patients with non-small cell lung cancer whose tumors harbored mutations in EGFR. It has also displayed efficacy in HNSCC (NCT00514943) and is being evaluated in ongoing studies using various paradigms (NCT01824823, NCT01415674, NCT01427478, NCT02979977). PI3K and ErbB inhibitor combination experiments were performed in four models with *PIK3CA* mutations (HSC-2, HSC-4, Detroit 562 and Cal-33, **Figure S1**) and one with high-level *PIK3CA* amplification (UM-SCC-59, 5 wild type copies) using a resazurin cell viability assay after 72 hour drug treatment and then validated by annexin V apoptosis assay (below). Our studies showed variable responses by cell line.

HSC-2, HSC-4 and Detroit 562 display a hotspot *PIK3CA* mutation (indicating activation of and likely dependence on the PI3K signaling pathway) but have limited responses to HS-173

and other PI3K inhibitors as monotherapies with  $IC_{50}$  close to or exceeding 1  $\mu$ M. In these three cell lines, we observed that the addition of afatinib to HS-173 resulted in dose-dependent improvements in the efficacy of PI3K inhibition (**Figure 1A-C**). These results represented drug synergy using the HSA model. This effect was also observed when pan-PI3K inhibitor BKM120 and another PI3K $\alpha$  inhibitor, BYL719, were titrated with afatinib (**Figure S2A-B**), but not when p110 $\beta$  inhibitor TGX-221 was tested in combination (**Figure S2C**) suggesting that the synergistic dose combination response specifically requires inhibition of PI3K $\alpha$ . Similarly, titrating afatinib into constant concentrations of HS-173 or BKM120 resulted in synergistic responses in combination-responsive *PIK3CA* mutant cell lines HSC-4 and Detroit 562 (**Figure S3**). In contrast, the data also demonstrated that one of the *PIK3CA* mutant HNSCC cell lines, Cal-33, as well as the *PIK3CA* amplified cell line, UM-SCC-59, showed little combination benefit (**Figure 1D-E**), suggesting that these models depend on alternative survival pathways.

After establishing that subsets of HNSCCs responded synergistically to HS-173 and afatinib, we examined the downstream signals in the PI3K and EGFR pathways in order to identify potential differences in signaling transduction pathways between two combination responsive models (HSC-2 and Detroit 562) and one combination non-responsive model (Cal-33). Thus, following a 6-hour treatment with vehicle (DMSO), HS-173 monotherapy, afatinib monotherapy, or HS-173 and afatinib combination therapy, we evaluated EGFR and ErbB2 phosphorylation as well as effector signaling through AKT, MEK and ERK (**Figure 2**). As expected, afatinib monotherapy was sufficient to inhibit EGFR and ErbB2 phosphorylation. While the degree to which afatinib reduced ErbB2 phosphorylation in lysates from treated Detroit 562 cells was fairly minimal here, more robust effects on ErbB2 phosphorylation are visible after shorter treatment times (likely due to transient effects on receptor phosphorylation, see **Figure 3C** below). Downstream of these effects on EGFR and ErbB2 signaling, ERK and MEK phosphorylation are similarly decreased in non-responsive Cal-33 and responsive HSC-2

cell lines. Detroit 562 cells display minimal changes in MEK phosphorylation following treatment at this dose and time point, yet ERK phosphorylation is reduced somewhat. AKT phosphorylation, used as a readout of primarily PI3K but also EGFR pathway activity, was reduced in HS-173 monotherapy treated samples in each cell line. In the responsive HSC-2 cell line, a further reduction in AKT phosphorylation was evidenced with the addition of afatinib to HS-173. Thus, in both non-responsive and responsive models, inhibition of PI3K's downstream signaling through AKT and inhibition of ErbB signaling both at the receptor level and downstream through MEK and ERK was achieved (**Figure 2**). This indicates that the combination effect was not limited to models with reductions in effector signaling.

#### *Responses to PI3K + EGFR Inhibition Vary Based on Inhibitor Type*

We further investigated the role of ErbB inhibition in HS-173 and afatinib combination response by testing PI3K $\alpha$  inhibitor HS-173 in combination with reversible EGFR inhibitor gefitinib in the responsive PI3K mutant HNSCC models Detroit 562 and HSC-2. Resazurin cell viability experiments performed as above displayed a much less marked effect with HS-173 and gefitinib as compared to co-treatment with HS-173 and afatinib (**Figure 3A-B**). These effects were confirmed using an orthogonal annexin V apoptosis assay. For example, in the Detroit 562 cell line (synergistically responsive to HS-173 and afatinib), we observed higher levels of FITC positive (apoptotic) cells following di-therapy compared to what would be expected from additive effects of HS-173 and afatinib monotherapies (adjusted p-value = 0.009, two-way ANOVA). Importantly, no significant change in cell death was seen in the non-synergistically responsive UM-SCC-59 model (adjusted p-value = 1, two-way ANOVA), and HS-173 combinations with gefitinib were ineffective in both cell lines (adjusted p-values = 1, two-way ANOVA) (**Figure 4**). These data suggested a significant difference in the ability of gefitinib and afatinib to induce synergistic cell kill in our models.

Given this surprising observation, we expanded our original analyses on 5 cell lines to a larger panel of HNSCC models. Here, we selected an additional nine models with genetic characteristics of tumors most likely to receive PI3K or EGFR inhibitors in a precision medicine setting, including those with either *PIK3CA* mutations or high-level gene amplifications (greater than four copies). As evidence of this, additivity between HS-173 and gefitinib was only observed in 4/14 (29%) of models.

Importantly, much more significant “further benefit,” which we define as including multiple synergistic dose combinations, was observed with HS-173 and afatinib combination therapy in 8/14 (57%) of models (**Figure 5A**). Of the four models that demonstrated additivity with gefitinib, three received further benefit with afatinib. The *in vitro* models that failed to display robust improvements in response to HS-173 with the addition of afatinib included Cal-33 (**Figure 1D**), UM-SCC-59 (**Figure 1E**), UM-SCC-19, UM-SCC-43, and UM-SCC-85 (**Figure 5A**). Interestingly, Cal-33, UM-SCC-19, UM-SCC-43, and UM-SCC-85, like some of the combination-responsive models discussed above, display activating mutations in *PIK3CA* (**Figure S1**). Cal-33 and UM-SCC-85 cells were among the most sensitive to PI3K inhibitor monotherapies, while UM-SCC-59 (with high-level amplification of wild-type *PIK3CA*) is one of the most resistant. Thus, neither *PIK3CA* mutation nor responsiveness to PI3K inhibitor monotherapy is a good predictor of responsiveness to HS-173 and afatinib co-treatment. Likewise, at least when considered as single variables, *PIK3CA* copy number (**Figure 5A**), *EGFR* copy number (**Figure 5A**), and ErbB protein expression (**Figure 5B**) are also poor indicators of combination response. Although mutations in *EGFR* have been shown to be closely linked to responses to EGFR inhibitors (Lynch et al., 2004; Paez et al., 2004; Thress et al., 2015; Wu et al., 2016; Yu et al., 2013; Yu et al., 2015), the cell lines used here did not display such variants. Thus, neither sensitivity nor resistance to EGFR inhibitor monotherapies or combination therapies can be explained by the presence of L858R or T790M/C797S mutations, respectively. After our resazurin assay determined that the HS-173 and gefitinib

combination was largely ineffective as compared to HS-173 and afatinib, we tested the combination of HS-173 and afatinib in UM-SCC-110 and patient-matched fibroblasts and demonstrated the inability of combination therapy to drive cell death in normal fibroblasts (**Figure S4**).

Together, these data strongly suggest important differences between afatinib- and gefitinib-based combinations in our model system. Given the differences between the inhibitors, we hypothesized that the greater effectiveness with afatinib over gefitinib may be due to 1) a broader spectrum of ErbB family member inhibition, and/or 2) irreversible as opposed to reversible inhibition of EGFR. To begin testing this hypothesis using combination responsive Detroit 562 cells, we performed a resazurin cell viability assay in which we compared the effects of HS-173 and gefitinib with or without ErbB2 specific inhibitor CP-724714 (**Figure 3B**). This demonstrated that CP-724714 was unable to add to HS-173 and gefitinib in this assay and the total effect of this tri-therapy combination remained much less substantial than the effect of HS-173 and afatinib. This result suggested the possibility that ErbB2 inhibition did not account for the differences between inhibitors or that CP-724714 could not sufficiently inhibit ErbB2 signaling in our system.

Consequently, to validate that the doses of CP-724714 used here could sufficiently inhibit ErbB2 signaling, we performed Western blot analysis on lysates harvested from Detroit 562 cells following CP-724714 or afatinib treatment. At doses equivalent to or less than those used in resazurin cell viability assays, we observed that both CP-724714 and afatinib treatment resulted in robust inhibition of ErbB2 phosphorylation after 15 or 60 minutes (**Figure 3C**). We also examined lysates from HSC-2 cells following 2 hour treatment with each mono- or di-therapy (**Figure 5C**). This demonstrated decreased EGFR phosphorylation in gefitinib and afatinib treated samples, with a slightly greater loss of EGFR phosphorylation with afatinib than gefitinib. Phosphorylation of ERK, Gab1, and MEK, downstream of EGFR, were similar for gefitinib and afatinib treatments; in addition, co-treatment with HS-173 and gefitinib or afatinib

did not reduce downstream ErbB signals beyond those levels seen with gefitinib and afatinib monotherapy treatments. Phosphorylation of PI3K pathway effector AKT was appropriately inhibited upon HS-173 treatment, but PDK1 and GSK3 $\beta$  phosphorylation remained unchanged. Together, these data suggest that ErbB2 inhibition alone may not be sufficient to explain differences between the gefitinib and afatinib combinations and therefore warrant further evaluation of differences between reversible and irreversible ErbB inhibitor combinations.

Thus, using a resazurin cell viability assay, we tested HS-173 in combination with three reversible ErbB inhibitors (erlotinib, BMS-566924, and CP-724714) and three irreversible ErbB targeting agents (TAK285, CUDC-101, and dacomitinib) in HSC-2 and Detroit 562 cells. While we observed that 0/4 (0%) reversible ErbB inhibitors displayed synergistic dose combinations in either cell line, 3/4 (75%) and 4/4 (100%) irreversible ErbB targeting drugs had synergistic dose combinations with HS-173 in Detroit 562 and HSC-2 cells, respectively (**Table 1, Figure S5**). These data add support to the hypothesis that irreversible inhibition of EGFR and/or its ErbB family members may be important for achieving the most significant growth inhibition with PI3K and ErbB inhibitor combinations.

## Discussion

Our data are consistent with previous studies showing the benefit of PI3K and EGFR inhibitor combination therapies (Anisuzzaman et al., 2017; D'Amato et al., 2014; Lattanzio et al., 2015; Michmerhuizen et al., 2016; Rebusci et al., 2011; Silva-Oliveira et al., 2017; Young et al., 2013) and also extend that work by discovering that PI3K inhibitors are much more effective in combination with irreversible than reversible EGFR inhibitors in HNSCC. In prior work comparing classes of EGFR targeting monotherapies in this cancer type, preclinical data demonstrated that irreversible EGFR inhibitors are superior to other EGFR targeting agents, including cetuximab (Ather et al., 2013; Silva-Oliveira et al., 2017) and reversible inhibitor gefitinib (Young et al., 2015). Similarly, previous work has shown that the addition of ErbB2

targeting antibodies pertuzumab (Erjola et al., 2006) or trastuzumab (Kondo et al., 2008) to gefitinib enhances its efficacy in HNSCC cell lines; however, our findings demonstrated that the broader specificity of irreversible inhibitors alone cannot explain these differences in sensitivity, as administering ErbB2 inhibitor CP-724714 with gefitinib and HS-173 did not enhance drug effects (**Figure 3**). Collectively, our data may suggest why greater improvements in patient survival following PI3K and EGFR combination therapies have not yet been observed in HNSCC and other cancers clinically and support the need for additional detailed studies of PI3K and EGFR combination therapies using irreversible ErbB inhibitors.

Of the published HNSCC studies evaluating PI3K and EGFR di-therapies, most have been performed with either cetuximab (D'Amato et al., 2014; Lattanzio et al., 2015; Rebutti et al., 2011) or the reversible EGFR inhibitors (e.g. gefitinib, erlotinib) (Anisuzzaman et al., 2017; Young et al., 2013). One exception is a recent report from Silva-Oliveira *et al.* that examined responses to PI3K pathway inhibitors (including AKT inhibitor MK-2206) with two different irreversible EGFR inhibitors (Silva-Oliveira et al., 2017). In this study, pharmacologic inhibition or siRNA knockdown of AKT resulted in improved sensitivity to afatinib and allitinib (a second irreversible EGFR inhibitor) in HN13 cells (Silva-Oliveira et al., 2017). The need to suppress AKT phosphorylation in responses to PI3K + EGFR drug combinations is supported by studies of both EGFR targeting antibodies (Benavente et al., 2009; Rebutti et al., 2011) and reversible inhibitors (Benavente et al., 2009; Rebutti et al., 2011; Silva-Oliveira et al., 2017; Young et al., 2013). Importantly, in lung cancer models, irreversible EGFR inhibitors have sustained reductions in EGFR phosphorylation and an improved ability to decrease effector AKT phosphorylation as compared to reversible inhibitors (Kwak et al., 2005). The inability of reversible EGFR inhibitors to sustain suppression of EGFR and AKT phosphorylation has been linked to altered receptor trafficking (Wiley, 2003), a mechanism that does not affect the activity of irreversible inhibitors. In contrast, we did not observe greater reductions in AKT phosphorylation with HS-173 and afatinib than with gefitinib dual-therapy (**Figure 5C**). These

data suggest that factors other than or in addition to the level of suppression of downstream EGFR effector signaling may be responsible for mediating combination benefit and/or that specific inhibitor combinations may be required to achieve synergistic cell death responses.

Of the emerging novel classes of PI3K and EGFR inhibitors that we evaluated here, several are already in advanced clinical development for HNSCC and other cancers as mono- and combination therapies. For example, BKM120 improved survival when administered with paclitaxel (versus paclitaxel and placebo) in a phase II HNSCC trial (Soulieres et al., 2017), and BYL719 monotherapy recently demonstrated safety in patients with solid tumors (Juric et al., 2018). Of the irreversible EGFR inhibitors that we evaluated, dacomitinib has shown efficacy beyond that of cetuximab in preclinical models (Ather et al., 2013) and is undergoing evaluation in phase II studies in recurrent and metastatic HNSCC patients (NCT00768664, NCT01449201). Afatinib, although still only indicated for use in lung cancer patients, has also demonstrated similar efficacy to cetuximab (Seiwert et al., 2014) in HNSCC patients; this result is very promising given that cetuximab was approved for use in HNSCC with radiation or cytotoxic chemotherapy after successful phase 3 trials (Bonner et al., 2006; Vermorken et al., 2008). Afatinib is currently undergoing evaluation in a variety of treatment paradigms in HNSCC (including NCT01824823, NCT01427478, NCT02979977 and NCT01783587) and has also been tested in other solid tumor types as part of a combination therapy with inhibitors targeting PLK (NCT01206816), Src (NCT01999985), insulin-like growth factor receptor (IGFR) (NCT02191891), MEK (NCT02450656), or multiple receptor tyrosine kinases (NCT00998296), but not yet with PI3K inhibitors.

Many irreversible EGFR inhibitors have activity against both wildtype and mutated forms of EGFR (including those with T790M and/or C797S resistance mutations), which may contribute to their improved clinical efficacy over reversible drugs like gefitinib and erlotinib. Thus, the use of irreversible EGFR inhibitors with PI3K inhibitors in HNSCC may lead to more durable responses than reversible EGFR inhibitor combinations by eliminating not only EGFR

mutations but also activation of compensatory signaling through PI3K as critical resistance mechanisms. Nevertheless, these combinations are still limited by other forms of resistance, including novel resistance mutations and co-dependent pathways, which will likely develop after prolonged exposure to even irreversible EGFR and PI3K inhibitor co-treatments.

Collectively, our work motivates the translation of specific PI3K and irreversible EGFR dual-therapies into xenograft mouse models and other more clinically relevant systems. If such studies confirm our *in vitro* findings, clinical trials evaluating these drug combinations will be warranted. More broadly, our data also motivate a need to develop additional biomarkers that can be used to determine not only if a drug inhibits its target, but also if the drug inhibits pivotal downstream effector pathways capable of rescuing cell survival. Indeed, our findings suggest that responses may be mediated by complex downstream signaling networks or other yet-undefined factors. Developing the next generation of adaptive biomarkers and rationally designed matched combination therapies may therefore be one key to improved survival for HNSCC patients.

### **Authorship Contributions**

Participated in research design: Michmerhuizen, Carey, Brenner

Conducted experiments: Michmerhuizen, Leonard, Matovina, Harris, Herbst

Performed data analysis: Michmerhuizen, Kulkarni, Zhai, Jiang

Wrote or contributed to the writing of the manuscript: Michmerhuizen, Brenner

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## Footnotes

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## Legends for Figures

### Figure 1. Responses to HS-173 + Afatinib Treatment in HNSCC Cell Lines.

(A) HSC-2, (B) HSC-4, (C) Detroit 562, (D) Cal-33, and (E) UM-SCC-59 were treated with increasing concentrations of PI3K $\alpha$  inhibitor HS-173 and/or EGFR/ErbB2 inhibitor afatinib for 72 hours. Cell viability was measured using a resazurin cell viability assay. Each point is the mean and s.d. of quadruplicate determinations from a single experiment. Each experiment was repeated independently at least three times with similar combination effects; representative data is shown along with analysis using Combenefit software (Di Veroli et al., 2016).

### Figure 2. Signaling responses to HS-173 + Afatinib in Combination Responsive and Non-Responsive HNSCC Cell Lines.

Western blot analysis of downstream PI3K and RAS-MEK-ERK pathway activation following six hour treatment with vehicle (DMSO), EGFR/ErbB2 inhibitor afatinib, PI3K $\alpha$  inhibitor HS-173, and combination in Cal-33, Detroit 562 and HSC-2 cell lines. HSP90 was used as a loading control. Representative images are shown.

### Figure 3. Responses to HS-173 + ErbB Inhibitor Treatment in *PIK3CA* Mutant HNSCC Cells.

(A) HSC-2 cells were treated with increasing concentrations of PI3K $\alpha$  inhibitor HS-173 and/or EGFR inhibitor gefitinib for 72 hours. Cell viability was measured using a resazurin cell viability assay. Each point is the mean and s.d. of quadruplicate determinations from a single experiment. Each experiment was repeated independently at least three times with similar combination effects; representative data is shown along with analysis using Combenefit software (Di Veroli et al., 2016). (B) Detroit 562 cells were treated with increasing

concentrations of PI3K $\alpha$  inhibitor HS-173 and/or EGFR gefitinib, ErbB2 inhibitor CP-724714, and/or EGFR/ErbB2 inhibitor afatinib for 72 hours. Cell viability was measured using a resazurin cell viability assay. Each point is the mean and s.d. of quadruplicate determinations from a single experiment. This experiment was repeated independently three times with similar combination effects; representative data is shown. (C) Western blot analysis of phosphorylated and total ErbB2 expression following treatment with vehicle (DMSO) or 15 or 60 minute treatment with either ErbB2 specific inhibitor CP-724714 or EGFR/ErbB2 inhibitor afatinib in Detroit 562 cells. HSP90 was used as a loading control.

**Figure 4. Cell Death Responses to HS-173 + Afatinib Treatment in Combination Responsive and Non-Responsive HNSCC Cell Lines.**

Combination non-responsive model UM-SCC-59 and combination responsive model Detroit 562 were treated with vehicle (DMSO), PI3K $\alpha$  inhibitor HS-173, reversible EGFR inhibitor gefitinib, and/or EGFR/ErbB2 irreversible inhibitor afatinib for 72 hours. Cell viability was measured using an annexin V apoptosis assay after cells were stained with FITC and PI. Data shown represents the mean and SD from 2-3 independent experiments. \*\* indicates significance with  $p < 0.01$  using two-way ANOVA to compare vehicle, HS-173, afatinib, and combination, as described above in Materials and Methods. Comparisons for HS-173 and gefitinib combinations in each cell line and for HS-173 and afatinib combination in UM-SCC-59 were performed, but are not shown given the lack of significant interaction term.

**Figure 5. Sensitivity of HNSCC Cell Lines to HS-173 and Gefitinib or Afatinib Combination Treatment**

(A) Table shows mutation and copy number data for cell lines tested for sensitivity to HS-173 and gefitinib or afatinib. *PIK3CA* mutations were confirmed via Sanger sequencing. No cell lines

displayed mutations in *EGFR*. *PIK3CA* and *EGFR* copy number were determined using the publicly available canSAR database (Bulusu et al., 2014; Halling-Brown et al., 2012) for Cal-33, HSC-2 and HSC-4 cells and using OncoPrint for UM-SCC cells. Detroit 562 *EGFR* copy number was reported as previously published (Young et al., 2013). Combinatorial effects of HS-173 and gefitinib or afatinib were determined using resazurin cell viability assays after 72 hour drug treatment. Experiments with quadruplicate replicates were performed 2-5 times and combination benefit was assessed using Combenefit software (Di Veroli et al., 2016) as described above. 4/14 (29%) cell lines displayed additive effects following HS-173 and gefitinib co-treatment; 8/14 (57%) of models responded more favorably to combination treatment with HS-173 and afatinib. **(B)** Protein isolated from each cell line in the panel was used to perform Western blot analysis for EGFR, ErbB2, and ErbB3.  $\beta$ -actin was used as a loading control. **(C)** Western blot analysis of downstream PI3K and RAS-MEK-ERK pathway activation following 2 hour treatment with vehicle (DMSO), PI3K $\alpha$  inhibitor HS-173, reversible EGFR inhibitor gefitinib, reversible ErbB2 inhibitor CP-724714, EGFR/ErbB2 inhibitor afatinib, or combinations in HSC-2 cells. HSP90 was used as a loading control. Representative images are shown.

**Table 1. Combinatorial Effects of PI3K + ErbB Inhibitors in HNSCC Cell Lines.**

<b>Inhibitor</b>	<b>Target</b>	<b>Reversible/ Irreversible</b>	<b>HSC-2 Synergy</b>	<b>Detroit 562 Synergy</b>
Gefitinib	EGFR	Reversible	No	No
Erlotinib	EGFR	Reversible	No	No
BMS-566924	EGFR/ErbB2	Reversible	No	No
CP-724714	ErbB2	Reversible	No	No
Afatinib	EGFR/ErbB2	Irreversible	Yes	Yes
TAK-285	EGFR/ErbB2	Irreversible	Yes	No
CUDC-101	EGFR/ErbB2/HDAC	Irreversible	Yes	Yes
Dacomitinib	EGFR/ErbB2/ErbB4	Irreversible	Yes	Yes

Combinatorial effects of PI3K $\alpha$  inhibitor HS-173 and reversible or irreversible ErbB targeting agents in HSC-2 and Detroit 562 HNSCC cell lines. Synergy was assessed using Combenefit software (Di Veroli et al., 2016). Synergy was not observed for PI3K $\alpha$  inhibitor HS-173 with any reversible inhibitor in either cell line. 4/4 and 3/4 irreversible EGFR inhibitors were synergistic with HS-173 in HSC-2 and Detroit 562 cells, respectively.

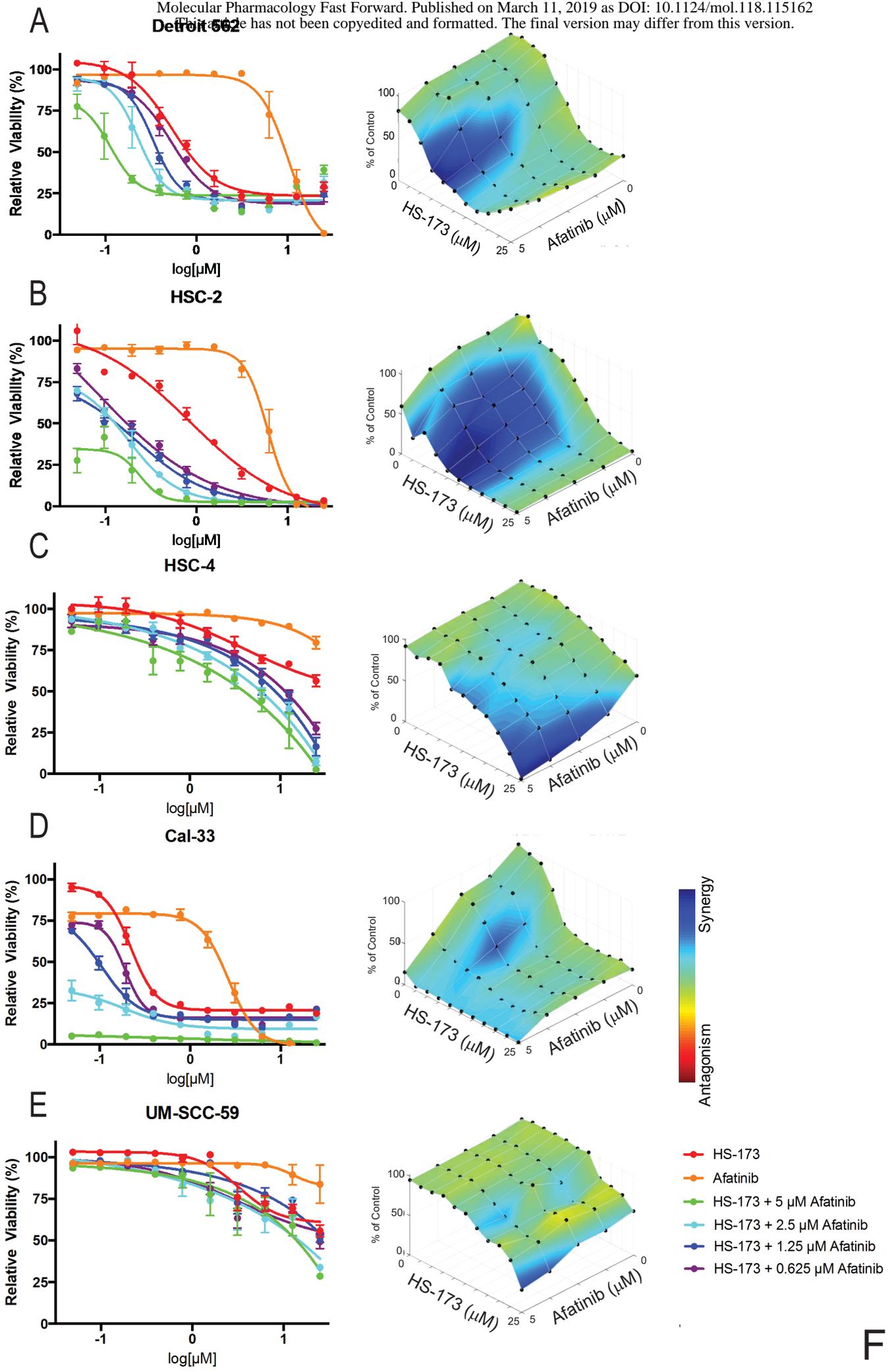


Figure 1

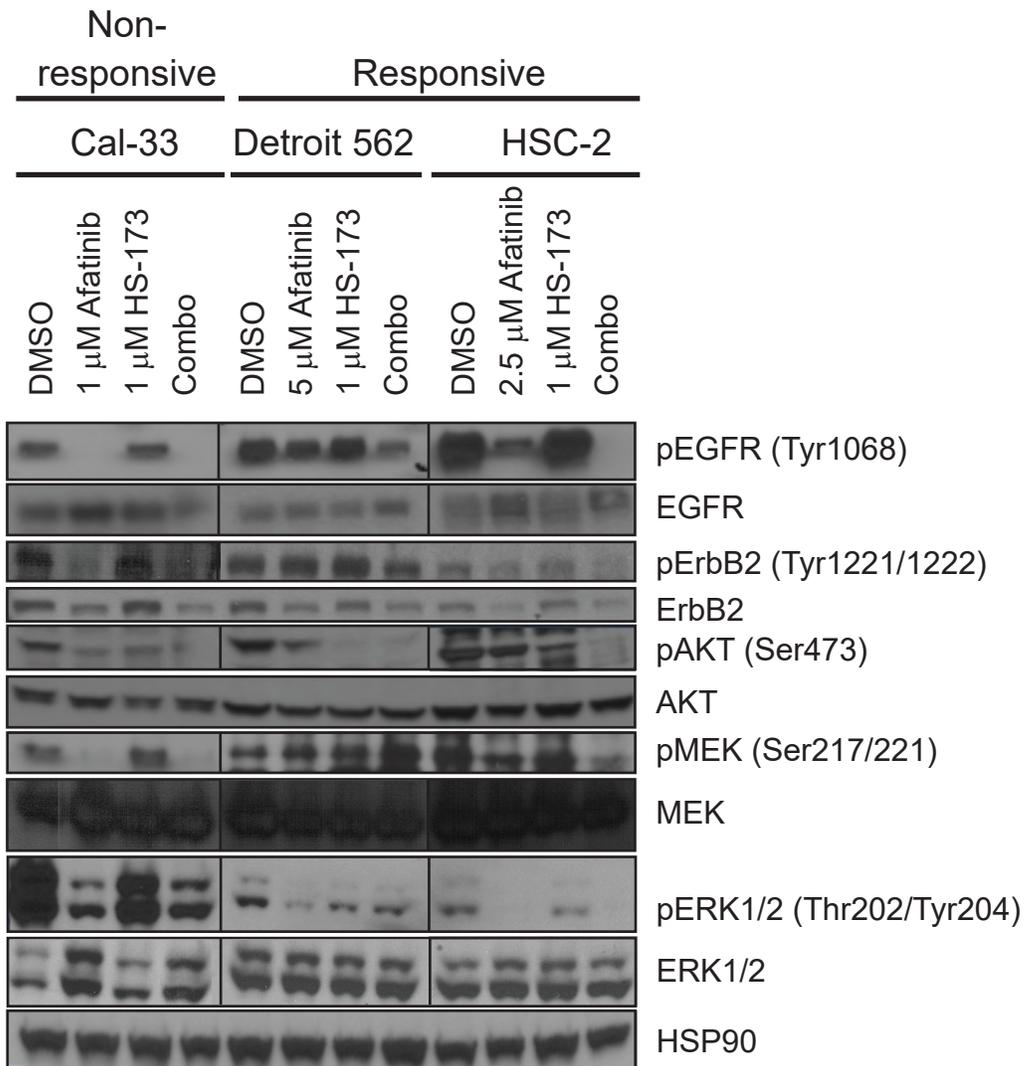


Figure 2

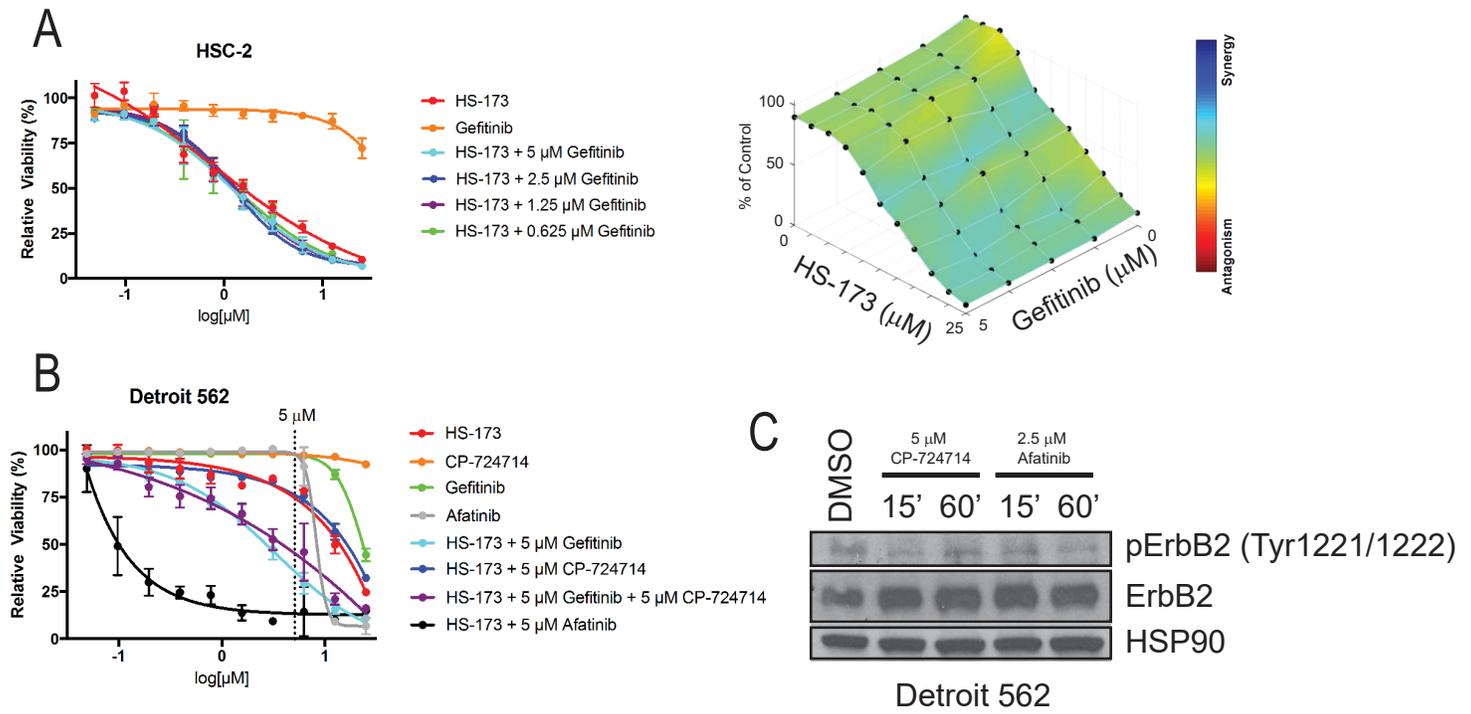


Figure 3

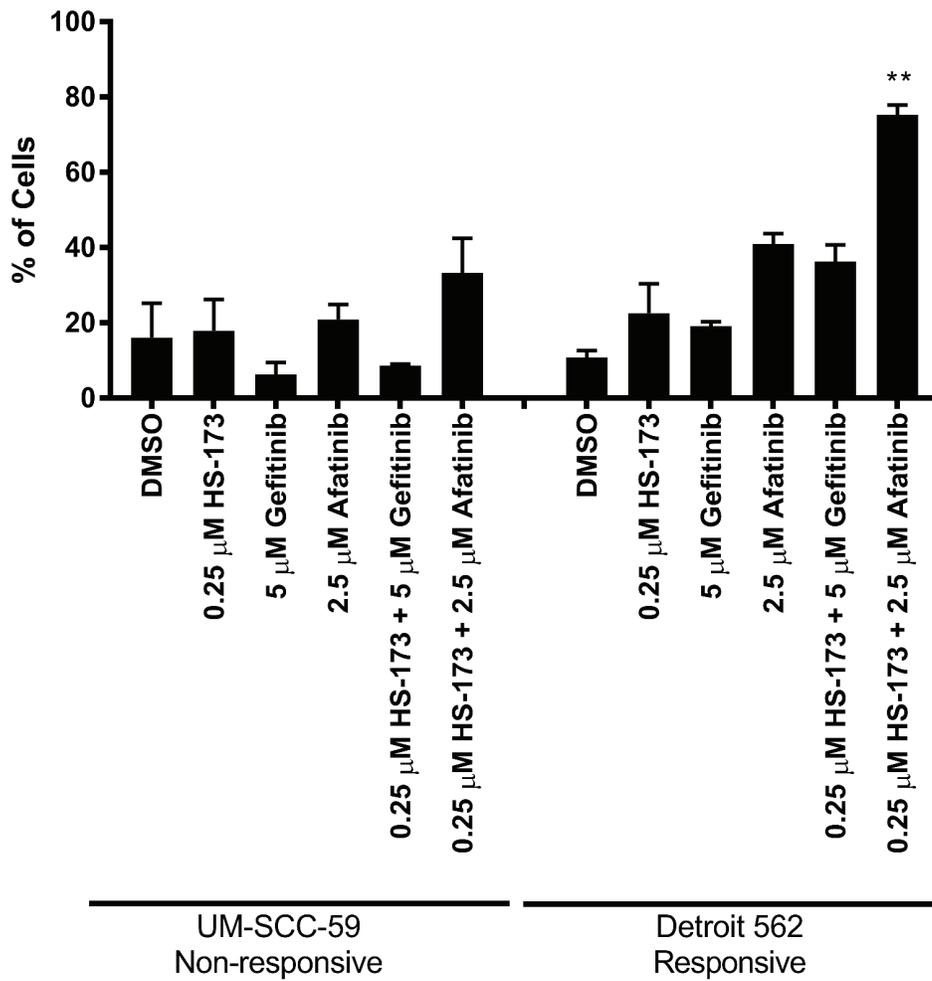


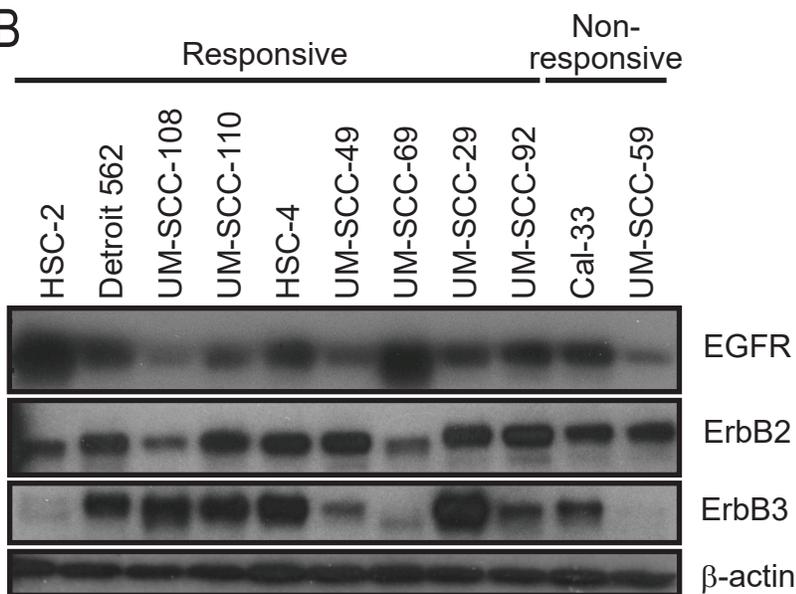
Figure 4

A

Cell Line	PIK3CA		EGFR	Response	
	Mutation	Copy Number		HS-173 + Gefitinib	HS-173 + Afatinib
Cal-33	H1047R	3	4		
Detroit 562	H1047R	Unknown	3		
HSC-2	H1047R	4	12		
HSC-4	E545K	3	6		
UM-SCC-19	H1047R	4	1		
UM-SCC-43	E545K	3	3		
UM-SCC-85	H1047R	2	4		
UM-SCC-29	WT	4	4		
UM-SCC-49	WT	5	5		
UM-SCC-59	WT	5	23		
UM-SCC-69	WT	6	15.67		
UM-SCC-92	WT	3	2.33		
UM-SCC-108	WT	4	3		
UM-SCC-110	WT	3	4		

	Not Synergistic
	Additive
	Further Benefit vs HS-173 + Gefitinib

B



C

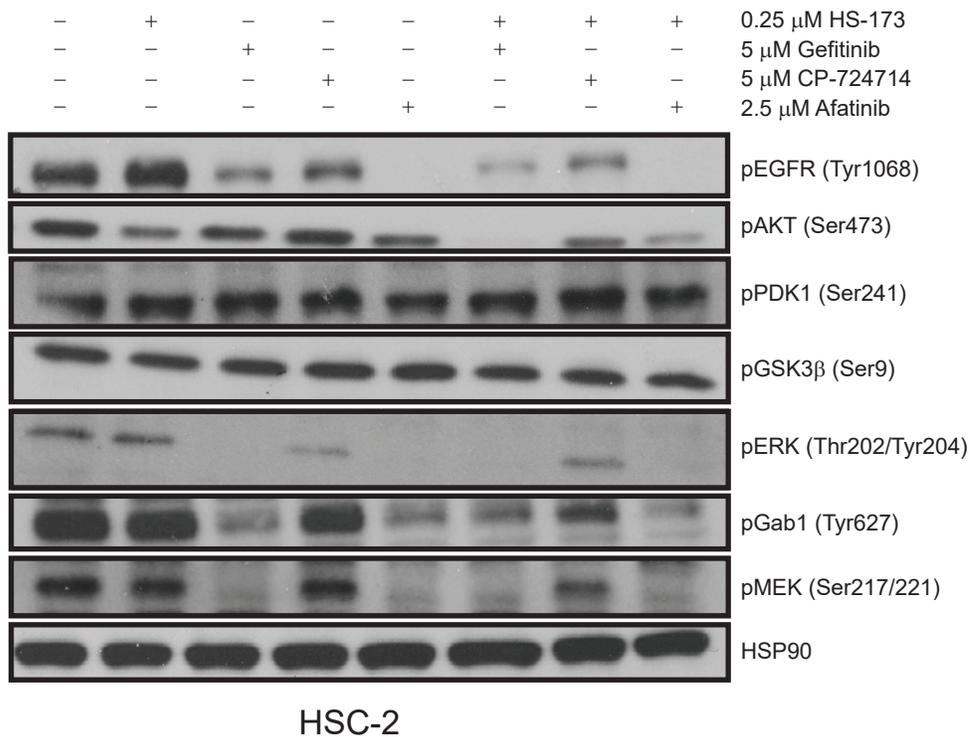


Figure 5