Combination of Anti-HER3 Antibody MM-121/SAR256212 and Cetuximab Inhibits Tumor Growth in Preclinical Models of Head and Neck Squamous Cell Carcinoma

Ning Jiang1,2, Dongsheng Wang2, Zhongliang Hu2, Hyung Ju C. Shin4, Guoqing Qian2, Mohammad Aminur Rahman2, Hongzheng Zhang2, A.R.M. Ruhul Amin2, Sreenivas Nannapaneni2, Xiaojing Wang3, Zhengjia Chen3, Gabriela Garcia5, Gavin MacBeath5, Dong M. Shin2, Fadlo R. Khuri2, Jun Ma1, Zhuo G. Chen2, and Nabil F. Saba2

Abstract
The EGFR monoclonal antibody cetuximab is the only approved targeted agent for treating head and neck squamous cell carcinoma (HNSCC). Yet resistance to cetuximab has hindered its activity in this disease. Intrinsic or compensatory HER3 signaling may contribute to cetuximab resistance. To investigate the therapeutic benefit of combining MM-121/SAR256212, an anti-HER3 monoclonal antibody, with cetuximab in HNSCC, we initially screened 12 HNSCC cell lines for total and phosphorylated levels of the four HER receptors. We also investigated the combination of MM-121 with cetuximab in preclinical models of HNSCC. Our results revealed that HER3 is widely expressed and activated in HNSCC cell lines. MM-121 strongly inhibited phosphorylation of HER3 and AKT. When combined with cetuximab, MM-121 exerted a more potent antitumor activity through simultaneously inhibiting the activation of HER3 and EGFR and consequently the downstream PI3K/AKT and ERK pathways in vitro. Both high and low doses of MM-121 in combination with cetuximab significantly suppressed tumor growth in xenograft models and inhibited activations of HER3, EGFR, AKT, and ERK in vivo. Our work is the first report on this new combination in HNSCC and supports the concept that HER3 inhibition may play an important role in future therapy of HNSCC. Our results open the door for further mechanistic studies to better understand the role of HER3 in resistance to EGFR inhibitors in HNSCC. Mol Cancer Ther; 13(7); 1826–36. ©2014 AACR.

Introduction
Head and neck cancer is the fifth most common form of cancer and causes more than 350,000 deaths globally each year (1). Patients with head and neck squamous cell carcinoma (HNSCC) account for approximately 90% of all head and neck malignancies (2).

Authors’ Affiliations: 1State Key Laboratory of Oncology in Southern China, Department of Radiation Oncology, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, People’s Republic of China; 2Department of Hematology and Medical Oncology, Winship Cancer Institute Emory University School of Medicine; 3Department of Biostatistics and Bioinformatics, Emory School of Public Health, Atlanta, Georgia; 4Quest Diagnostics, Tucker, Georgia; and 5Merrimack Pharmaceuticals, Inc., Cambridge, Massachusetts

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Note: All of the experiments reported in this article were performed at Emory University.

Corresponding Authors: Zhuo G. Chen, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365-C Clifton Road, Suite C3086, Atlanta, GA 30322. Phone: 404-778-3977; Fax: 404-778-5520; E-mail: gzchen@emory.edu; and Nabil F. Saba, 1365-C Clifton Road, Suite C2110, Atlanta, GA 30322, E-mail: nfsaba@emory.edu

doi: 10.1158/1535-7163.MCT-13-1093
©2014 American Association for Cancer Research.
Recently, an increasing body of literature has suggested that resistance to anti-EGFR therapy arises frequently through activation of alternative signaling pathways that bypass the original target (13, 14). Compensatory HER3 signaling and sustained PI3K/AKT activation are associated with sensitivity and resistance to anti-EGFR–targeted therapies, especially in HNSCC (13–16). Unlike other HER receptors, HER3 has diminished intracellular kinase activity but has known ligands. These characters make HER3 an obligate heterodimerization partner for other HER receptors (16). HER3 contains six PI3K-binding sites that are crucial for PI3K/AKT pathway activation (16). A preclinical study reported an association between sensitivity to gefitinib and the overexpression of HER3 in HNSCC cell lines (17). Furthermore, after sustained exposure to gefitinib or erlotinib, cells showed upregulated HER3 and AKT phosphorylation, which correlated with HER3 translocation from the nucleus to the membrane (15). Increased expression of heregulin, a potent HER3 ligand, also provided a possible mechanism of cetuximab resistance in colorectal cancer (18). There is a recent evidence reported that HER3 signaling plays an important role in acquired resistance to cetuximab, perhaps a more crucial one in comparison with MET in HNSCC and non–small cell lung cancer (13). Direct targeting of HER3 by siRNA in cetuximab-resistant cells has been shown to restore cetuximab sensitivity (13). These data suggest an opportunity to develop combinatorial strategies by using cetuximab and anti-HER3 agent in HNSCC.

MM-121 (SAR256212) is a fully human antibody that directly binds to the extracellular domain of HER3 (19, 20) and induces receptor downregulation resulting in the inhibition of downstream HER3-dependent pathways. As MM-121 has not previously been tested in HNSCC, we were interested in exploring its activity as a single agent and in combination with cetuximab in preclinical models of HNSCC. Overall, we found that HER3 was active in the majority of HNSCC cell lines, a combination of EGFR and HER3 inhibition provided improved antitumor activity relative to either inhibitor alone, and the combination effectively inhibited signaling through both ERK and PI3K/AKT pathways in vitro and in vivo.

Materials and Methods

Cell lines and reagents

Cetuximab was obtained from ImClone and MM-121/SAR256212 was provided by Merrimack Pharmaceuticals and Sanofi. The human HNSCC Tu212 cell line was provided by Gary L. Clayman (University of Texas MD Anderson Cancer Center, Houston, TX) in 2002 (21, 22). Human HNSCC cell lines SCC2, SCC47, JHU-012, 93-VU-147T, PCI-13, PCI-15A, PCI-15B, and UM22B were kindly given by Dr. Robert Ferris in 2012 and SCC090 by Dr. Susanne Gollin from the University of Pittsburgh (Pittsburgh, PA) in 2010, respectively. Human HNSCC cell lines SCC1483 and SQCCY1 were obtained from Dr. Shi-Yong Sun at Emory University (Northeast Atlanta, GA) in 2012. SCC2, SCC47, SCC090, 93-Vu-147T, and SCC1483 cells are positive for human papilloma virus (HPV; refs. 23, 24). Most cell lines were maintained in DMEM/F12 (1:1), SCC090 in minimum essential media and JHU-012 in RPMI-1640 medium all supplemented with 10% FBS at 37°C, 5% CO2. All cells were routinely screened for mycoplasma contamination by the MycoAlert Mycoplasma Detection Kit (Lonza Ltd., Allendale, NJ). The authenticity of cell lines Tu212, SCC2, SCC47, SCC090, 93-VU-147T, JHU-012, PCI-13, SCC1483, and SQCCY1 was verified through the genomic short tandem repeat (STR) profile by the Research Animal Diagnostic Genomics Core (EIGC) in October 2013, respectively. Authenticity of PCI-15A, PCI-15B, and UM22B was not done by the authors, but reported by Zhao and colleagues in 2011, using the same STR profile (22).

Colony formation assay

Cells were plated in 6-well culture plates at the concentration of 200 per well. After 24 hours incubation, cells were treated with PBS, 2 μg/mL cetuximab, 20 μg/mL MM-121, or the cetuximab and MM-121 combination (CM combination) for 9 days to form colonies as previously described (25). The dose of cetuximab was chosen from our previous study (25) and the dose of MM-121 was chosen from escalating serial doses, which showed similar trend of synergistic effect in combination with cetuximab (data not shown). Medium was changed every 3 days. The colonies were then stained with 0.2% crystal violet with buffered formalin (Sigma). Colony numbers were manually counted using ImageJ software. Cell numbers ≥50 were considered as a colony.

Cell proliferation assay

The inhibition of cell proliferation by cetuximab and MM-121 was analyzed by a cell proliferation assay as previously described (26). Briefly, 2.5 × 10^3 cells were seeded in 60-mm dishes and incubated overnight. Cells were then treated with PBS, 62 μg/mL cetuximab, 125 μg/mL MM-121, and the combination for 72 hours. The dose of MM-121 and cetuximab was chosen based on previous studies (19, 25) and our sulfonamide B-cell proliferation assay results (Supplementary Fig. S1). Cells were harvested by trypsinization and counted using a cell counter (Beckman Coulter). All the experiments were performed in triplicate.

Flow cytometry analysis of cell cycle and apoptosis

Cells were treated with the two drugs and their combination for 24, 48, 72, and 96 hours, respectively. The doses were chosen based on the previous studies (19, 25) and were consistent with the cell proliferation assay. Cell-cycle and apoptosis analysis using a fluorescence-
activated cell sorting flow cytometer (Becton Dickinson) were described previously (25). Briefly, for cell-cycle analysis, cells were trypsinized and fixed in precooled 70% ethanol for at least 30 minutes. After spinning down, the cells were resuspended and incubated in 500 μL PI/RNASE staining buffer (BD Pharmingen) for 15 to 30 minutes at room temperature before analysis. For apoptosis assay, cells were collected and prepared by using the PE Annexin V Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer’s instructions. FlowJo software was used for data analysis.

Western blot analysis
Cells were treated with the two drugs and their combination with doses consistent with the cell proliferation assay for the indicated time. Cells lysates and xenograft tissue lysates were generated using lysis buffer as previously reported (27). The lysate was centrifuged at 16,000 × g at 4°C for 10 minutes. Fifty micrograms of total protein for each sample was separated by 10% SDS–PAGE and transferred onto a Westran S membrane (Whatman Inc.). Desired proteins were probed with corresponding antibodies. Rabbit anti-human AKT, pAKT, pERK, pS6, and -actin (1:10,000 dilutions) were purchased from Cell Signaling Technology, mouse anti-human AKT (1:10,000 dilution) from Sigma, anti-human EGFR and HER3 antibodies from Santa Cruz Biotechnology, and anti-human pEGFR antibody from Millipore. Horseradish peroxidase–conjugated secondary anti-mouse and anti-rabbit IgG (H+L) was obtained from Promega. Bound antibody was detected using the SuperSignal West Pico Chemoluminescence system (Pierce, Inc.). ImageJ software was used for blot quantification.

In vivo xenograft treatment study
The animal experimental protocol was approved by the Institutional Animal Care and Use Committees of Emory University. In brief, 4 × 10^6 Tu212 and 2 × 10^6 SCC47 cells (1:1 in Matrigel) were injected subcutaneously into female nude mice (athymic nu/nu) ages 4 to 6 weeks. Mice were randomly divided into six groups after tumor formation. PBS control, cetuximab 6.25 mg/dose, MM-121 300 μg/dose (MM-121.LD), MM-121 600 μg/dose (MM-121.HD), combination with low-dose MM-121 (comb. LD), and combination with high-dose MM-121 (comb. HD; n = 7 for each treatment group). Doses were chosen based on previous studies (19, 25). Drugs were given by intraperitoneal (i.p.) injection twice a week. Tumor volume and bodyweight were measured three times a week. Tumor volume was calculated using the formula: \( V = \pi/6 \times \text{larger diameter} \times \text{smaller diameter}^2 \) as reported previously (25). Major organs were harvested for toxicity evaluation by hematoxylin and eosin (H&E) staining.

Immunohistochemistry staining and analysis
Xenograft tissues were harvested, fixed in 10% buffered formalin, and embedded in paraffin. Areas of necrosis were quantified under ×40 as a percentage on routinely H&E-stained slides. Staining of Ki67 (prediluted; Biomedica), TUNEL (Promega), and CD34 (Abcam) was performed as previously described (25). Staining and fluorescent signals from each assay were visualized by Olympus BX41 microscope. For Ki67 and TUNEL, the percentage of nuclei labeled cells was counted in five randomly and sequentially selected areas from each slide at ×100 magnification. Positive CD34 signals were counted in five random fields under ×100 magnification and microvessels were quantified as described previously (28). Briefly, only vessels containing apparent lumen that were positively stained with CD34 were counted. Necrotic areas were excluded from analysis. These quantifications were determined by at least 2 individuals blindly and independently.

Statistical analyses
Comparison of means from multiple treatment groups was carried out using one-way ANOVA or the Kruskal–Wallis test to determine the significance of tumor growth inhibition among treatment groups. A Bonferroni correction was introduced to correct for multiple comparisons. The pairwise comparison was used to compare mean tumor volumes of cell growth inhibition between the different groups over time. Mean values of in vitro colony formation assay, cell proliferation assay, and in vivo tumor volumes were used for calculation of the corresponding synergistic indices (SI) using the methods described before (29). An SI of greater than one indicates a synergistic effect. Statistical analyses were conducted using SPSS version 20. All P values were two-sided and P values less than 0.05 were considered significant.

Results
HER3 is widely expressed and activated in HNSCC cell lines
To determine the total and phosphorylated expression levels of HER3 in HNSCC, 12 HNSCC cell lines were screened by immunoblotting in our study. All 12 cell lines expressed total HER3 to variable levels. Phosphorylated HER3 was found in nine of 12 cell lines (Fig. 1A). Total and phosphorylated levels of the other HER receptors (EGFR, HER2, and HER4) were also detected by immunoblotting (Supplementary Fig. S2A). As our results indicated that HER3 is widely expressed and activated in most HNSCC cell lines, two representative cell lines, SCC47 and Tu212, were chosen for further study as they both exhibited expression of total and phosphorylated HER3.

MM-121 alone inhibits activation of HER3 and AKT in HNSCC cancer cells
As the activity of MM-121 has not previously been tested in HNSCC, its inhibitory effect on HER3 activation and downstream signaling through AKT was assessed. Inhibition of HER3 phosphorylation was observed at a...
dose of 5 μg/mL and higher in both SCC47 and Tu212 cell lines (Fig. 1B). Treatment with MM-121 at a dose of 125 μg/mL for 24 hours completely blocked the activation of HER3 and AKT in both cell lines (Fig. 1B). The results showed that MM-121 has a strong inhibitory activity on phosphorylation of HER3 and AKT as a single drug in HNSCC cancer cell lines. The inhibition of ERK phosphorylation, however, was not observed by MM-121 treatment alone (Supplementary Fig. S2B).

The combination of cetuximab and MM-121 (CM) provides dual inhibition of PI3K/AKT and ERK signaling pathways

HER family receptors activate a number of important signal transduction pathways, including PI3K/AKT and ERK pathways, which are critical for cell growth and survival and are implicated as major factors in many types of cancer. To investigate the combined effect of the HER3 inhibitor MM-121 and the EGFR inhibitor cetuximab on PI3K/AKT and ERK activity, we treated SCC47 and Tu212 cells with MM-121 alone, cetuximab alone, and a combination of the two agents. MM-121 potently blocked HER3 phosphorylation at a concentration of 125 μg/mL, whereas cetuximab partially inhibited EGFR and HER3 activation at a concentration of 62 μg/mL in both cell lines (Supplementary Fig. S3). The CM combination substantially inhibited phosphorylation of both HER3 and EGFR after 72 and 96 hours of treatment (Supplementary Fig. S3). Only CM combination simultaneously decreased both AKT and ERK activation after 72 (Fig. 2) and 96 hours (Supplementary Fig. S4) compared with single antibody treatments. Activation of AKT/mTOR signaling as determined by phosphorylated S6 ribosomal protein (pS6) levels has previously been implicated in progression and poor prognosis of HNSCC (30). Interestingly, in our study, substantial deduction of the pS6 level was only observed in the CM combination group (Fig. 2). These results suggest that CM combination substantially inhibits EGFR and HER3 activation and consequently blocks both of the downstream PI3K/AKT and ERK signaling pathways.

Figure 1. A, total and activated HER3 expression in HNSCC cell lines. Western blot analysis was used to determine the expression of HER3 and pHER3 expression in 12 HNSCC cell lines. B, MM-121 alone inhibits HER3 and AKT activation in vitro. HNSCC cell lines SCC47 and Tu212 were treated with escalating doses of MM-121 as indicated for 12 and 24 hours. Cell lysates were immunoblotted to detect pHER3 (Tyr1289), pAKT (Ser473), total HER3, and AKT. Experiments were repeated three times.

Figure 2. The cetuximab and MM-121 (CM) combination simultaneously inhibits PI3K/AKT and ERK signaling pathways. SCC47 and Tu212 cells were treated with 125 μg/mL MM-121, 62 μg/mL cetuximab, and the combination for 48 and 72 hours. Cell lysates were collected and immunoblotted as indicated.
The CM combination effectively inhibits HNSCC cell growth in vitro

The PI3K/AKT and ERK pathways are critical for cell growth and survival. As our study has shown that the combination of cetuximab and MM-121 inhibits PI3K/AKT and ERK signaling pathways, we speculate that the CM combination may provide an improved strategy to inhibit the growth and survival of HNSCC cancer cells. To evaluate this, we initially accessed growth inhibition by either MM-121 or cetuximab alone in eight HNSCC cell lines. Both antibodies alone showed marginal inhibition of cell growth in vitro with concentrations up to 500 µg/mL (Supplementary Fig. S1). These findings are consistent with previous studies that showed that cetuximab alone have limited growth inhibition effect on certain cancer cell lines in vitro with concentrations up to 150 µg/mL (25, 31).

A colony formation assay was then carried out to determine the long-term growth-inhibitory activity of the CM combination treatment. In both cell lines, the CM combination showed significantly greater inhibition of colony formation in comparison with each single drug or with control after 9 days of treatment (CM vs. control, \( P < 0.001 \) or vs. CM single drug, \( P < 0.05 \); Fig. 3A). The SI of colony formation assay were 8.25 for Tu212 and 3.92 for SCC47 (Supplementary Table S1), indicating a synergistic inhibitory effect of cetuximab and MM-121 on colony formation ability (29). Cetuximab alone also showed greater growth inhibition in both cell lines compared with control (\( P < 0.001 \)). In addition, similar effects were observed by short-term cell proliferation assay after 72 hours of treatment. The growth of the two cell lines was significantly inhibited by the CM combination compared with single-drug treatments (\( P < 0.05 \)). Compared with control, MM-121 and cetuximab as single agents both showed growth inhibition in SCC47 cells (CM vs. control, \( P < 0.05 \)) and only cetuximab showed growth inhibition in Tu212 cells (\( P < 0.001 \); Fig. 3B). Again, a synergistic inhibitory effect was found with SI of 1.46 (Tu212) and 1.64 (SCC47; Supplementary Table S1).
The CM combination induces cell-cycle arrest and apoptosis in HNSCC cells

To determine the underlying mechanism of inhibitory effect of CM combination on tumor cell growth, cell-cycle analysis and apoptosis assay were carried out. Cell-cycle analysis was used to determine whether the observed antitumor effect reflected any change in cell-cycle distribution. We observed an increased proportion of G1-phase cells and a decreased proportion of S-phase cells as early as 24 hours after the CM treatment in both SCC47 and Tu212 cell lines (data not shown). As shown in Fig. 4A, after 72 hours of treatment in the SCC47 cell line, the CM combination induced significantly greater numbers of G0–G1-phase (\( P < 0.01 \)) but reduced S (\( P < 0.01 \)) and G2–M-phase cells (\( P < 0.05 \)) compared with control. Similarly, an increase in G0–G1 cells (\( P < 0.01 \) vs. control) and a decrease in S-phase cells (\( P < 0.001 \) vs. control) were observed in Tu212 cells treated with CM combination (Fig. 4A). Overall, the CM treatment most effectively induced cell-cycle arrest in G1 relative to control or either agent alone. To investigate the molecular mechanism underlying these changes, we monitored the levels of cyclin D1, and cyclin E by immunoblotting in both cell lines. Consistent with our in vitro observations, treatment with the CM combination resulted in downregulation of these two proteins (Fig. 4B).

To assess the effect of the CM combination on apoptosis, phycoerythrin Annexin V was used as a marker for the early features of apoptosis, whereas 7-AAD staining indicated late-stage apoptosis. Compared with other groups, the CM combination induced significantly more early- and late-stage apoptosis after 96 hours of treatment in the two cell lines (Fig. 4C, \( P < 0.05 \)). Consistent with these findings, increased levels of cleaved PARP and caspase-3 were also observed in both cell lines (Fig. 4D). These results indicate that the observed antiproliferative effects...
of the CM combination likely arise from a combination of cell-cycle arrest and apoptosis in HNSCC cell lines.

**The CM combination shows potent antitumor effect in HNSCC xenograft models**

To expand our findings into the *in vivo* setting, two xenograft models using HNSCC cell lines (SCC47 and Tu212) were established as previously described (32, 33). Mice were randomly assigned to six treatment groups: PBS control, cetuximab (C), MM-121.LD, MM121.HD, comb. LD, and comb. HD and were treated twice a week through i.p. injection.

Consistent with our *in vitro* observations, the CM combination showed the greatest tumor growth inhibition in Tu212 xenografts. As shown in the tumor volume measurement result, treatment with both high and low doses of CM combination significantly suppressed tumor growth as compared with the PBS control and cetuximab.

**Figure 5.** The CM combination inhibits HNSCC xenograft tumor growth *in vivo*. Mice bearing subcutaneous SCC47 and Tu212 tumors of approximately 100 mm³ were treated by i.p. injection twice per week for 4 weeks with: PBS (phosphate-buffered saline) control, cetuximab (6.25 mg/dose), MM-121 at low dose (300 μg/dose, MM-121.LD), MM-121 at high dose (600 μg/dose, MM-121.HD), the combination with LD MM-121 (comb. LD), and the combination with HD MM-121 (comb. HD). A, tumor volumes were measured three times a week. B, tumors were harvested and weighed 26 (Tu212) and 29 (SCC47) days after the first treatment. C, major organs were harvested for toxicity evaluation by H&E (magnification, ×100). Error bars, mean ± SE of 7 mice from each group; **, *P* < 0.01 versus control; †, *P* < 0.05 versus cetuximab.
alone (Fig. 5A; \( P < 0.0001 \) in both cases). Comb. HD showed greater tumor growth inhibition than both doses of MM-121 alone (\( P < 0.05 \)), whereas comb. LD showed greater tumor suppression than MM-121.LD (\( P < 0.01 \)). By using the endpoint tumor volumes for calculation (29), a synergistic inhibitory effect was found with the SI of 1.67 (comb. LD) and 1.56 (comb. HD; Supplementary Table S1). After 26 days of treatment, the comb. LD and comb. HD inhibited Tu212 tumor growth by 87\% and 89\%, respectively, compared with control (defined as tumor weight; Fig. 5B).

The comb. HD combination was also highly effective in SCC47 xenografts based on tumor volume measurement (Fig. 5A). All of the treatment groups had significantly reduced tumor growth rates compared with the control group (\( P < 0.05 \)). Comb. HD treatment significantly inhibited tumor growth as compared with control (\( P < 0.001 \)), cetuximab (\( P = 0.04 \)), and MM-121.LD (\( P < 0.01 \)). Comb. LD also inhibited tumor growth over time compared with control (\( P < 0.001 \)) and cetuximab (\( P = 0.06 \)). No significant difference was observed between the MM-121 single-agent groups and the comb. LD group (Fig. 5A). After 29 days of treatment, comb. HD significantly decreased xenograft tumor weight as compared with control (\( P < 0.001 \)) and cetuximab (\( P = 0.04 \); Fig. 5B).

The antitumor activity of CM treatment was not accompanied by any side effect or treatment-related weight loss (Supplementary Fig. S5). No cellular abnormalities were observed in the examined organs, including heart, lung, liver, kidney, and spleen (\( n = 3 \) mice/group), derived from both xenograft mouse models (Fig. 5C).

**The CM combination has the greatest effect on HER3/EGFR signaling, tumor proliferation, apoptosis, and angiogenesis in vivo**

The levels of HER3/\( \text{pHER3} \), EGFR/\( \text{pEGFR} \), and downstream proteins in fresh xenograft tissues were analyzed by Western blot analysis (\( n = 3 \)/group). Consistent with our *in vitro* findings, HER3, EGFR, AKT, and ERK activation were significantly inhibited by the CM combination (Fig. 6A and Supplementary Fig. S6). The pS6 level was also decreased in CM combination-treated tissues (Fig. 6A). A similar trend of decreased \( \text{pHER3} \) and \( \text{pAKT} \) expression was observed in xenograft tissues by immunohistochemical staining (Supplementary Fig. S7). We then studied Tu212 xenograft tissues and found that both high- and low-dose combination groups showed significantly increased necrotic areas as compared with the untreated control (\( P < 0.01 \)). Biomarkers of cell proliferation, apoptosis, and angiogenesis (Ki67, TUNEL, and CD34, respectively) were also examined in the xenograft tissues. Significant reduction in Ki67 signals and increase in TUNEL signals were observed in all treatment groups (\( P < 0.05 \) vs. control), especially in combination groups (\( P < 0.01 \) vs. control). Both high- and low-dose combinations significantly diminished microvessel density measured by CD34 staining as compared with the control (\( P < 0.01 \); Fig. 6B).

**Discussion**

Although better tolerated than cytotoxic chemotherapy, approximately 80\% of patients showed *de novo* resistance and an increasing number of patients become acquired resistant to anti-EGFR therapy in HNSCC (34). Potential strategies to overcome resistance are, therefore, highly needed. Hyperactivation of HER3 has previously been reported to negatively correlate with response to anti-EGFR therapy (15, 35). Moreover, tumor cells that escape from cetuximab inhibition exhibit EGFR upregulation-dependent HER3 activation (13). Dual inhibition of both EGFR and HER3 is, therefore, an attractive clinical strategy for treating HNSCC. Anti-HER3 antibody MM-121 alone or in combination with other antitumor drugs has been studied previously in several cancers (19, 20, 36–39).

In this study, we explored the activity and the underlying mechanism of MM-121 combined with the anti-EGFR antibody cetuximab in the treatment of preclinical HNSCC models. Our results indicated that the combination of the two antibodies significantly inhibited HNSCC tumor cell growth both *in vitro* and *in vivo*. In addition, compared with either antibody alone, the combination inhibited the activation of both EGFR and HER3 and subsequently blocked the activation of AKT, ERK, and S6. Taken together, these findings suggest that the combination of cetuximab with MM-121 may provide potential clinical benefits for patients with HNSCC.

HER3 protein is reportedly overexpressed in patients with HNSCC and its membranous expression is correlated with decreased survival (40). In this study, we assessed the total and phosphorylated levels of all four HER family members in 12 HNSCC cell lines. HER3 was expressed in all 12 cell lines, and \( \text{pHER3} \) was observed with EGFR and pEGFR in nine of the 12 cell lines (Fig. 1A and Supplementary Fig. S2A). Our *in vitro* activity studies showed that neither HER3 nor EGFR inhibition alone was sufficient to inhibit tumor cell growth and ERK activation in HNSCC (Figs. 2, 3, and Supplementary Fig. S1). As ERK generally promotes cell proliferation, the lack of inhibition on ERK phosphorylation may provide an explanation for the limited growth inhibition. Only dual inhibiting EGFR and HER3 through combined treatment of cetuximab and MM-121 could simultaneously block both PI3K/AKT and ERK pathways in HNSCC (Fig. 2 and Supplementary Fig. S4), which is consistent with a recent study using the dual HER3/EGFR–targeting antibody MEHD7945A (31). Together, these data support the hypothesis that improved tumor inhibition activity can be achieved through simultaneously inhibiting both EGFR and HER3 in HNSCC. Interestingly, several studies reported that IGF (insulin-like growth factor receptor) activation is involved in resistance to EGFR inhibitors through activating the PI3K/AKT pathway (41–44). It would be of interest to determine the IGF expression level and target the IGF and HER pathways together in HNSCC models in future.

Ribosomal protein S6 (S6) is a substrate of ribosomal protein S6 kinase (S6K), which is one of the best-
characterized downstream effectors of the PI3K/AKT/mTOR pathway. Phosphorylation of S6 is required for translation initiation and controls cell size, growth, and proliferation in a cell-cycle-independent fashion (45). In HNSCC, pS6 overactivity persists during tumor progression and cytoplasmic staining of pS6 was detected and positively correlated with poor differentiation (30, 46). Moreover, inhibiting this pathway by rapamycin resulted in rapid tumor regression concomitant with a marked decrease in cell proliferation, enhanced apoptosis both in vitro and in vivo in HNSCC (47). Interestingly, we also observed inhibition of S6 phosphorylation after treatment with CM combination in our study. Moreover, pS6 decreasing was initially observed in MM-121–treated SCC47 and cetuximab-treated Tu212 cells at 48 hours. However, this effect was diminished after 72 and 96 hours of treatment regardless of the inhibition status of pAKT at these time points (Fig. 2 and Supplementary Fig. S4). Only the CM combination continuously inhibited S6 phosphorylation from 48 to 96 hours. These findings suggest that only simultaneously inhibited EGFR and HER3 could totally and continuously block pathways that phosphorylate S6 and, thus, contribute to tumor growth inhibition and apoptosis in HNSCC.

HPV is recognized to play a role in the pathogenesis of a subset of HNSCC (48). It is known that this subgroup of patients have a better prognosis compared with their HPV-negative counterparts and could be unnecessarily
over treated by traditional cytotoxic therapy (49, 50). Despite the fact that EGFR expression is inversely correlated with HPV status, current clinical trials are exploring the role of the less-toxic cetuximab in treating HPV-positive disease (50). The fact that HER3 was expressed and activated in all five HPV-positive HNSCC cell lines in our study and the observation of an increased tumor growth inhibition with CM in comparison with C or M alone in the HPV-positive SCC47 cell line model, suggests that HER3 inhibition alone or in combination with EGFR inhibition deserves further investigation in HPV-positive HNSCC.

Another important issue frequently raised is the toxicity related to single or combined targeted therapy. In our study, the doses of cetuximab and MM-121 were chosen as 312.5 μg/kg per dose and 15 or 30 mg/kg per dose twice a week, respectively, based on previous studies (19, 25). We found neither treatment to be associated with body weight loss nor evidence of organ damage in nude mice after treatment for almost a month (Fig. 5C and Supplementary Fig. 5S). To translate this combination to clinical use, the doses of the two agents need to be further optimized. Furthermore, as combing MM-121 with cetuximab coordinately blocked the downstream signaling pathways of EGFR and HER3, it is not surprising that we observed a significant reduction in microvessel density after the combined treatment (Fig. 6B), which is consistent with our previous observation of angiogenesis inhibition by EGFR blockade in both HNSCC and lung cancer xenograft models (21).

In conclusion, we have demonstrated that simultaneous blockade of the PI3K/AKT and ERK pathways in HNSCC cell line models can be achieved by combining cetuximab and MM-121, which act together to inhibit tumor growth. Although several ongoing clinical trials are investigating MM-121 alone or in combination with other systemic agents in treating cancer, including a phase I trial currently recruiting patients with advanced solid tumors, the mechanism underlies their therapeutic effect remains to be elucidated. Our study provides a molecular mechanism for the anti-tumor effects of combined EGFR and HER3 inhibition in models of HNSCC and supports the clinical investigation of the combination therapy in HNSCC.

Disclosure of Potential Conflicts of Interest

G. MacBeath has ownership interest (including patents) in Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Jiang, D. Wang, Z. Hu, H.J.C. Shin, G. Qian, H. Zhang, S. Nannapaneni, Z.G. Chen, N.F. Saba
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Rahman, D.M. Shin, F.R. Khuri, J. Ma, N.F. Saba
Study supervision: A.R.M.R. Amin, J. Ma, Z.G. Chen, N.F. Saba

Acknowledgments

The authors thank Dr. Anthea Hammond for her critical reading and editing of the article.

Grant Support

This study was supported by funding from Merrimack Pharmaceuticals Inc. (to Z.G. Chen and N.F. Saba) and partially by grant from the Specialized Program of Excellence (SPORE) in Head and Neck Cancer (P50CA128613; to D.M. Shin).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 21, 2013; revised March 24, 2014; accepted April 10, 2014; published OnlineFirst April 18, 2014.

References


Molecular Cancer Therapeutics

Combination of Anti-HER3 Antibody MM-121/SAR256212 and Cetuximab Inhibits Tumor Growth in Preclinical Models of Head and Neck Squamous Cell Carcinoma

Ning Jiang, Dongsheng Wang, Zhongliang Hu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-1093

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/04/23/1535-7163.MCT-13-1093.DC1

Cited articles
This article cites 49 articles, 19 of which you can access for free at:
http://mct.aacrjournals.org/content/13/7/1826.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/13/7/1826.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/13/7/1826.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.