Pan-HER Inhibitor Augments Radiation Response in Human Lung and Head and Neck Cancer Models

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Abstract

Purpose: Aberrant regulation of the EGF receptor family (EGFR, HER2, HER3, HER4) contributes to tumorigenesis and metastasis in epithelial cancers. Pan-HER represents a novel molecular targeted therapeutic composed of a mixture of six monoclonal antibodies against EGFR, HER2, and HER3.

Experimental Design: In the current study, we examine the capacity of Pan-HER to augment radiation response across a series of human lung and head and neck cancers, including EGFR inhibitor-resistant cell lines and xenografts.

Results: Pan-HER demonstrates superior antiproliferative and radiosensitizing impact when compared with cetuximab. The mechanisms underlying these effects appear to involve attenuation of DNA damage repair, enhancement of programmed cell death, cell-cycle redistribution, and induction of cellular senescence. Combined treatment of Pan-HER with single or fractionated radiation in human tumor xenografts reveals a potent antitumor and regrowth delay impact compared with Pan-HER or radiation treatment alone.

Conclusions: These data highlight the capacity of Pan-HER to augment radiation response in lung and head and neck cancer models and support investigation of Pan-HER combined with radiation as a promising clinical therapeutic strategy.

Introduction

Members of the EGF receptor family (EGFR/HER1, ErbB-2/HER2, ErbB-3/HER3, and ErbB-4/HER4) play an important role in the differentiation, proliferation, and survival of human cells. Ligand binding promotes homo- and heterodimerization of HER receptors, resulting in activation of the RAS/RAF/MEK/ERK, PI3K/AKT, and/or PLC/PKC downstream signaling pathways (1). However, aberrant expression or overactivation of the ErbB receptor network is commonly associated with tumorigenesis and progression of human epithelial cancers. Indeed, overexpression of individual HER members such as EGFR has been associated with poor clinical prognosis (2). In the early 1980s, Mendelsohn and colleagues identified EGFR as a viable molecular target for inhibition with a monoclonal antibody against the extracellular receptor domain (3). Since then, HER targeting and inhibition has been intensively pursued as a clinically validated cancer therapy over several decades.

Approximately 60% of all patients with cancer receive radiation treatment during the course of their disease. Radiation can be quite effective when an adequate dose can be delivered with limited exposure to surrounding normal tissues. Whereas dose escalation holds potential for improving local tumor control, it is less likely to significantly impact survival for patients with very bulky tumors or systemic metastatic disease (4). A compelling rationale exists to investigate novel molecular targeting agents that may sensitize tumor cells to radiation. Cetuximab (ICM-225, Erbitux) is a chimeric monoclonal antibody that binds to extracellular domain III of the EGFR. Extensive preclinical investigation has demonstrated the potential for cetuximab to enhance tumor cell sensitivity to radiation (5, 6). In a randomized phase III clinical trial published in 2006, the addition of cetuximab to radiation resulted in an absolute survival benefit over radiation alone in patients with locally advanced head and neck squamous cell carcinoma (HNSCC) (7). Furthermore, cetuximab has been shown to improve overall survival when added to cisplatin and fluorouracil in patients with recurrent or metastatic head and neck cancer (8). These mature clinical results reveal the benefit of combining a molecular targeted agent against EGFR with radiation or chemotherapy in advanced HNSCC malignancies.

Despite these advances, many patients do not respond effectively to EGFR inhibition (9). Many patients manifest intrinsic resistance, and some who initially respond to treatment subsequently develop progressive disease (acquired resistance). Accumulating evidence suggests that potential mechanisms of acquired resistance involve HER signaling plasticity, amplification, and/or compensatory signaling of other receptor tyrosine kinases (RTK) in the presence of single receptor inhibition (10, 11). The majority of FDA-approved HER family monoclonal antibodies target single receptors (cetuximab and panitumumab against EGFR, trastuzumab and pertuzumab against HER2). Several clinical trials have failed to demonstrate significant benefit...
Translational Relevance

Despite substantial progress in the clinical development of EGFR cancer therapeutics, only a subset of patients respond effectively to single anti-EGFR agents. Crosstalk among EGFR/HER family members represents a major factor affecting clinical efficacy of EGFR-targeted therapy. It remains critical to advance complementary therapeutic approaches to enhance anti-EGFR treatment efficacy. Pan-HER, a novel mixture of antibodies against three EGFR/HER receptor family members, is shown to augment radiation response and overcome acquired resistance to cetuximab in lung and head and neck tumors. Mechanistic studies further elucidate involvement of cell-cycle progression, DNA damage repair, and senescence as underlying mechanisms for the antitumor activity of Pan-HER. With its unique mode of action and the potential to overcome cetuximab resistance, our data provide strong background for advancing clinical trial designs of Pan-HER in combination with radiation.

when EGFR targeted therapies are added to standard chemotherapy or radiation (12, 13). Refinement of HER targeted therapeutics is warranted to address resistance and maximize potential benefit for individual patients.

Pan-HER is a novel mixture of six monoclonal antibodies directed against distinct, nonoverlapping, extracellular epitopes on EGFR, HER2, and HER3, yielding simultaneous inhibition of all three receptors. Preclinical investigation has demonstrated that combinations of antibodies against nonoverlapping epitopes on HER members are more efficacious in overall receptor inhibition and cell growth inhibition compared with agents that target an individual receptor alone (14). Such antibody mixtures maintain efficacy even in the presence of extracellular HER activating ligands. Early-phase clinical trials illustrate the capacity of antibody mixtures to markedly downregulate EGFR, resulting in antitumor activity in patients with advanced HNSCC (15). Indeed, the combination of antibodies against EGFR, HER2, and HER3 that comprise Pan-HER has been shown to limit compensatory upregulation of alternative RTKs and exerts a superior antitumor impact when compared with antibodies that target one or two members of the HER family (16). In the current study, we investigated whether comprehensive HER inhibition with Pan-HER would impact radiation response in human non–small cell lung (NSCLC), HNSCC, cetuximab-resistant, and patient-derived xenograft (PDX) cancer models.

Materials and Methods

Reagents and antibodies

Pan-HER was provided by Symphogen A/S and engineered as a bivalent IgG1. All other antibodies were obtained from Cell Science and maintained in a laminar airflow cabinet under aseptic conditions. The care, handling, and treatment of experimental animals were conducted in accordance with an animal institutional animal care and use committee (IACUC) of the contributing institution. The humoral immune response to each antibody was monitored regularly by enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS) analyses.

Cell lines

The human lung H226 cell line was provided by Drs John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX), and the NCI-H292 cell line was obtained from the ATCC. The human HNSCC SCC1 (UM-SCC1), SCC6 (UM-SCC6), and SCC104 (UM-SCC104) cell lines were kindly provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI), and the SCC1483 cell line was provided by Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA). NSCLC cells were maintained in RPMI-1640 (Corning Life Sciences) supplemented with 10% FBS. HNSCC cells were cultured in DMEM cell culture media supplemented with 10% FBS and 1 µg/mL hydrocortisone. The acquired cetuximab-resistant clones of H226 and SCC1 were developed following long-term exposure to cetuximab as described previously (17). The Authenticity of these cell lines was regularly verified on the basis of cell morphology and genomic short tandem repeat (STR) profile of each cell line. All cell lines were tested for authenticity in accordance with ATCC guidelines 4 to 6 months prior to the initiation of our investigation.

Ionizing radiation, clonogenic survival, and cell viability assay

Cells were irradiated in vitro using a 137Cs-irradiator (JL Shepherd & Associates Model 109). In vitro clonogenic survival following radiation was defined as the ability of cells to maintain their clonogenic capacity and form colonies greater than 50 cells, as described previously (18). Cell viability assays were carried out by crystal violet staining as described previously (17). Following drug treatment, surviving attached cells were fixed/stained with 0.5% crystal violet in methanol. Plates were air-dried overnight and dye was eluted with 0.1 mol/L sodium citrate (pH 4.2) in ethanol (1:1). Elution was transferred to 96-well plates, and the absorbance was read at 540 nm to determine cell viability.

Flow cytometric analysis

Cells from all treatment groups were fixed in ice-cold 70% ethanol followed by rehydration for 10 minutes at 4°C in PBS containing 1% BSA and 0.2% Triton X-100. To investigate treatment impact on DNA damage repair, cells were then sequentially labeled with anti-phospho-Histone H2AX (γH2AX) (Ser139) monoclonal antibody (Clone JBW301, EMD Millipore) overnight at 4°C followed by AlexaFluor 488–conjugated secondary monoclonal antibody for 1 hour. Following centrifugation, cells were resuspended in a propidium iodide (PI) solution to determine cell-cycle phase as described previously (19). Data were analyzed using FlowJo Software. Apoptosis was detected using an Annexin V/PI dual staining kit from BD Biosciences Pharmingen as described previously (17).

Senescence-associated β-galactosidase detection

Cells (2 × 10⁴) were plated in 6-well plates and treated with Pan-HER and/or radiation. Following 72 hours of treatment, cells were stained for senescence-associated β-galactosidase using the Senescence Detection Kit (Cell Signaling Technology).

Radiation response in human tumor xenografts

Athymic nude mice were obtained from Harlan Bioproducts for Science and maintained in a laminar airflow cabinet under aseptic conditions. The care, handling, and treatment of experimental animals were conducted in accordance with an animal institutional animal care and use committee (IACUC) of the contributing institution. The humoral immune response to each antibody was monitored regularly by enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS) analyses.
protocol approved by the institutional animal care and use committee. Tumor cells were injected subcutaneously into the dorsal flank of the mice. For the PDX, freshly resected tissue from a patient with a p16-negative, T2N0M0 squamous cell carcinoma of the floor of mouth was implanted in athymic nude mice following one serial passage through SCID mice. Tumor size was monitored by direct measurement with calipers. Tumor volume was approximated using the formula: \( V = \frac{4}{3} \pi r^3 \times \text{(large diameter)} \times \text{(small diameter)} \). Pan-HER and cetuximab were administered via intraperitoneal injection at the specified doses and intervals. Radiation treatment was delivered by a cabinet X-ray biological irradiator X-RAD 320 from Precision X-Ray Inc. Mice were immobilized using custom-designed lead jigs that exposed the tumor-bearing dorsal flank to radiation while minimizing the exposure of non-tumor-bearing normal tissue. Toxicity was monitored by body condition index, animal weight, skin appearance, and posture. Tumor growth delay was defined as the average time for tumors to reach a volume of 500 or 1,000 mm³.

Statistical analysis
Data were analyzed using IBM SPSS for Macintosh, Version 22.0. In general, comparisons between means were assessed using ANOVA to determine differences for both main effects (e.g., radiation doses or presence/absence of drug) and the nonadditive interaction of these factors. The statistical significance of post hoc assessment of differences between multiple group means was determined using the Bonferroni method. When multiple assessments were obtained over time, specifically with regard to regrowth of tumors over time, repeated-measures ANOVA was performed. In all cases, alpha was set at \( P < 0.05 \) as the determinant of statistical significance.

Results
Pan-HER downregulates EGFR, HER2, and HER3 and inhibits tumor cell proliferation
To confirm the mechanism of action of Pan-HER in vitro, we examined protein levels of EGFR, HER2, and HER3 by immunoblotting following differential dose exposure to Pan-HER treatment for 48 hours across a spectrum of NSCLC (H226, H292) and HNSCC (SCC1483, SCC1, SCC6) cell lines. As shown in Fig. 1A, Pan-HER downregulated all HER family protein levels and downregulated EGFR, HER2, and HER3 in colorectal cancer cell lines. Characterization of baseline EGFR, HER2, and HER3 levels in these cell lines is shown in Supplementary Fig. S1A. Collectively, we observed a pronounced inhibition of cancer cell proliferation in a dose-dependent manner as shown in Fig. 1B. However, this inhibition of cellular proliferation was observed in cell lines with higher baseline levels of EGFR, HER2, and possibly HER3 (H226, H292, SCC1483, SCC1, SCC6; all \( P < 0.05 \)). Cell lines with comparatively lower baseline levels of these HER proteins (A549, HCT116, and H460) were largely resistant to the antiproliferative effect of Pan-HER. These results suggest that Pan-HER may be more efficacious in cell lines that overexpress EGFR, HER2, and/or HER3. Importantly, Pan-HER also demonstrated the capacity to inhibit the proliferation of tumor cells that were refractory to cetuximab treatment (Fig. 1C). Using previous established cetuximab-resistant HNSCC and NSCLC cells (18), we found a more profound antiproliferative impact of Pan-HER than that from cetuximab in both cetuximab-resistant H226 cells (\( P < 0.001 \)) and cetuximab-resistant SCC1 cells (\( P = 0.034 \)). These cell lines retain Pan-HER sensitivity despite remaining comparatively resistant to cetuximab. These data reveal a strong antitumor capacity of Pan-HER not only in primary NSCLC and HNSCC but also in tumors with acquired resistance to cetuximab.

Pan-HER enhances tumor cell radiosensitivity
We next investigated the capacity of Pan-HER to augment radiation response using in vitro clonogenic survival analysis in NSCLC and HNSCC cell lines. As shown in Fig. 2, pretreatment with 10.0 μg/mL Pan-HER 72 hours prior to graded doses of radiation significantly reduced clonogenic survival when compared with cells treated with radiation alone (\( P < 0.05 \) for all cell lines). The radiation dose enhancement ratios for survival at 10% (ER10) induced by Pan-HER were modest but consistent between 1.18 and 1.45. In addition, Pan-HER significantly enhanced the radiosensitivity of cell lines resistant to cetuximab (cet-resistant H226 and cet-resistant SCC1 (\( P < 0.001 \) in each cell line)). These data reveal the capacity for Pan-HER to combine with radiation and augment radiation response in NSCLC, HNSCC, and cetuximab-resistant cell lines.

We next investigated the capacity of Pan-HER to enhance radiation-induced inhibition of cell cycle progression. Cell cycle analysis using flow cytometry of PI-stained cells revealed that pretreatment with Pan-HER followed by radiation resulted in significant cell cycle arrest in \( G_0/G_1 \) phase across all cell lines tested, compared with cells treated with either Pan-HER or radiation alone (Supplementary Fig. S1B). Interestingly, we also observed cell cycle redistribution in \( G_2/M \) phases in SCC1483 cells treated with Pan-HER and radiation compared with individual modality-treated cells. As a higher proportion of cells accumulate in either \( G_0/G_1 \) or \( G_2/M \) phase, tumors may be more responsive to radiation treatment than cells in the relatively radioresistant \( S \) phase. These data suggest that Pan-HER may inhibit \( G_1 \) to \( S \) and/or \( G_2 \) to \( M \) phase progression, resulting in greater cell cycle arrest following radiation, and the potential for increased response to subsequent radiation treatments.

Pan-HER and radiation attenuates DNA damage repair
Cellular responses to DNA damage are coordinated primarily through two distinct signaling cascades, the ATM-CHK2 and ATR-CHK1 pathways, which are activated by single-strand (SSB) and double-strand DNA breaks (DSB; ref. 20). Studies have shown that the association of EGFR with the catalytic subunit of DNA-dependent protein kinase (DNA-PK) is a central component of the nonhomologous end-joining (NHEJ) repair pathway (21). Recent studies have also identified a potential role of HER2 in activating ATM/ATR signaling (22), thus highlighting a potential advantage of cancer agents capable of inhibiting multiple HER family members simultaneously. To investigate the impact of Pan-HER on DNA damage repair, we examined the profile of DNA damage at different time points and cell cycle phases via flow cytometric analysis of phosphorylated histone 2AX foci (\( \gamma H2AX \)), a marker for DNA DSB. The dotplots reveal that radiation alone (XRT) induced a dramatic increase of \( \gamma H2AX \)-positive H226 cells at 1 hour following treatment with subsequent decline by 48 hours suggesting DNA damage repair (Fig. 3A). In contrast, the combination of Pan-HER with radiation (Pan-HER + XRT) induced a
durable increase in γH2AX-positive cells in G2/M phase that persisted at 24 hours ($P < 0.05$) and 48 hours ($P < 0.01$). Similar results were observed in other cell lines including H292, SCC1, and SCC1483 (Supplementary Fig. S2A). The one exception was with SCC1483 where the combination of drug with radiation was significantly greater at 24 hours ($P < 0.05$) but not at 48 hours. Importantly, only when Pan-HER was combined with radiation did we observe a sustained increase in γH2AX foci in all cell lines tested. These results combined with the clonogenic survival data in Fig. 2 support previous findings that durable retention of γH2AX foci is more indicative of lethal DNA damage than initial γH2AX peaks (23).

To further examine the impact of Pan-HER on DNA damage repair, we investigated individual DNA repair proteins in cells treated with Pan-HER or PBS prior to graded doses of radiation. As illustrated in Fig. 3B, proteins involved in DNA repair such as BRCA1, ATM, DNAPK, NBS1, Rad52, and DNA Ligase IV showed increased expression in response to radiation. However, this activation was attenuated in cells pretreated with Pan-HER prior to radiation. This finding corroborates the more profound inhibition of clonogenic survival that was observed in cells treated with both Pan-HER and radiation in Fig. 2. Additional cell lines demonstrated a similar inhibition in DNA damage repair proteins following pretreatment with Pan-HER compared with control cells (Supplementary Fig. S2B). Taken together, these data suggest that Pan-HER may augment radiation response in part through inhibition of DNA damage repair in tumor cells.

Pan-HER enhances radiation-induced programmed cell death

Tumor cells with impaired ability to repair DNA damage are more prone to programmed cell death through apoptosis or necrosis. Therefore, we examined the capacity of Pan-HER to induce programmed cell death in response to radiation using Annexin V/PI flow cytometric analysis. This technique identifies cells in early (Annexin V–positive/PI-negative) or...
late (Annexin V-positive/PI-positive) phases of apoptosis. As depicted in Fig. 4A, a profound increase in early-phase apoptotic cells was observed 72 hours after combined treatment with Pan-HER and radiation (PX) compared with control cells (C) or cells treated with Pan-HER (P) or radiation (X) alone (P < 0.001). The 2-dimensional flow dot plots demonstrate a higher proportion of cells in the bottom right quadrant (Annexin V-positive, PI-negative) that received a combination of Pan-HER and radiation, corresponding to more cells undergoing early-phase apoptosis following combined treatment. We observed similar results across a variety of additional NSCLC and HNSCC cell lines (P < 0.05 for each cell line; Supplementary Fig. S3). In accordance with these results, we also observed the capacity of Pan-HER to upregulate the proapoptotic proteins BAD and Bax in multiple cell lines (Fig. 4B) when combined with radiation, indicating that Pan-HER may enhance programmed cell death through upregulation of proapoptotic proteins in response to radiation. Taken together, these data suggest another mechanism through which Pan-HER may augment radiation response in cancer cells.

Pan-HER induces cellular senescence in response to radiation

Persistent DNA damage and dysfunctional telomeres can exhaust the proliferative potential of an individual cell and, as a result, induce a state of cellular senescence (24). Given this association between γH2AX foci and senescence, we investigated the capacity of Pan-HER to enhance radiation-induced senescence. By examining the activity of senescence-associated β-galactosidase (SA-β-Gal), we observed that the combination of Pan-HER and radiation (PX) resulted in significantly more SA-β-Gal-positive cells compared with control cells (C) or cells that received Pan-HER (P) or radiation (X) alone (Fig. 5A; P < 0.05 for each cell line). This observation was noted in all HNSCC and NSCLC cell lines examined. To further validate these findings, we examined the capacity of Pan-HER to downregulate the transcription factor forkhead box M1 (FOXM1), a negative regulator of senescence (25). Using immunoblotting, we observed that H226, H292, and SCC1 cells treated with Pan-HER before varying doses of radiation resulted in decreased FOXM1 protein expression compared with untreated cells, suggesting a potential role of FOXM1 in mediating Pan-HER-induced cellular senescence (Fig. 5B). These results corroborate other reports where EGFR inhibitors induce an antitumor impact through cellular senescence (26) and suggest an additional mechanism by which Pan-HER may increase radiosensitization of NSCLC and HNSCC cells.

Pan-HER augments radiation response in human tumor xenografts

To expand our in vitro results to investigation in the in vivo setting, we examined the capacity of Pan-HER to augment radiation response in NSCLC, HNSCC, cetuximab-resistant cell lines, and xenograft models. Using clonogenic survival analysis, we observed that the combination of Pan-HER and radiation resulted in significantly more colony formation compared to control cells or cells treated with Pan-HER or radiation alone (Fig. 2). We also observed a profound increase in colony formation in cetuximab-resistant cell lines (Cet-resistant H226, Cet-resistant SCC1) following combined treatment compared with control cells. These findings suggest that Pan-HER may serve as a radiosensitizer in clinical settings where EGFR inhibitors are in use.
HNSCC, and PDX models. In an effort to capture interaction between Pan-HER and radiation, treatment doses and schedules were based upon previous studies from this laboratory and others such that the tumor response to each individual modality would be modest.

Using human tumor xenografts with primary (SCC1483 and H226) cell lines, the combination of Pan-HER and fractionated radiation resulted in a significant antitumor impact and regrowth delay at 1,000 mm$^3$ (>50 days for SCC1483 or >68 days for H226) when compared with mice that received vehicle (22 or 40 days), Pan-HER (36 or 50 days), or radiation alone (34 or 50 days; $P < 0.001$ for each cell line; Fig. 6A and B). These in vivo data corroborate the capacity of Pan-HER to augment radiation response across NSCLC and HNSCC models in vitro.

We next evaluated the capacity of Pan-HER to overcome resistance to cetuximab in a xenograft model using the cetuximab-resistant HNSCC cell line SCC1. Notably, the combination of Pan-HER and radiation inhibited tumor growth profoundly, compared with mice that received individual treatment modalities or mice that received cetuximab and radiation ($P < 0.01$) (Fig. 6D). More importantly, Pan-HER was found to induce regression of tumors that were highly refractory to cetuximab. In contrast, cetuximab did not inhibit the growth of tumors that were previously treated with pan-HER. As shown in Fig. 6D, mice that initially received cetuximab and radiation were retreated with Pan-HER and radiation. Immediately following this second round of treatment, tumors showed dramatic and rapid regression. In contrast, mice that initially received Pan-HER alone and were retreated with cetuximab experienced no such regression in tumor volume. These data confirm the capacity of Pan-HER to overcome acquired resistance to cetuximab and radiation in similar fashion to that previously demonstrated in vitro.

**Figure 3.**
Effect of Pan-HER and radiation on DNA damage repair. A, radiation-induced DNA damage throughout the cell cycle was determined using anti-γH2AX antibody and PI staining as described in Materials and Methods. Cells were treated with 20.0 μg/mL Pan-HER (Pan-HER), 2-Gy radiation (XRT), or the combination of 20.0 μg/mL Pan-HER and 2-Gy radiation (Pan-HER + XRT). Left, bivariant cytograms of γH2AX levels and DNA content in H226 cells obtained 0 to 48 hours after treatment. The populations of γH2AX-labeled cells in G2–M stage were gated in each cytogram and quantified in the right bar graph. B, immunoblotting depicts the inhibition of DNA repair proteins on cells treated with 20.0 μg/mL Pan-HER for 24 hours prior to different doses of radiation compared with cells treated with radiation alone. *, $P < 0.05$. 

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Finally, we examined the capacity of Pan-HER to augment the radiation response of xenografted tumors from a patient-derived human tumor using a previously described method (27). Remarkably, a single dose of Pan-HER demonstrated a profound capacity to sensitize cells to a single fraction of radiation (PX), resulting in a robust regrowth delay (>153 days to reach 500 mm³) when compared with PBS-treated mice (50 days) and mice that were treated with either a single dose of Pan-HER (94 days) or a single fraction of radiation (95 days; \( P < 0.001 \); Fig. 6C).

In summary, Pan-HER demonstrated a potent capacity to augment radiation response in both standard tumor cell line assessments and PDX. Notably, we observed no differences in body weight (Supplementary Fig. S4), skin appearance, or activity level of mice receiving Pan-HER alone or Pan-HER combined with radiation across any of the dose levels and treatment schedules tested.

**Discussion**

While many tumors respond initially to molecular targeting of individual HER family members, durable overall response is frequently limited by acquired resistance (28). Compensatory activation of other HER network members often sustains signaling pathways critical for tumor cell proliferation and survival (29). This has stimulated the development of agents that target multiple HER family receptors using bi- or dual-specific antibodies (30, 31). HER2 amplification has been identified as a potential mechanism of acquired resistance to EGFR tyrosine kinase inhibitors in a subset of lung cancers and is associated with poorer disease-free survival (32). HER3 hyperactivation has been observed following inhibition of EGFR or HER2, indicating a potential role of HER3 in mediating resistance to anti-EGFR or anti-HER2 therapeutics (33). This upregulation of HER3 is also a poor prognostic factor and is associated with poorer response to anti-EGFR therapy and worse survival rates (34). In this study, we investigated the capacity of Pan-HER to augment radiation response in NSCLC, HNSCC, and cetuximab-resistant cancer models both in vitro and in vivo. The broad efficacy of this agent to complement radiation, resulting in stronger anticancer efficacy, holds promise to address the challenge of acquired resistance to HER-targeted agents.

Another mechanism of resistance to HER-targeted agents derives from amplification of MET that occurs in patients with NSCLC who exhibit resistance to the EGFR RTK inhibitors...
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gefitinib or erlotinib (35). This MET amplification results in EGFR crosstalk and HER3 activation, resulting in persistent, downstream activation of AKT. MET is also highly expressed in HER2-positive breast cancers, suggesting a cooperative interaction between MET and HER2 in cancer progression and treatment resistance. Recent studies using multiple four-in-one antibodies have demonstrated that only comprehensive inhibition of EGFR, HER2, and HER3 can prevent crosstalk between the HER network and MET that potentially contributes to acquired resistance (36). Therefore, ongoing investigations in our laboratory are directed at evaluating the efficacy of Pan-HER to augment radiation response in NSCLC cell lines that are resistant to erlotinib, as well as defining the use of combining Pan-HER with MET inhibition in EGFR-T790M mutated models.

Our initial in vitro data demonstrate the capacity of Pan-HER to significantly inhibit tumor cell proliferation across a broad range of NSCLC, HNSCC, and cetuximab-resistant cell lines (Fig. 1). We observed that Pan-HER was most effective in inhibiting proliferation in cell lines that exhibited higher baseline levels of EGFR, HER2, and HER3 (Supplementary Fig. S1). Given the known association between HER receptor expression level and poor clinical outcome in a variety of tumor types, the observation that Pan-HER is most effective in cell lines with high baseline HER levels suggests its potential for patients with a poor prognosis. We are currently investigating the efficacy of Pan-HER combined with radiation in NSCLC cell lines that have individual HER proteins knocked-down, such as H520 (EGFR-null). Such work could provide insight into the relative importance of each HER member in the response to Pan-HER and thereby yield predictive information regarding which cancers might be most susceptible to the radiosensitizing effect of Pan-HER inhibition.

Our in vitro results are strengthened by findings in mouse models, where we observed a significant capacity of Pan-HER to augment the radiation effect in NSCLC, HNSCC, cetuximab-resistance HNSCC, and PDX models. We observed a durable regrowth delay in xenografted tumors from a T2N0M0, p16-negative squamous cell carcinoma of the floor of mouth treated with a single dose of Pan-HER and 12-Gy radiation. PDX may more closely reflect the heterogeneity of human tumors and offer a useful preclinical model for investigation of novel therapeutics (37). Additional PDX experiments to examine tumor characteristics that show significant response to Pan-HER and radiation in vitro may help identify biomarkers that predict therapeutic response.

Locally advanced HNSCC is characterized by aggressive behavior with a propensity for recurrence and variable response to treatment. This clinical heterogeneity may reflect the inherent molecular heterogeneity of each individual tumor. Current risk stratification for HNSCC using disease anatomic location, stage, and histologic characteristics may fail to capture individual molecular alterations responsible for acquired treatment resistance, metastatic progression, and/or recurrence of disease. Recent efforts by The Cancer Genome Atlas (TCGA) have been directed at generating integrated genomic annotations of molecular alterations in HNSCC (38). These genomic analyses may be critical in identifying oncogenic driver events, thereby allowing for more precise treatments that specifically target individual mutations. Current investigations in our laboratory are concentrating on HNSCC PDX samples treated with both Pan-HER and radiation in an effort to identify molecular signatures within the HER pathway among tumor samples. These investigations may allow for the establishment of unique treatment regimens and the identification of malignant phenotypes that help predict individual responsiveness to Pan-HER and radiation.

In addition to downregulating molecules involved in cancer growth, there is increasing evidence to suggest that therapeutic monoclonal antibodies, such as rituximab, trastuzumab, and cetuximab, are capable of initiating an immune response by engaging with Fc receptors on innate immune cells and eliciting an effector response through antibody-dependent cellular cytotoxicity (ADCC) against tumor cells (39). These antibodies are routinely used in patients treated with radiation for various malignancies, and there is growing evidence to suggest that radiation may sensitize tumor cells to such an immune response (40). Despite these findings, a mechanistic interaction

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of Pan-HER and radiation on cellular senescence. A, cells were treated with 20.0 μg/mL Pan-HER, 2-Gy radiation (XRT), or the combination of 20.0 μg/mL Pan-HER and 2-Gy radiation (Pan-HER + XRT) for 72 hours and subsequently stained for SA-β-Gal. The bar graph depicts the average number of SA-β-Gal-positive cells from three randomly selected images containing similar cell confluency. B, immunoblotting depicts the inhibition of FOXM1 on cells treated with 20.0 μg/mL Pan-HER for 24 hours prior to different doses of radiation compared with cells treated with radiation alone. *, P < 0.05.
between radiation and antibody-directed immune response has not yet been clarified. Furthermore, antibody mixtures have been shown to activate complement-dependent cytotoxicity (CDC) in addition to ADCC (41). Therefore, a compelling rationale exists to investigate the capacity of Pan-HER in combination with radiation to elicit ADCC and CDC both in vitro and in vivo.

Collectively, these results reveal the significant capacity of Pan-HER to augment radiation response across NSCLC, HNSCC, cetuximab-resistant, and PDX tumor models. This combination holds translational implications given the well-established use of radiation and molecular targeted agents in treating a variety of malignancies. In addition, the data suggest that the unique mechanism of action of Pan-HER in inhibiting multiple HER members offers a promising treatment strategy to address acquired resistance to individual HER family inhibitory agents. Contemporary cancer drug development strongly emphasizes demonstration of monotherapeutic efficacy in metastatic disease prior to comparison with standard-of-care treatments in the definitive setting. The overwhelming majority of clinical trials investigate radiation or a molecular targeted therapeutic individually. This paradigm may unintentionally overlook molecular targeted agents that work most effectively when combined with radiation and may limit advances in the treatment of locally advanced malignancies (42). The strong interaction of Pan-HER combined with radiation warrants systematic clinical trial investigation and represents a promising radiosensitization strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.M. Francis, S. Huang, C. Li, M. Kragh, P.M. Harari
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Francis, S. Huang, E.A. Armstrong, L.R. Werner, A.D. Swick, P.M. Harari
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Francis, S. Huang, E.A. Armstrong, L.R. Werner, C. Li, Z.S. Morris, A.D. Swick, M. Kragh, J. Lanitto, R.J. Kimple, P.M. Harari

Figure 6. Pan-HER augments radiation response in human tumor xenografts. A–D, mice with tumor xenografts from NSCLC, HNSCC, cetuximab-resistant HNSCC, and patient-derived tumor were treated with Pan-HER, radiation (XRT), or both modalities (Pan-HER + XRT, cetuximab + XRT) during the time interval indicated by the dotted gray line in each figure (n = 8–12 mice per group). *, P < 0.05; **, P < 0.001.
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Received July 13, 2015; revised September 2, 2015; accepted September 3, 2015, published OnlineFirst September 29, 2015.
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