Antitumor Effects of MEHD7945A, a Dual-Specific Antibody against EGFR and HER3, in Combination with Radiation in Lung and Head and Neck Cancers

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Abstract

Human epidermal growth factor receptor family members (EGFR, HER2, HER3, and HER4) play important roles in tumorigenesis and response to cancer therapeutics. In this study, we evaluated the capacity of the dual-target antibody MEHD7945A that simultaneously targets EGFR and HER3 to modulate radiation response in lung and head and neck cancer models. Antitumor effects of MEHD7945A in combination with radiation were evaluated in cell culture and tumor xenograft models. Mechanisms that may contribute to increased radiation killing by MEHD7945A, including DNA damage and inhibition of EGFR–HER signaling pathways, were analyzed. Immunohistochemical analysis of tumor xenografts was conducted to evaluate the effect of MEHD7945A in combination with radiation on tumor growth and microenvironment. MEHD7945A inhibited basal and radiation-induced EGFR and HER3 activation resulting in the inhibition of tumor cell growth and enhanced radiosensitivity. MEHD7945A was more effective in augmenting radiation response than treatment with individual anti-EGFR or anti-HER3 antibodies. An increase in DNA double-strand breaks associated γ-H2AX was observed in cells receiving combined treatment with MEHD7945A and radiation. Immunohistochemical staining evaluation in human tumor xenografts showed that MEHD7945A combined with radiation significantly reduced the expression of markers of tumor proliferation and tumor vasculature. These findings reveal the capacity of MEHD7945A to augment radiation response in lung and head and neck cancers. The dual EGFR/HER3-targeting action of MEHD7945A merits further investigation and clinical trial evaluation as a radiation sensitizer in cancer therapy.

Introduction

Radiotherapy is a central cancer treatment modality delivered to over half of all cancer patients during the course of their disease. However, normal tissue tolerance is often a limiting factor for high-dose radiation delivery in various anatomic sites. Combining radiation with cytotoxic chemotherapy or molecular-targeted therapies offers the potential to improve therapeutic outcome (1–4).

The epidermal growth factor receptor (EGFR) family is a critical target in tumorigenesis and progression, and it has been extensively studied with clinical application during the last decade (5, 6). The EGFR family consists of four members, including EGFR (ErbB1/HER1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). These receptors differ in their ligand-binding and substrate specificity. They form homo- or heterodimers following ligand activation. Tumor overexpression of EGFR family members is associated with poor clinical response and outcome (7, 8).

Inhibition of EGFR using either monoclonal antibodies (mAb) or small-molecule tyrosine kinase inhibitors sensitizes tumor cells to radiation in a variety of tumor types. The most mature clinical data are derived from studies with the combination of anti-EGFR mAb cetuximab with radiotherapy in head and neck squamous cell carcinoma (HNSCC; refs. 9–11). Less attention has been paid to HER3 due to its lack of active kinase activity. However, emerging evidence has placed HER3 in the spotlight as a key signaling hub in several clinical contexts (12, 13). HER3 has been identified as a contributor to acquired resistance to EGFR and HER2 therapy by activating a compensatory PI3K–AKT survival pathway for EGFR or HER2 inhibitors. HER3 signaling contributes to carcinoma cell survival following radiation (14, 15). Overexpression and activating mutations in HER3 have been identified in multiple cancer types, including breast, gastric, colon, melanoma, ovarian, pancreatic, bladder, and non–small cell lung cancers (NSCLC; ref. 16). Herein, simultaneous blockade of EGFR and HER3 may specifically improve the outcome of radiotherapy. In the present study, we examined the antitumor capacity of MEHD7945A (EGFR and HER3 dual inhibitor) in combination with radiation in NSCLC and HNSCC cell lines.

Materials and Methods

Cell culture

Five HNSCC cell lines (UM-SCC1, UM-SCC4, UM-SCC6, UM-SCC11A, and UM-SCC38) were provided by Dr. Thomas
Clonogenic survival assay
Survival following irradiation was defined as the ability of cells to maintain their clonogenic capacity and to form colonies. A specified number of cells were seeded in 6-well plates. Following a 4 hours pretreatment of MEHD7945A, cells were irradiated with a $^{137}$Cs-iradiator (Model 109, J. L. Shepherd & Associates). Following 10 to 14 days incubation, cells were fixed in 10% neutral-buffered formalin and the surviving fractions were calculated after counting the numbers of colonies containing at least 50 cells. Survival curves were generated after normalizing to the samples without radiation in control and MEHD7945A-treated groups.

Cell proliferation assay
Cells were seeded in 96-well plates and exposed to doses of MEHD7945A for 72 hours. Cell proliferation was tested by the product datasheet. The expression of phosphorylated protein was determined following quantification of scanned images by ImageJ software.

Human phospho-MAPK array
Phospho-MAPK array was purchased from R&D systems. The human phospho-MAPK array kit was used to screen the activation of 26 kinases, including ERK, JNK, P38, and AKT families. Assays were performed following the protocols as described in the product datasheet. The expression of phosphorylated protein was determined following quantification of scanned images by ImageJ software.
antibody. Slides were then incubated with streptavidin peroxidase and visualized using the Dakocytomation Liquid DAB+Substrate Chromogen System. A TUNEL kit from BD Biosciences was used for the detection of apoptosis. Images were quantified via taking the average staining intensity measured from two tumors per treatment group.

**Fluorescence imaging of vasculature in xenografted tumors**

Twenty–four hours following the final treatments, tumors were excised and frozen. Thereafter, frozen sections were fixed in acetone at 4°C. After rehydration, tumor sections were stained for proliferation (Ki67) and vasculature (9F1) as previously described (18).

**Results**

**MEHD7945A inhibits EGFR/HER3 signaling and cell proliferation**

Because MEHD7945A is a dual-specific antibody against EGFR and HER3, we first examined the expression level of EGFR and HER3 in multiple cell lines derived from NSCLC and HNSCC (Fig. 1A). We found that even though most cell lines express both EGFR and HER3, the levels of phosphorylated HER3 vary. Using immunoblotting, we confirmed that MEHD7945A significantly inhibited EGFR and HER3 phosphorylation and its downstream MAPK and AKT signaling at a concentration of 1 to 100 μg/mL in these cell lines (Supplementary Fig. S1). We then evaluated the growth inhibitory effect of MEHD7945A on these cell lines using a CCK8 cell counting kit. As shown in Fig. 1B, MEHD7945A inhibited cell growth in a dose-dependent manner. Notably, UM-SCC6, which expresses high levels of pHER3 and exhibited significant inhibition of p-HER3 and p-AKT by MEHD7945A treatment, was more sensitive to MEHD7945A than other cell lines. The inhibitory effects of MEHD7945A on cell signal transduction, cell growth, and colony formation were observed to be more effective than those observed with anti-EGFR or anti-HER3 antibody alone on UM-SCC6 cells (Supplementary Fig. S2A–S2C). Moreover, the impact of MEHD7945A on inhibition of

**Figure 1.**

A, EGFR/HER3 expression profiles in multiple HNSCC and NSCLC cell lines. Six HNSCCs (UM-SCC1, UM-SCC4, UM-SCC6, UM-SCC11A, UM-SCC38, and SCC1483 cells) and five NSCLC cell lines (NCI-H226, NCI-H292, NCI-H358, NCI-H520, and A549) were cultured in relevant media. Whole-cell lysates were obtained and separated by SDS-PAGE and immunoblotted with the indicated antibodies. B, MEHD7945A inhibits growth of cells. Cells were exposed to serial concentrations of MEHD7945A for 72 hours. Thereafter, growth of tumor cells was determined by cell proliferation analysis.
colony formation and radiosensitization was similar to that from the combination of individual anti-EGFR and anti-HER3 antibodies (Supplementary Fig. S2C and S2D). With the greater simplicity of single drug administration for clinical use, these results suggest a potential advantage of MEHD7945A when compared with the combination of two distinct antibodies to effectively target both EGFR and HER3.

MEHD7945A enhances radiosensitivity

To determine the effect of dual inhibition of EGFR and HER3 by MEHD7945A on cell radiosensitivity, we performed clonogenic survival assays with UM-SCC6 and NCI-H226 cells following treatments. As shown in Fig. 2A, treatment with 5 μg/mL MEHD7945A before radiation reduced clonogenic survival when compared with control without MEHD7945A. The radiation dose enhancement ratios for survival at 10% (ER10) induced by MEHD7945A were modest but consistent at 1.37 and 1.19 for UM-SCC6 and NCI-H226, respectively. Similar to the results observed in the proliferation analysis above, MEHD7945A was more potent in inhibiting clonogenic survival than single-agent treatment with anti-EGFR or anti-HER3 mAb.

MEHD7945A increases DNA DSBs

To further assess the impact of MEHD7945A on cell radiation response, we examined the radiation-induced DNA damage profile by measuring levels of γ-H2AX, a marker of DNA DSBs using flow cytometry and immunocytochemistry staining analysis. As shown in the bottom of Fig. 2B, which displays the representative two-dimensional DNA histogram plots following treatment, a robust increase of γ-H2AX–positive UM-SCC6 cells was observed at 1 hour following radiation treatment followed by a subsequent decline (bar graph). MEHD7945A augmented radiation-induced γ-H2AX signals at 1 hour and caused a significant decline delay at 4, 8, and 24 hours ($P < 0.05$). Further analysis using the fractional product method indicated that this impact is additive for MEHD7945A combined with radiation. Consistently, we found similar results by

**Figure 2.** A, MEHD7945A can sensitize cells to radiation. UM-SCC6 and NCI-H226 were incubated with 5 μg/mL of cetuximab, HER3 antibody, and MEHD7945A for 4 hours, and radiated with indicated doses. Clonogenic assays were performed as described previously. Control curves were exposed to radiation without drug treatment. B, combination of MEHD7945A with radiation can increase DNA damage. UM-SCC6 and NCI-H226 cells were incubated with MEHD7945A (20 μg/mL) for 24 hours before 4 Gy radiation treatments. γ-H2AX was analyzed by flow cytometry as described in Materials and Methods at 1, 4, 8, and 24 hours following radiation. The populations of γ-H2AX–labeled cells in G0, S, and G0–M stages were gated in each bivariant cytogram and quantitated by FlowJo software. Bottom, representative cytogram obtained at 4 hours following indicated treatment. C, representative images of γ-H2AX foci in the nucleus at 4 hours following indicated treatment. Bar graph, average number of γ-H2AX foci of 100 cells; *, $P < 0.05$ and normalized to radiation alone.
determining the γ-H2AX foci in the nucleus as shown in Fig. 2C. MEHD7945A in combination with radiation significantly enhanced γ-H2AX foci formation at 4 hours following radiation treatment. These results suggest that the increased radiosensitivity may result in part from enhancement of DNA damage induced following exposure to MEHD7945A and radiation. It is also possible that MEHD7945A is serving to inhibit prosurvival and proliferative signaling in these cells, thereby contributing to the enhanced radiation response.

MEHD7945A blocks radiation-induced activation of EGFR and HER3

Given the impact of MEHD7945A on clonogenic survival, we further investigated underlying mechanisms of MEHD7945A on radiosensitivity. Acknowledging the activation of multiple kinases by radiation, we analyzed the radiation-induced EGFR and HER3 activation in UM-SCC6 and NCI-H226 cells by Western blot assay. As shown in Fig. 3A, radiation activated EGFR and HER3 by 1.8- to 2.5-fold in UM-SCC6 cells, by 5.9- to 6.9-fold in NCI-H226 cells, and respective downstream kinases MAPK and AKT by 1.8- to 4.6-fold in UM-SCC6 cells and 1.8- to 3.7-fold in NCI-H226 cells within 60 minutes following radiation exposure. Cells pretreated with 50 μg/mL MEHD7945A for 24 hours before radiation exhibited a significant decrease in activated EGFR and HER3 as well as MAPK and AKT. Activation of EGFR and HER3 resolved 24 hours after radiation, while AKT and MAPK activation remained. As compared with cells treated with radiation alone, activation of MAPK and AKT was markedly diminished in cells pretreated with MEHD7945A. We then analyzed cell-cycle phase distribution of UM-SCC6 and NCI-H226 following MEHD7945A and/or radiation treatments. Figure 3 demonstrated that MEHD7945A enriched the cell-cycle population in G1 and radiation enriched the population in G2-M. As expected, the combination of MEHD7945A and radiation resulted in enhanced cell populations in either G1 and/or G2–M accompanied by a significant reduction in the percentage of cells in S-phase. These data suggest that combined MEHD7945A and radiation treatment inhibit EGFR/HER3 signaling through MAPK and AKT that result in cell-cycle redistribution.
Exposure of cells to radiation can induce compensatory activation of multiple signaling pathways that promote tumor cell survival and repopulation. To explore the impact of MEHD7945A affects activation of relevant molecules, we used a human phospho-MAPK array that simultaneously detects the active phosphorylated form of 26 different MAPK and AKT family members. As summarized in Fig. 4, radiation induced the activation of multiple MAPK/AKT kinases. Pretreatment with MEHD7945A (20 μg/mL) significantly decreased the phosphorylation of these molecules, including ERK, JNK, p38, MKK, MSK, AKT, GSK, GREG, S6, and RSK, all of which are involved in cell survival regulation and apoptosis. Although several molecules, such as JNK1, p38, MSK2, and TOR were increased when MEHD7945A was combined with radiation, we found a consistent inhibition in the phosphorylation of all AKT family members. These results highlight a role of the PI3K–AKT pathway in regulating MEHD7945A-induced radiosensitivity.

MEHD7945A augments radiation response in tumor xenografts

To further evaluate the capacity of MEHD7945A to augment radiation response in vivo, human tumor xenograft experiments in athymic nude mice were carried out using UM-SCC6, SCC1483, and NCI-H226 cells. Following the establishment of tumor xenografts, mice were treated with either single or fractionated doses of radiation and/or MEHD7945A. In single-dose experiments, mice were treated with 16 Gy of radiation and/or 8 mg/kg of MEHD7945A. The antitumor impact of treatment was determined by tumor growth delay as shown in Fig. 5 and using Kaplan–Meier survival as in Supplementary Fig. S3. As depicted, the combination of MEHD7945A and radiation exhibited a greater antitumor impact than that observed with single modality treatment in UM-SCC6, SCC1483, and NCI-H226 xenograft tumors. Similarly, the combination of fractionated radiation and MEHD7945A also induced a significant regrowth delay in SCC1483 and NCI-H226 (P < 0.05). Although there were slightly different response profiles between the single and fractionated treatment regimens, the consistent inhibitory impact of combined treatment supports the previous in vitro findings and reveals a profound capacity of MEHD7945A to augment radiation response in HNSCC and NSCLC tumors. In addition, combination of 16 Gy radiation with MEHD7945A showed more potent regrowth delay than 22 Gy radiation alone in NCI-H226 xenografts (Supplementary Fig. S4), suggesting the potential use of MEHD7945A to modulate radiation side effects by adjusting radiation dose.

We then examined the histology of UM-SCC6 tumor specimens from the single-dose xenograft experiments for functional validation. We used immunohistochemical staining to examine the levels of several markers involved in cell proliferation, DNA damage repair, and apoptosis. As shown in Fig. 6, we found that treatment with MEHD7945A alone or in combination with radiation induced a marked reduction of proliferation marker PCNA. This was accompanied by a decreased level of p-MAPK and p-S6, which are critical in survival signaling pathways. Moreover, combined MEHD7945A and radiation caused a significant expression increase of the DNA damage marker γ-H2AX and apoptosis marker p-caspase-3, but not apoptotic TUNEL at this time point. Overall, these IHC results correlate well with the antitumor capacity of combined MEHD7945A and radiation shown in the xenograft results.

MEHD7945A affects tumor xenograft vasculature

Radiation response of tumors is influenced in part by the microenvironment. We therefore performed immunofluorescent staining analysis following injection of systemic labels to examine tumor vasculature and proliferation characteristics in whole tumor cross-sections from UM-SCC6 xenografts. An antiendothelial antibody (9F1) was used to delineate vascular structures and Ki67 was used as a proliferation marker. As shown in Supplementary Fig. S5, a decrease of 9F1 density and Ki67 staining was observed in tumors receiving combined treatment of MEHD7945A and radiation when compared with control and single treatments. These results imply that the impact of MEHD7945A in augmenting radiation killing may in part reflect inhibition of tumor repopulation and vascular function.

Discussion

The EGFR/HER network consists of an interconnected family of receptors that influence tumor cell growth and response to therapy (19, 20). Blockade of one HER receptor can often be functionally compensated by another HER family member. Therefore, targeting the EGFR alone may ultimately have modest impact, particularly for tumors with multiple oncogenic drivers within and beyond the HER system. Combinatorial strategies to target multiple HER family members have been increasingly explored in recent years (21–26). Many aggressive tumors express both EGFR and HER3, and HER3 has been identified as a key contributor to acquired resistance to EGFR inhibitors (27–29). We have previously demonstrated the capacity of MEHD7945A to overcome acquired resistance to EGFR inhibitors (30). In this report, we further identify MEHD7945A as a promising therapeutic approach combined with radiation in lung and head and neck cancer models.

MEHD7945A is shown to inhibit the growth of lung and head and neck cancer cells with different expression levels of EGFR and/or HER3 (Fig. 1). Among multiple cell lines tested, we found that MEHD7945A was most effective in inhibiting the proliferation of UM-SCC6 cells, which have the highest basal and phosphorylated levels of HER3. In addition, MEHD7945A induced the most significant inhibition of p-HER3 and p-AKT in UM-SCC6 cells. These results suggest the importance of the HER3–Akt pathway in regulating MEHD7945A-mediated antiproliferative effects. Moreover, the inhibitory effects of MEHD7945A on cell signal transduction and cell growth was more potent than those observed with anti-EGFR or anti-HER3 alone. We observed a slightly greater (not statistically different) response to single agent MEHD7945A versus the combination of anti-EGFR and anti-HER3 to inhibit cell clonogenicity (Supplementary Fig. S2). This may reflect the fact that in the case of MEHD7945A, both EGFR and HER3 are targeted by a single molecule, rather than requiring two distinct antibody molecules that may induce steric hindrance for ease of target access.

Exposure of cells to ionizing radiation leads to activation of the EGFR family, which subsequently stimulates downstream signaling cascades that regulate DNA damage repair, cell proliferation, and survival (31, 32). We found that MEHD7945A-induced inhibition of HER3-AKT signaling translated into the augmentation of radiation response. Although MEHD7945A enhanced radiosensitivity in both UM-SCC6 and NCI-H226 cells (Fig. 2A), statistical analysis revealed a significant impact of MEHD7945A and radiation in UM-SCC6, which exhibits strong...
Figure 4.
MEHD7945A can inhibit radiation-induced activation of multiple MAPK effector molecules, including ERK, JNK, p38, and AKT. Human phospho-MAPK array was conducted on UM-SCC6 cells treated with MEHD7945A (20 μg/mL, pretreated 24 hours before XRT), XRT (4 Gy), or a combination of both. Proteins were harvested 24 hours after radiation. Quantitation of phosphorylated proteins was completed using scanned images from ImageJ software. Data points, mean of duplicate spots.
MEHD7945A in combination with radiation can delay the growth of xenograft tumors. UM-SCC6, SCC1483, and NCI-H226 xenografts were performed as described in Materials and Methods. A, mice were treated with single doses of IgG, MEHD7945A (8 mg/kg, pretreated 24 hours before XRT), XRT (16 Gy), or MEHD7945A with radiation. B, fractionated treatments of IgG, MEHD7945A, radiation, or MEHD7945A in combination with radiation. Treatment and doses were described in Materials and Methods. A, mice were treated with single doses of IgG, MEHD7945A (8 mg/kg, pretreated 24 hours before XRT), XRT (16 Gy), or MEHD7945A with radiation. B, fractionated treatments of IgG, MEHD7945A, radiation, or MEHD7945A in combination with radiation. Treatment and doses were as shown in the figures. Data points, mean tumor size ± SEM; *P < 0.05 compared with radiation alone. The graph shows the delay in tumor growth after treatment with MEHD7945A, radiation, or MEHD7945A in combination with radiation.

Figure 5.

Interestingly, we found that MEHD7945A may augment radiation response through interference with tumor–stromal interactions, such as angiogenesis. Using the endothelial specific marker 9F1, we found a reduction of tumor vascular density in tumors following MEHD7945A and radiation treatment (Supplementary Fig. S5). A body of preclinical and clinical data has emerged in support of combining angiogenesis inhibitors with radiation. Studies have suggested that antiangiogenic agents may serve to transientlynormalize tumor vasculature to offer more efficient oxygen and drug delivery (33–35). In addition, by determining the optimal scheduling and dose of antiangiogenic agents, the combination of radiation with antiangiogenic agents may improve therapeutic outcome via vessel normalization (36). Therefore, a detailed investigation regarding the temporal changes of the tumor microenvironment induced by MEHD7945A may be valuable for our understanding of MEHD7945A and radiation interactions.

In conclusion, our data show that MEHD7945A is a promising HER family dual-target inhibitor that can augment radiation response in lung and HNSCC tumors. Consistent with previous studies of other EGFR targeting agents (37, 38), our mechanistic studies indicate that MEHD7945A augments radiation response via the induction of cell-cycle arrest followed by the induction of apoptosis and cell death likely reflecting inhibitory effects on DNA damage repair machinery. Moreover, HER3–AKT signaling emerges as an important determinant of MEHD7945A-regulated cell survival and radiosensitivity. Our data suggest that patients...
MEHD7945A in combination with radiation can inhibit proliferation and cell survival signaling in tumor xenografts. UM-SCC6 xenograft tumors with single-dose treatment of IgG, MEHD7945A (8 mg/kg), radiation (16 Gy), or MEHD7945A in combination with radiation. Tumors were harvested 24 hours after treatment. IHC labeling was performed as described in Materials and Methods. Cell proliferation was detected by PCNA staining. Cell survival signaling was detected by p-MAPK/p-s6 staining. DNA damage was evaluated by population of γ-H2AX-positive cells. Apoptosis was demonstrated by TUNEL-positive cells and p-caspase-3 staining. Images were quantified via taking the average staining intensity measured from 10 fields of two tumors per treatment group.

Figure 6.

MEHD7945A Augments Radiation Response

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with tumors harboring amplification or overexpression of both EGFR and HER3 may be expected to show favorable response to MEHD7945A. Several clinical trials of MEHD7945A are underway in HNSCC and other cancer sites. This work provides strong rationale to examine the impact of MEHD7945A in combination with radiotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Li, S. Huang, M.X. Sliwkowski, P.M. Harari
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