

Dual Targeting of EGFR and HER3 with MEHD7945A Overcomes Acquired Resistance to EGFR Inhibitors and Radiation

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Abstract

EGF receptor (EGFR) inhibition is efficacious in cancer therapy, but initially sensitive tumors often develop resistance. In this study, we investigated the potential to overcome acquired resistance to EGFR inhibitors with MEHD7945A, a monoclonal antibody that dually targets EGFR and HER3 (ErbB3). In cancer cells resistant to cetuximab and erlotinib, we found that MEHD7945A, but not single target EGFR inhibitors, could inhibit tumor growth and cell-cycle progression in parallel with EGFR/HER3 signaling pathway modulation. MEHD7945A was more effective than a combination of cetuximab and anti-HER3 antibody at inhibiting both EGFR/HER3 signaling and tumor growth. In human tumor xenograft models, we confirmed the greater antitumor potency of MEHD7945A than cetuximab or erlotinib. MEHD7945A retained potent activity in tumors refractory to EGFR inhibitor alone. Furthermore, MEHD7945A also limited cross-resistance to radiation in EGFR inhibitor-resistant cells by modulating cell-cycle progression and repair processes that control apoptotic cell death. Taken together, our findings confirm an important role of compensatory HER3 signaling in the development of acquired resistance to EGFR inhibitors and offer preclinical proof-of-concept that MEHD7945A can effectively overcome EGFR inhibitor resistance. *Cancer Res*; 73(2); 824–33. ©2012 AACR.

Introduction

Members of the ErbB/HER receptor family (EGFR, HER2, HER3, HER4) play an important role in tumorigenesis and have been studied intensively in cancer therapeutics. Blockade of the EGF receptor (EGFR) using either monoclonal antibody (mAb) or small-molecule tyrosine kinase inhibitor (TKI) offers a promising approach that has been well validated over the last decade (1, 2). Unfortunately, many patients who initially respond to EGFR inhibitor treatments eventually manifest tumor progression (3–5). Hence, efforts to better understand underlying mechanisms of acquired resistance to EGFR inhibitors, and potential strategies to overcome resistance, are highly needed.

To understand underlying mechanisms of acquired resistance to EGFR inhibitors, we previously established a series of resistant clones to 2 different classes of EGFR inhibitors, cetuximab (mAb) and erlotinib (TKI), from sensitive tumor

cell lines without EGFR and KRAS mutations following long-term EGFR inhibitor exposure (6, 7). Following systematic screening, we identified a significant increase of p-EGFR and p-HER3 in these resistant clones. Depletion of HER3 by siRNA restored sensitivity to the EGFR inhibitor cetuximab (8). Further analysis of these clones revealed an increase of EGFR–HER3 dimerization and subsequent EGFR-dependent activation of HER3. Consistent with this observation, several studies indicated that acquired resistance to EGFR inhibitors may derive, in part, from activation of HER3 to effectively bypass the effect of EGFR inhibition (9–11). As HER3 is an obligate heterodimerization partner, these findings provide a rationale for the evaluation of combinatorial EGFR/HER3 targeting approaches in tumors manifesting acquired resistance to EGFR inhibitors.

As an inactive tyrosine kinase, HER3 is not amenable to inhibition with ATP analogues. MEHD7945A is a recently identified dual target antibody against EGFR and HER3 that exhibits dual action by inhibiting ligand dependent EGFR- and HER3-mediated downstream signaling (12). MEHD7945A shows profound antitumor activity *in vitro* and *in vivo* across a variety of tumor cell types when compared with the respective monospecific antibodies. In addition, MEHD7945A is effective in facilitating antibody-dependent cell-mediated cytotoxicity but appears to induce less skin toxicity in comparison to cetuximab in nonclinical studies. In the current study, we sought to investigate the capacity of MEHD7945A to overcome acquired resistance to EGFR inhibitors in our established cetuximab- and erlotinib-resistant tumor cells derived from lung and head and

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neck cancers. In addition, as previous studies suggested cross-resistance to radiation in these resistant cells (7), we examined the effect of MEHD7945A in regulating radiation response in EGFR inhibitor-resistant cells.

Materials and Methods

Reagents and antibodies

MEHD7945A and anti-HER3 (DL3.6b) were provided by Genentech, Inc. Cetuximab (Erbix) was provided by ImClone Systems Inc., and erlotinib (Tarceva) was provided by OSI Pharmaceuticals. Antibodies against EGFR, p-EGFR (Y1173), HER3, and Histone 3 were obtained from Santa Cruz Biotechnology Inc., and anti-p-DNAPK and Ku80 were obtained from Thermal Scientific Lab Vision. Anti- α -tubulin was obtained from Calbiochem. All other antibodies were obtained from Cell Signaling Technology, and all other chemicals were purchased from Sigma.

Primary and EGFR inhibitor-resistant tumor cells

The primary human non-small cell lung carcinoma (NSCLC) H226 cells were provided by Drs John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX) and were maintained in RPMI with 10% FBS. The human head and neck squamous cell carcinoma (HNSCC) SCC6 (UM-SCC-6) cells were provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and were cultured routinely in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% FBS and 1 μ g/mL hydrocortisone. These cells were tested and authenticated by the provider. The acquired cetuximab- and erlotinib-resistant clones of H226 and SCC6 were developed following long-term exposure to cetuximab or erlotinib as described previously (6, 7). All cell culture media and supplements were obtained from Life Technologies, Inc.

Cell proliferation assay

Viable growing cells was determined by crystal violet staining as described previously (7).

Cell-cycle analysis

Tumor cells were harvested by trypsin followed by ethanol fixation. After centrifugation, cells were incubated with phosphate-citric acid buffer (0.2 mol/L Na_2HPO_4 , pH 7.8, 4 mmol/L citric acid) at room temperature for 45 minutes. Thereafter, cells were stained with a solution containing 33 μ g/mL propidium iodide (PI), 0.13 mg/mL RNase A, 10 mmol/L EDTA, and 0.5% Triton X-100 at 4°C for 4 hours. Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA content was analyzed by Modfit (Verity Software House Inc.) to determine the proportion of cells in sub- G_0 , G_0 - G_1 , S, and G_2 -M phases of the cell cycle.

EGFR inhibitor-resistant tumor xenografts

Athymic nude mice (3- to 4-week-old male) were obtained from Harlan Bioproducts for Science and maintained in a laminar airflow cabinet under aseptic conditions. The care and treatment of experimental animals was in accordance with Institutional Guidelines. Cetuximab- or erlotinib-resistant

tumor cells ($\sim 1 \times 10^6$) were injected subcutaneously into the dorsal flank area of the mice. Following the establishment of tumor, cetuximab or MEHD7945A was administered via intraperitoneal (i.p.) injection twice per week, and erlotinib was given by oral gavage 5 d/wk. Radiation treatment was delivered by a cabinet X-ray biological irradiator X-RAD 320 from Precision X-Ray, Inc. Mouse was immobilized using custom-designed jigs that only exposed the dorsal flank with tumor xenograft to irradiation without exposing non-tumor-bearing normal tissues. Tumor volume was determined by direct measurement with calipers and calculated by the formula: $\pi/6 \times (\text{large diameter}) \times (\text{small diameter})^2$.

Immunofluorescent staining of γ H2AX foci

Cells were plated on chamber slides and exposed to 10 μ g/mL of drugs for 1.5 hours before irradiation. Twenty-four hours following 3 Gy radiation, cells were fixed in 2% paraformaldehyde and permeabilized in 0.2% Triton X-100. The cells were then probed with anti- γ H2AX antibody (Upstate) followed by Alexa Fluor 594-conjugated secondary antibody (Invitrogen). Fluorescent γ H2AX foci were then captured using a Zeiss Axioplan fluorescent microscope. To quantitate γ H2AX foci, visual scoring of foci in 200 randomly chosen intact nuclei from irradiated samples was determined after subtracting the background numbers of foci from unirradiated samples.

Radiation survival

Survival following radiation exposure was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, after exposure to radiation, cells were trypsinized, counted, and seeded for colony formation in 35-mm dishes at 50 to 5,000 cells/dish. Following 10 to 14 days, colonies were stained with crystal violet and manually counted. Colonies consisting of 50 cells or more were scored, and 4 to 10 replicate dishes containing 10 to 150 colonies/dish were counted for each treatment.

Cellular fractionation and immunoblotting analyses

Cellular fractionation was conducted as described previously (13). Detailed information is provided in the Supplementary Materials and Methods.

Apoptosis assessment

Apoptosis was assessed by the loss of plasma membrane asymmetry as one of the earliest features of apoptosis using Annexin V/PI kit from BD Biosciences Pharmingen. Detailed information is provided in the Supplementary Materials and Methods.

Statistical analysis

Student *t* test was used to evaluate the significance of differences between 2 samples, and ANOVA was used to evaluate differences among 3 or more groups in tumor xenograft studies. Differences between samples were considered statistically significant when $P < 0.05$. To assess additive or synergistic effects, we used the fractional product method as described previously (14). Briefly, the observed fractional tumor volume (FTV) is equal to the mean tumor volume of

each treated group divided by the mean tumor volume of the control group at each time point. The synergy assessment was determined by calculating the ratio of FTV (drug) \times FTV (radiation)/FTV (drug + radiation). A ratio greater than 1.0 suggests that the combined treatment effects are synergistic.

Results

MEHD7945A inhibits growth of cetuximab-resistant tumor cells

We previously established acquired resistant clones to 2 distinct classes of EGFR inhibitor following long-term exposure to cetuximab or erlotinib in NSCLC and HNSCC tumor cells. We first compared the *in vitro* antiproliferative effect of MEHD7945A with cetuximab in cetuximab-resistant clones from H226 and SCC6. As shown in the left of Fig. 1A, both cetuximab and MEHD7945A exhibited similar capacity to inhibit the growth of parental H226 cells (small box figure). Notably, MEHD7945A significantly inhibited tumor growth of H226-CetR cells that remained refractory to cetuximab treatment. Similar results were observed in the cetuximab-resistant clone of SCC6 as shown in the right of Fig. 1A. While parental SCC6 cells responded well to both antibody treatments (small box), SCC6-CetR only responded to MEHD7945A. In analysis of cell-cycle progression, we found that MEHD7945A induced a significant G₀-G₁ arrest accompanied by a reduction in the percentage of cells in S-phase compared with control or

cetuximab-treated, cetuximab-resistant cells (Supplementary Fig. S1A). These results corresponded well to the antiproliferative effect of MEHD7945A shown in Fig. 1A. Further immunoblotting revealed a significant inhibition of the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathways by MEHD7945A but not cetuximab (Supplementary Fig. S1B). Maintenance of PI3K/AKT signaling was observed in our resistant cells and is known to be a critical factor for acquired resistance to EGFR inhibitors in several previous studies (15), this result suggests that MEHD7945A may overcome acquired resistance to cetuximab, in part, via inhibition of HER3/PI3K/AKT signaling.

We next compared the antiproliferative effect of MEHD7945A against the combination of 2 monospecific antibodies, EGFR (cetuximab) and HER3 (DL3.6b), which is the corresponding HER3 antibody in MEHD7945A (12). Using the same concentration (10 μ g/mL) of each antibody, we found MEHD7945A more potent to inhibit cellular proliferation than the combination of cetuximab and anti-HER3 antibody in both cetuximab-resistant clones (Fig. 1B). Consistently, we found MEHD7945A more effective than the combination of cetuximab and HER3 antibody to inhibit EGFR/MAPK and HER3/PI3K/AKT signaling as shown in Fig. 1C. These results suggest favorable clinical potential to investigate this dual target antibody approach rather than multidrug combination therapy with monospecific EGFR and HER3 antibodies.

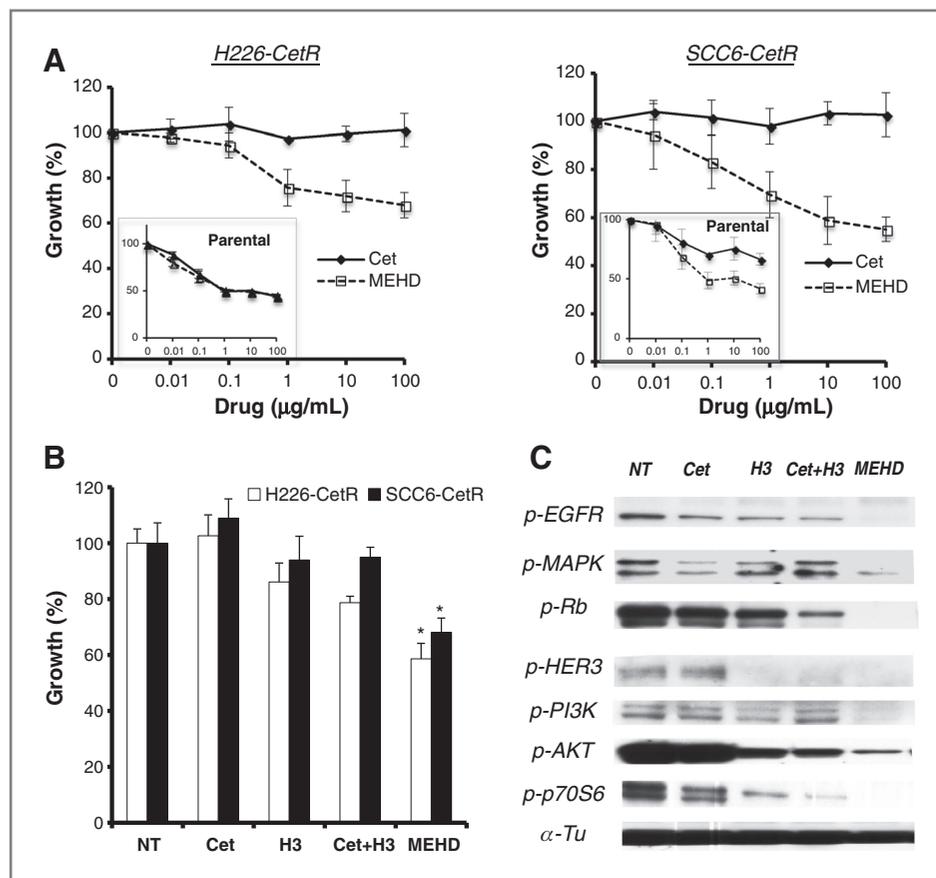


Figure 1. MEHD7945A inhibits growth of cetuximab-resistant cells. A, cetuximab-resistant H226 (H226-CetR) or SCC6 (SCC6-CetR) cells were exposed to serial concentrations of cetuximab (Cet) or MEHD7945A (MEHD) for 72 hours. Thereafter, growth of tumor cells was determined by cell proliferation analysis. Response of parental H226 to both drugs is shown in the box as a reference. B, a stronger antiproliferative efficacy of MEHD7945A than the combination of cetuximab and anti-HER3 (H3) antibody. Results are expressed as percentage of cell growth relative to nontreated controls (NT) *, $P < 0.05$. C, immunoblotting showed that MEHD7945A is more efficient than the combination of cetuximab and anti-HER3 antibody to inhibit EGFR and HER3 signaling. The α -Tubulin (α -Tu) serves as a loading control.

To further explore whether MEHD7945A could inhibit tumor cell growth in another EGFR inhibitor-resistant setting, we examined the effect of MEHD7945A on our erlotinib-resistant cells. As expected, erlotinib inhibited the growth of parental cells in a dose-dependent manner but had little effect on erlotinib-resistant H226 cells as shown in the left of Fig. 2A. This confirmed the resistant phenotype of erlotinib-resistant cells. Interestingly, MEHD7945A inhibited the growth of both parental and erlotinib-resistant cells as shown in the right of Fig. 2A. Similar results were observed in the parental and erlotinib-resistant SCC6 cells (Fig. 2B). While both parental and erlotinib-resistant SCC6 cells responded differently to erlotinib, they both responded well to MEHD7945A with a similar pattern. As anticipated, parental cells responded better to MEHD7945A than erlotinib resistant cells likely reflecting the high expression of EGFR and HER3 in the resistant cells as shown in our previous study (8). Taken together, these results consistently show that MEHD7945A overcomes acquired resistance to both classes of EGFR inhibitors.

MEHD7945A overcomes EGFR inhibitor resistance in human tumor xenografts

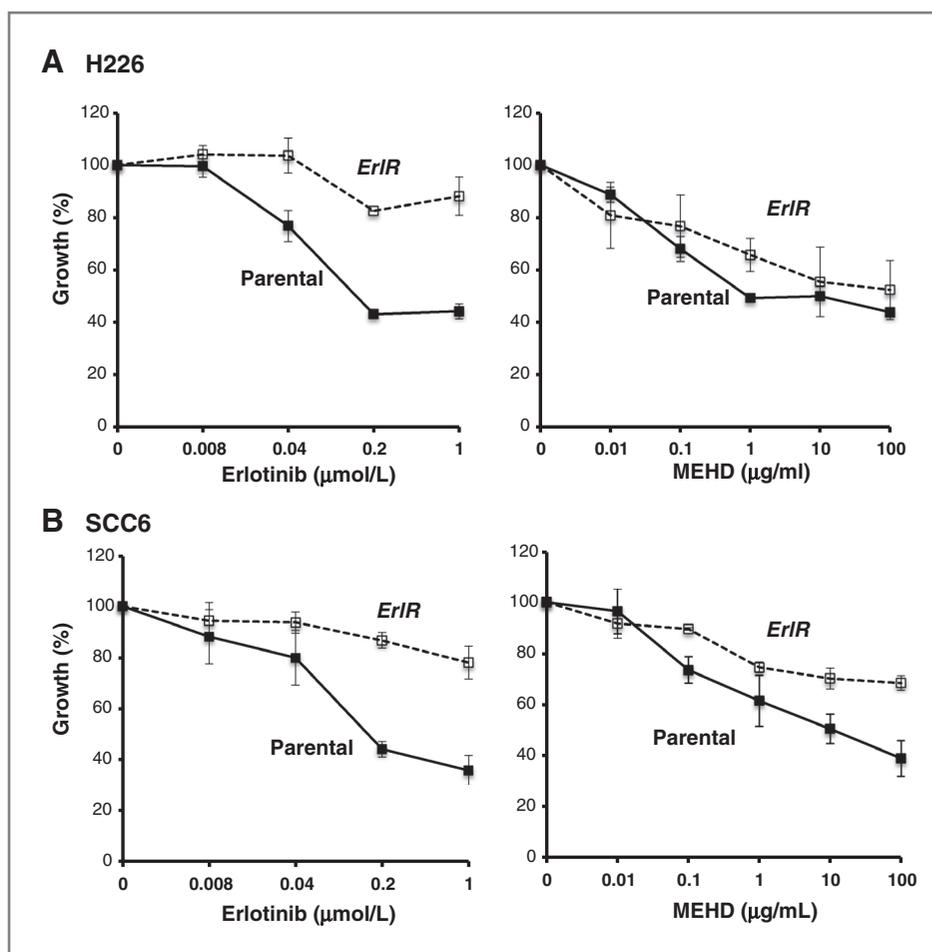
To extend these *in vitro* findings, we inoculated cetuximab-resistant or erlotinib-resistant SCC6 tumor cells into athymic

mice. Following establishment of tumors (150–200 mm³), mice were treated with the same dose of cetuximab or MEHD7945A. Treatment with MEHD7945A, but not cetuximab, induced significant growth delay of cetuximab-resistant tumors when compared with untreated controls as shown in the left of Fig. 3. More importantly, MEHD7945A was found to induce regression of tumors that were highly refractory to cetuximab as shown at days 51 to 58. In contrast, cetuximab did not inhibit the growth of tumors that were previously treated with MEHD7945A. Similarly, erlotinib-resistant tumors responded with growth delay to initial challenge with MEHD7945A, but not erlotinib treatment. MEHD7945A also induce very brisk regression in those tumors that remained highly refractory to erlotinib treatment. These results confirm and extend the previous *in vitro* findings and indicate a profound capacity of MEHD7945A to overcome acquired resistance to both cetuximab and erlotinib *in vivo*.

MEHD7945A overcomes cross-resistance to radiation

Our previous studies found a cross-resistance to radiation in EGFR inhibitor-resistant cells following long-term exposure to EGFR inhibitors (7). We therefore examined whether MEHD7945A could overcome this cross-resistance to radiation in EGFR inhibitor-resistant cells. We compared the radiation

Figure 2. MEHD7945A inhibits growth of erlotinib-resistant cells. Parental or erlotinib-resistant H226 (A) or SCC6 (B) cells were exposed to serial concentrations of erlotinib (Erl) or MEHD7945A (MEHD) for 72 hours. Thereafter, growth of tumor cells was determined by cell proliferation analysis.



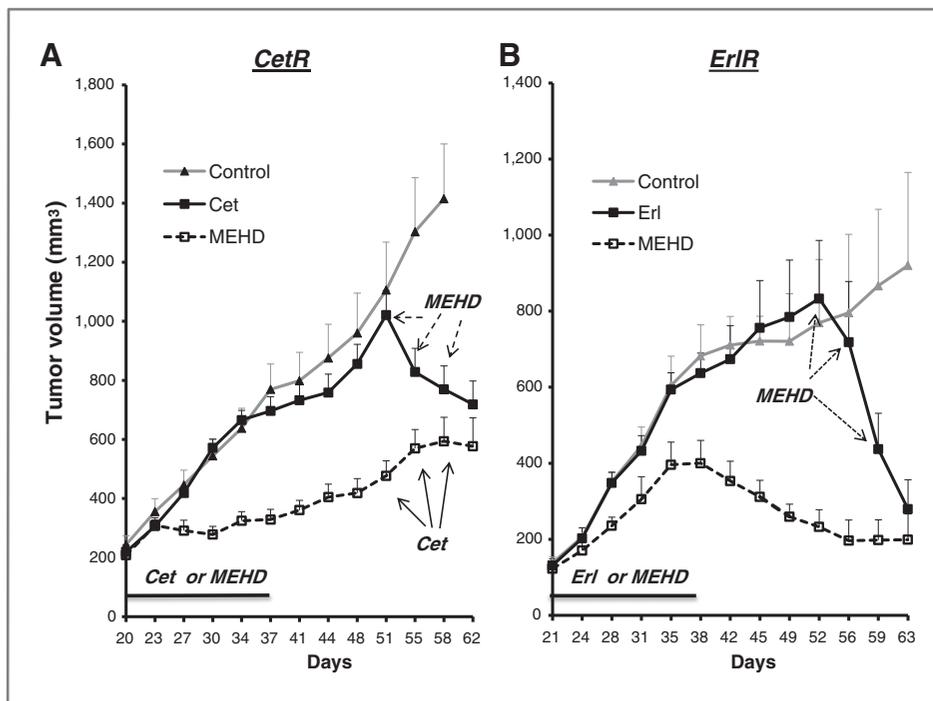


Figure 3. MEHD7945A overcomes resistance to cetuximab or erlotinib in human tumor xenografts. Cetuximab- or erlotinib-resistant SCC6 cells were inoculated into the dorsal flank of athymic mice. (A) Mice with CetR xenografts were initially treated with cetuximab or MEHD7945A at 2.2 mg/kg/dose twice weekly from days 20 to 37. Thereafter, 4.4 mg/kg/dose of MEHD7945A was applied to mice that previously received cetuximab treatment, and cetuximab (4.4 mg/kg/dose) was applied to MEHD7945A-treated mice at day 51 for 3 consecutive doses. B, Mice with ErlR xenografts were initially treated with 3 mg/kg/dose of MEHD7945A or 60 mg/kg/wk of erlotinib from days 21 to 38. At day 52, erlotinib-treated mice were challenged with 6 mg/kg/dose of MEHD7945A for 3 consecutive doses. Tumor volume was monitored and values represent mean tumor size (mm^3) \pm SEM ($n = 8$ per group).

response in cetuximab-resistant clones challenged with MEHD7945A or cetuximab. Using clonogenic survival analysis, we found that pretreatment of cetuximab did not change the response profile when compared with radiation-alone control (NT). In contrast, treatment with MEHD7945A significantly reduced cell survival following radiation exposure in cetuximab-resistant H226 and SCC6 clones (Fig. 4A). In addition, we characterized the DNA damage profile following 3 Gy radiation by examining the activation of the histone-variant H2AX that becomes phosphorylated (γ H2AX) following a reaction on radiation-induced DNA double-strand breaks (DSB). As shown in Fig. 4B, a significant increase of γ H2AX foci was observed in MEHD7945A-pretreated, but not cetuximab-pretreated, cells by a factor of 1.2 to 2.2 when compared with untreated control. Interestingly, we found that MEHD7945A augmented radiation-induced DNA damage more significantly in SCC6-CetR than that of H226-CetR cells. This result is consistent with a more profound cell killing in MEHD7945A-treated SCC6-CetR cells when compared with H226-CetR cells determined by clonogenic survival analysis (Fig. 4A). To further extend these *in vitro* findings, we compared the capacity of cetuximab and MEHD7945A to augment radiation response in our cetuximab-resistant tumor xenograft model system. As shown in Fig. 4C, SCC6-CetR tumors respond modestly to treatment with cetuximab, MEHD7945A, or radiation when compared with control tumors. As expected, the combination of cetuximab and radiation (XRT + C) did not produce a significant treatment benefit in the cetuximab-resistant tumors when compared with the corresponding single treatment with cetuximab ($P > 0.05$) or radiation ($P > 0.05$) up to day 66. However, tumor response to MEHD7945A was considerably stronger than that observed with cetuximab, and the combination of MEHD7945A and radiation (XRT + M) showed a significant

tumor growth inhibition resulting in substantial growth delay when compared with the corresponding single modality treatment with radiation ($P < 0.01$) or MEHD7945A ($P < 0.05$) from day 44 or 51, respectively, to the end of the experiment on day 66. Additional statistical analysis using the fractional product method (14) confirmed that the combined MEHD7945A and radiation treatment exhibited a strong synergy assessment ratio between 1.2 and 1.47 from day 37 to the end of the experiment on day 66. Although a modest enhancement of radiosensitivity was observed in MEHD7945A-treated cells from the *in vitro* clonogenic survival analysis (Fig. 4A), we observed a profound impact of MEHD7945A to augment radiation response in the cetuximab-resistant tumor xenografts. This result extends the *in vitro* findings and confirms that MEHD7945A can augment radiation response in cells that remain refractory to cetuximab.

MEHD7945A inhibits radiation-induced survival and damage repair pathways

To further investigate underlying mechanisms for the effect of MEHD7945A on radiation response, immunoblotting was conducted to examine the expression and activity of proteins involved in regulating survival and DNA damage repair. As shown in Fig. 5A, we observed the activation of HER family members and their downstream MAPK and AKT signaling 24 hours after exposure to 6 Gy radiation in cetuximab-resistant H226 cells. This radiation-induced survival signaling correlated well with an increase of p-Rb that serves as a key factor to stimulate G_1 to S-phase transition. However, treatment with MEHD7945A was superior to cetuximab to inhibit radiation-induced survival signaling and the level of p-Rb. We also observed a significant increase of phosphorylated p53 that is critical in regulating cell-cycle arrest and apoptosis in

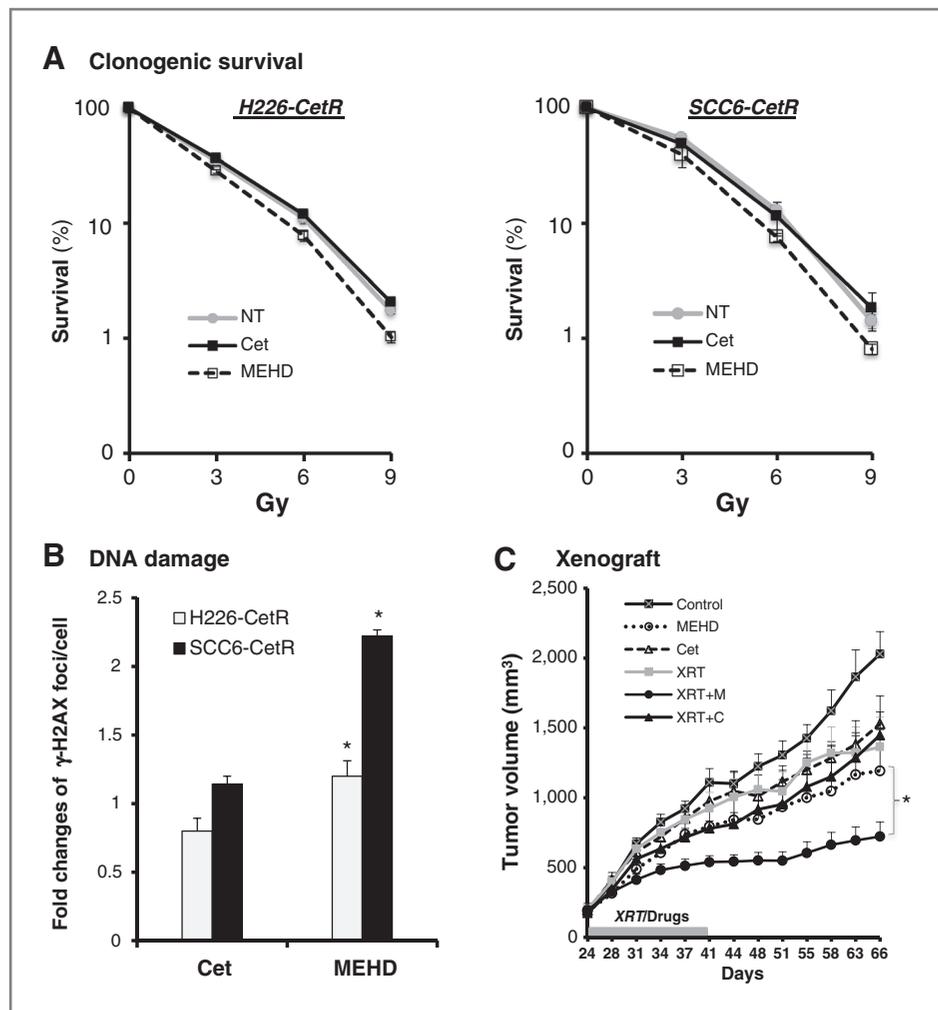


Figure 4. MEHD7945A is more potent than cetuximab to augment radiation response in EGFR inhibitor-resistant cells. **A**, radiosensitivity of drug-free control (NT), 10 μ g/mL of cetuximab (Cet) or MEHD7945A (MEHD) pretreated (72 hours) CetR cells were examined by clonogenic survival analysis as described in Materials and Methods. Results were expressed as the percentage of colony formation relative to controls without radiation treatment. Data points are represented as mean \pm SD. **B**, radiation-induced DNA damage was determined by examining γ H2AX foci in the nucleus by immunofluorescent staining as described in Materials and Methods. Results were expressed as the fold change of foci relative to controls without drug treatment. Data points are represented as mean \pm SD. *, $P < 0.05$. **C**, radiation response was determined in a human xenograft model. Mice with SCC6-CetR tumor xenografts were treated with either single or combined drug and radiation (XRT) treatment starting at day 21. Cetuximab or MEHD7945A were delivered at a dose of 1 mg/kg and radiation was delivered at 2 Gy twice per week for 3 consecutive weeks. The grey box along the x-axis indicates the treatment interval from days 24 to 41. Tumor volume was monitored twice weekly and values represent mean tumor size (mm³) \pm SEM ($n = 8$ per group). *, $P < 0.05$ when compared with single modality treatment groups.

MEHD7945A-treated cells. Consistently, a significant increase of cleaved PARP and caspase-3 that resulted from the activation of apoptosis was found in MEHD7945A-treated cells 48 hours after radiation.

As DNA damage response is initiated with the recognition of damage and often results in cell-cycle arrest for repair, we next examined whether MEHD7945A could inhibit repair capacity by examining several key proteins involved in the repair of lethal DSB. DNA protein kinase (DNAPK), Ku80, and XRCC-4 like factor (XLF) are involved in nonhomologous end joining, and BRCA1 is essential for initiating homologous recombination repair. Mre11, Rad50, and Nbs1 form a complex in DSB sites and act as crucial elements for DSB repair and cell-cycle checkpoints (16). Inhibition of these molecules is known to

sensitize tumor cells to radiation. Following nuclear fractionation, we found that the level of most repair proteins in the nucleus is increased following radiation exposure in the control nontreated cells (Fig. 5B), such as XCF, BRCA1, and pNBS1. Treatment with MEHD7945A was more effective than cetuximab to reduce the level of all the tested repair proteins in cells exposed to radiation. Interestingly, we found that a significant decrease of nuclear DNAPK in the MEHD7945A-treated cells was accompanied by an increase of DNAPK in the non-nuclear fraction. This result is consistent with previous findings suggesting that EGFR agents inhibit repair capacity by disrupting nuclear import of functional DNAPK (17). The lack of nuclear DNAPK import inhibition in the cetuximab-treated cells is consistent with the cross-resistance to radiation observed in

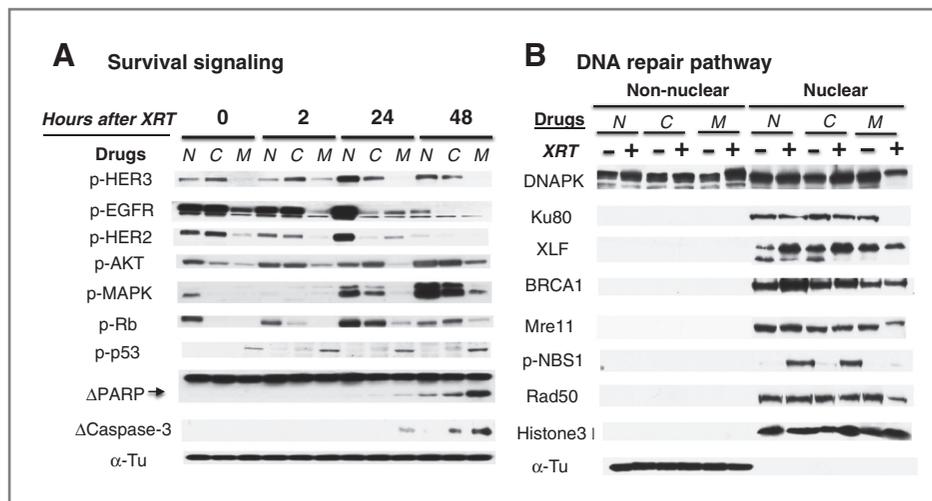


Figure 5. MEHD7945A inhibits radiation-induced survival and damage repair signaling. **A**, H226-CetR cells were either nontreated (N) or pretreated with 10 μ g/mL of cetuximab (C) or MEHD7945A (M) for 24 hours followed by 6 Gy radiation. Thereafter, cells were harvested at 0, 2, 24, or 48 hours after radiation and lysed for Western blot analysis. Δ represents cleaved fragment of PARP or caspase-3. **B**, the effect of cetuximab and MEHD7945A on the expression of nuclear DNA damage proteins 48 hours following radiation. Histone 3 and α -Tu serve as loading and purity controls of nuclear and non-nuclear fractions, respectively. Figure is a representative of 2 to 3 independent experiments with similar result.

our cetuximab-resistant cells. Similar results were observed in cetuximab-resistant SCC6 cells (data not shown). Taken together, these results suggest 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.

MEHD7945A regulates cell-cycle progression and apoptosis following radiation

To further investigate the effect of MEHD7945A on radiation-induced cell-cycle progression, we examined the cell-cycle phase distribution of cetuximab-resistant cells 1 or 2 days following exposure to 6 Gy radiation treatment. As shown in Fig. 6A, the S-phase cell-cycle populations were enhanced 1 day after radiation in the untreated control of both EGFR inhibitor-resistant clones. This result correlates well with previous observations of an increase of proliferative and survival signaling 24 hours after radiation (Fig. 5A). However, treatment with MEHD7945A, but not cetuximab, induced a robust increase of cell arrest in G_0 - G_1 phase. Interestingly, we found that MEHD7945A-treated cells resumed a similar cell-cycle phase distribution to control cells 2 days after radiation. Further analysis of cells with sub- G_0 DNA content comprising apoptotic cells and debris fractions identifies a robust increase of cells in MEHD7945A-treated but not cetuximab-treated cells 2 days after radiation (Supplementary Fig. S2).

To further validate this observation of apoptosis, we applied another approach using Annexin V/PI flow cytometry analysis. Annexin V serves as a marker for the loss of plasma membrane asymmetry representing an early feature of apoptosis. As shown in Fig. 6B, there is a significant increase of early apoptotic cells in the MEHD7945A-treated H226-CetR cells 2 days (D2) after radiation when compared with control and cetuximab-treated cells. There is only a modest increase of apoptotic cells in MEHD7945A-treated group 1 day (D1) after

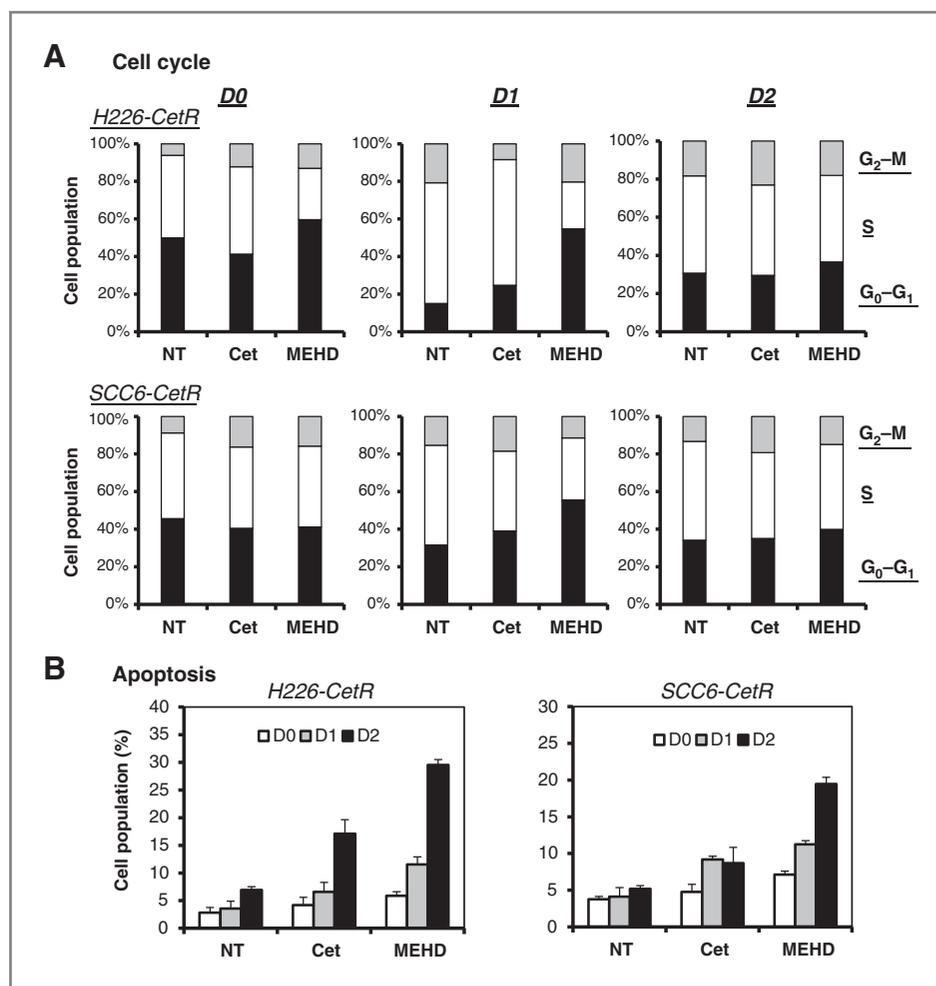
radiation. Similar results were observed in the cetuximab-resistant SCC6 cells. These results again indicate that MEHD7945A is inducing cell-cycle arrest early following exposure to radiation. With the capacity to inhibit DNA repair pathways, MEHD7945A appears to augment radiation effect via apoptosis induction in these unrepaired cells at a latter stage.

Discussion

In the current study, we provide evidence that MEHD7945A can overcome acquired resistance in 2 distinct EGFR inhibitor-resistant model systems. Using established cetuximab- or erlotinib-resistant cells from NSCLCs and HNSCCs, we find that MEHD7945A, but not EGFR inhibitors alone, effectively inhibits MAPK and PI3K/AKT survival pathways that play a key role in regulating acquired resistance to EGFR inhibitors (Fig. 1). Furthermore, MEHD7945A exhibits more potent antitumor capacity than cetuximab or erlotinib in human tumor xenograft systems and effectively shrinks tumors that remain highly refractory to cetuximab or erlotinib (Fig. 3). In addition, MEHD7945A overcomes cross-resistance to radiation in these EGFR inhibitor-resistant cells. This latter finding is noteworthy, as most EGFR inhibitor combinations with cytotoxic chemotherapy have shown limited clinical benefit (5, 18). Although the combination of cetuximab and radiotherapy has shown improved 5-year survival in patients with head and neck cancer, a substantial proportion of patients eventually manifest tumor recurrence (19). As both EGFR and HER3 are activated after radiation (Fig. 5A), MEHD7945A targeting of both receptors offers a promising approach to overcome acquired resistance in clinical therapy strategies that use EGFR/radiation combinations.

Although specific mechanisms resulting in acquired clinical resistance to EGFR inhibitors and radiation are not fully understood, increasing evidence indicates that crosstalk

Figure 6. MEHD7945A induces cell-cycle arrest and apoptosis following radiation. A, H226-CetR or SCC6 cells were either nontreated (NT) or pretreated with 10 μ g/mL of cetuximab (Cet) or MEHD7945A (MEHD) for 24 hours followed by 6 Gy radiation. Cells were then harvested at day 0 (D0), day 1 (D1), or day 2 (D2) following radiation and processed for cell cycle or Annexin V/PI apoptosis analysis by flow cytometry as described in Materials and Methods. A, cell populations in G₀-G₁, S, and G₂-M phase following radiation. B, the percentage of early apoptotic cells (Annexin V-positive; PI-negative). Columns, mean values of duplicate samples.



among HER family members represents a major factor affecting clinical efficacy of HER-targeted therapy (20–22). Blockade of one HER receptor can be functionally compensated by another HER family member. Early studies in breast cancer cells showed that trastuzumab inhibited signaling from HER2 but did not disrupt activation of dimerization between HER2 and other HER family members (11). Considerable evidence points to HER3 and/or EGFR as key contributors to acquired resistance against HER2 targeting agents (23). Similarly, we and other investigators have shown that HER3 is involved in regulating acquired resistance to EGFR inhibitors (8, 9, 24). In addition, Engleman and colleagues identified that c-Met, another receptor tyrosine kinase, induced acquired resistance to gefitinib via coupling to HER3 and activation of HER3/PI3K/AKT signaling. (10). Interestingly, we found that the impact of MEHD7945A was more potent than dual agent blockade of EGFR by the combination of cetuximab and erlotinib (Supplementary Fig. S3), which shuts down EGFR and has shown to be superior to either agent alone in our previous study (6). These findings reveal an important role of HER3 as a signaling hub for the HER family that results in compensatory pathways for EGFR inhibitors. These findings also highlight the potential

value to inhibit functions of multiple HER family members to achieve the broadest clinical efficacy to overcome acquired resistance to EGFR inhibitor therapy. The capacity of MEHD7945A to overcome the primary (intrinsic) resistance to EGFR inhibitor therapy still needs to be examined, as the role of HER3 in regulating intrinsic resistance to EGFR inhibitors is not yet well characterized. A separate study is near completion to investigate the effect of MEHD7945A on the growth and radiation response of primary lung and head and neck tumor cell lines with variable sensitivities to cetuximab (25).

This 2-in-1 MEHD7945A targets both EGFR and HER3, thereby offering a combinatorial targeted therapy (12). Interestingly, we found that MEHD7945A was more effective than the combination of individual anti-EGFR and anti-HER3 antibody to inhibit tumor cell growth and MAPK and PI3K/AKT signaling pathways in cetuximab-resistant cells (Fig. 1B and C). Beyond a difference in binding affinity between MEHD7945A and the monospecific antibodies, the ability of MEHD7945A to simultaneously target EGFR/HER3 in close proximity or cluster could be another crucial factor to explain the superior effect of MEHD7945A over the combination of cetuximab and anti-HER3 antibody. Increasing evidence has shown the existence of

EGFR dimers or clusters in cells even in the absence of ligand stimulation (26, 27). This alternate dimer is an important intermediate form in the transition of the inactive receptor to the active, untethered dimer (28). As heterodimerization of EGFR was known to produce the most profound downstream signaling, MEHD7945A could then be more powerful than combination of individual targeting agents to shut down the proliferation signaling via its ability to bind to EGFR/HER3 clusters. Furthermore, it was recently established that combination of noncompetitive anti-EGFR antibodies synergistically reduce surface receptor level and lead to enhanced tumor cell killing and prolonged survival in a variety of mouse models (29, 30). Friedman and colleagues proposed that synergism results from the formation of large clusters of receptors on the cell surface following combination antibody treatment (29). With the potential of MEHD7945A to increase the formation of EGFR/HER3 clusters, it will be of interest to further investigate whether MEHD7945A can enhance EGFR and HER3 internalization and degradation in tumor cells.

HER2 also appears to be involved in regulating acquired resistance to EGFR inhibitors. Following long-term exposure to cetuximab *in vitro*, Yonesaka and colleagues found amplification of HER2 gene and/or increased neuregulin concentration in cetuximab-resistant clones of lung and colorectal cancers (31). Further analysis suggested that aberrant HER2 signaling, either through HER2 gene amplification or through autocrine neuregulin activation of HER3, led to persistent MAPK signaling and consequently to cetuximab resistance. Quesnelle and Grandis also identified activated HER2, but not HER2 gene amplification as the underlying mechanism for acquired resistance to cetuximab in bladder tumor cells established from an *in vivo* model system (32). Interestingly, we found that MEHD7945A could also inhibit phosphorylation of HER2 in our resistant cells (Fig. 5A). It would be of interest to explore the capacity of MEHD7945A to overcome EGFR inhibitor resistance in tumors with activated HER2. In addition, it may also be valuable to explore whether MEHD7945A is applicable to HER2-overexpressing breast cancers refractory to trastuzumab, as EGFR and HER3 have been implicated as key factors to regulate trastuzumab resistance.

Acquired resistance presents a considerable challenge to the optimal clinical advancement of EGFR molecular targeting agents. In addition, resistance to EGFR agents may co-associate with resistance to other cancer drugs and radiation. Data from the current study suggest that MEHD7945A is able to overcome cross-resistance to radiation via inhibition of radiation-induced survival signaling and DNA damage repair that results in the induction of apoptosis (Figs. 4–6). Treatment with MEHD7945A, but not cetuximab, inhibited radiation-induced survival signaling and resulted in cell-cycle arrest within 24 hours after radiation. Following inhibition of DNA repair machinery following exposure to MEHD7945A, most unrepaired cells entered apoptosis by 48 hours after radiation. Furthermore, we identified a significant increase of p-p53 in cells treated with MEHD7945A (Fig. 4B). These data are notable, as recent work reveals p53 as a critical factor in regulating acquired resistance to EGFR inhibitors and radia-

tion (15). Knocking down wild-type p53 in sensitive H226 cells, we observe a reduction in the sensitivity to cetuximab and radiation. In contrast, with reconstitution of functional p53, cetuximab-resistant cells show sensitivity to both treatments (15). Hence, it is possible that MEHD7945A overcomes acquired resistance to EGFR inhibitors and radiation via p53-regulated pathways. Beyond radiotherapy, p53 and AKT pathways have been shown to regulate acquired resistance to several chemotherapeutic agents (33, 34). It will be of interest to examine the potential of MEHD7945A to overcome resistance to the combined administration of EGFR inhibitors and chemotherapy. Additional experiments are underway to explore this question.

In conclusion, MEHD7945A, a dual targeting antibody against both EGFR and HER3, shows the capacity to overcome acquired resistance to EGFR inhibitors and radiation. Results from the current work suggest that MEHD7945A offers a promising therapeutic approach for combinatorial molecular target therapy. The single-agent simplicity of MEHD7945A also provides an opportunity to combine with other agents that have been shown to regulate resistance to EGFR therapy. Our improved understanding of HER family signaling biology suggests that agents such as MEHD7945A may prove highly valuable to advance the overall impact of EGFR therapy in cancer and help address the challenge of acquired resistance. Clinical trials are in development with MEHD7945A that will further investigate several of these important questions.

Disclosure of Potential Conflicts of Interest

P.M. Harari holds a laboratory research agreement with Genentech. L.C. Amler and M.X. Sliwkowski are employed by Genentech, Inc, a member of the Roche Group, as Director of Oncology Biomarker Development and Sr. Staff Scientist, respectively, and are shareholders in Roche. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Huang, E.A. Armstrong, C.R. Peet, J. Saker, P.M. Harari
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Huang, C.R. Peet, J. Saker, P.M. Harari
Writing, review, and/or revision of the manuscript: S. Huang, L.C. Amler, M. X. Sliwkowski, P.M. Harari
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Huang, C. Li, P.M. Harari
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Dual Targeting of EGFR and HER3 with MEHD7945A Overcomes Acquired Resistance to EGFR Inhibitors and Radiation

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