Sym004, a Novel Anti-EGFR Antibody Mixture, Augments Radiation Response in Human Lung and Head and Neck Cancers

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Abstract
Sym004 represents a novel EGF receptor (EGFR)-targeting approach comprising a mixture of two anti-EGFR antibodies directed against distinct epitopes of EGFR. In contrast with single anti-EGFR antibodies, Sym004 induces rapid and highly efficient degradation of EGFR. In the current study, we examine the capacity of Sym004 to augment radiation response in lung cancer and head and neck cancer model systems. We first examined the antiproliferative effect of Sym004 and confirmed 40% to 60% growth inhibition by Sym004. Using clonogenic survival analysis, we identified that Sym004 potently increased cell kill by up to 10-fold following radiation exposure. A significant increase of γH2AX foci resulting from DNA double-strand breaks was observed in Sym004-treated cells following exposure to radiation. Mechanistic studies further showed that Sym004 enhanced radiation response via induction of cell-cycle arrest followed by induction of apoptosis and cell death, reflecting inhibitory effects on DNA damage repair. The expression of several critical molecules involved in radiation-induced DNA damage repair was significantly inhibited by Sym004, including DNAPK, NBS1, RAD50, and BRCA1. Using single and fractionated radiation in human tumor xenograft models, we confirmed that the combination of Sym004 and radiation resulted in significant tumor regrowth delay and superior antitumor effects compared with treatment with Sym004 or radiation alone. Taken together, these data reveal the strong capacity of Sym004 to augment radiation response in lung and head and neck cancers. The unique action mechanism of Sym004 warrants further investigation as a promising EGFR targeting agent combined with radiotherapy in cancer therapy.

Introduction
The EGF receptor (EGFR) plays an important role in human tumorigenesis. Targeting EGFR with molecular inhibitors has been intensely pursued as a cancer treatment strategy following landmark studies by Mendelsohn and colleagues who showed the antitumor effect of a monoclonal antibody (mAb) to EGFR (1). To date, four EGFR targeting agents (cetuximab, panitumumab, gefitinib, and erlotinib) from two distinct drug classes have gained U.S. Food and Drug Administration (FDA) approval (2). These include monoclonal antibodies (mAbs) directed against the extracellular ligand-binding domain of EGFR and small molecules tyrosine kinase inhibitors (TKI) directed against the cytosolic catalytic domain of the EGFR. Among these, cetuximab (Erbitux) and panitumumab (Vectibix) are approved mAbs for the treatment of metastatic colorectal cancer and cetuximab for the treatment of head and neck cancer.

A series of preclinical studies provide proof-of-principle that cetuximab and panitumumab can enhance radiation response in a variety of tumor model systems (3–5). Although selected patients receive clear benefit from anti-EGFR mAbs, overall single-agent response rates are on the order of 10% (6). Favorable response rates and improved survival is achieved when cetuximab is combined with radiotherapy as in the landmark phase III clinical trial (7, 8), although a substantial proportion of patients do not respond to anti-EGFR therapy reflecting primary or acquired resistance. Moreover, many patients who initially respond well to treatment still manifest subsequent tumor recurrence (9, 10). Hence, the advancement of next-generation EGFR inhibitors and further refinement of complementary therapeutic approaches are valuable to further enhance anti-EGFR treatment efficacy.

When two mAbs against distinct receptor epitopes are combined, a rapid and more efficient receptor internalization is observed and followed by EGFR degradation (11). The mixture antibody treatment is also more effective.
than single antibodies in inhibiting signaling and tumor growth in tissue culture and animal models (12, 13). Furthermore, antibody mixtures have been shown to activate complement-dependent cytotoxicity that may contribute to a more effective antitumor capacity than single mAbs (14). Sym004 was developed following screening of more than 400 different mAb combinations based on the highest capacity to inhibit cell growth (15). Sym004 is a mixture of anti-EGFR mAb 992 and 1024 that targets nonoverlapping epitopes (epitope 992 vs. 1024) in EGFR extracellular domain III. Interestingly, mAb 992 and 1024 work highly synergistically when combined. These two epitopes are different from the epitopes of cetuximab and panitumumab. Similar to cetuximab, Sym004 can inhibit tumor growth by blocking ligand-induced receptor activation and signaling. However, Sym004 induces a highly efficient degradation of EGFR that is not observed with cetuximab (16). It is postulated that the rapid and efficient EGFR internalization results from clustering of receptor–antibody complexes at the cell membrane. Preliminary studies show that Sym004 exhibits superior antitumor capacity in comparison with cetuximab or panitumumab in both in vitro and in vivo models (16). Furthermore, Sym004 inhibits growth of cancer cells with acquired resistance to cetuximab resulting from increased EGFR ligand production. These findings highlight Sym004 as a promising strategy to maximize EGFR inhibition that may induce more potent tumor suppression than current clinically used EGFR targeting mAbs.

Materials and Methods

Reagents and antibodies

Sym004 was provided by Symphogen A/S. Antibodies against EGFR, p-EGFR (Y1173), BAD, Importinβ1, and histone 3 were obtained from Santa Cruz Biotechnology Inc. and anti-DNAPK was obtained from Thermal Scientific Lab Vision. Anti-α-tubulin was obtained from Calbiochem, and anti-Sec61β was obtained from Upstate. All other antibodies were obtained from Cell Signaling Technology, and all other chemicals were purchased from Sigma.

Cell lines

The primary human non–small lung carcinoma (NSCLC) H226 cell line was provided by Drs John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX) 10 years ago and H292 cell line was obtained from the American Type Culture Collection in 2005. The human head and neck squamous cell carcinoma SCC1 (UM-SCC1) cell line was provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and SCC1483 cell line was provided by Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA) in 2004. NSCLC SCC1483 cell line was provided by Dr. Jennifer Grandis (University of Michigan, Ann Arbor, MI) and head and neck squamous cell carcinomas (HNSCC) were cultured in Dulbeccos’ Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1 µg/mL hydrocorti-

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sone. 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 culture media and supplements were obtained from Life Technologies, Inc.

Cell proliferation assay

Viable growing cells were determined by crystal violet staining as described previously (17).

Quantification of EGFR mRNA expression

EGFR mRNA level was quantified with real-time PCR (RT-qPCR) using a Bio-Rad iQ5 RT-qPCR Detection System and SsoFast EvaGreen Supermix reagent as recommended by manufacturer (Bio-Rad Laboratories). Detailed information is provided in the Supplementary Materials and Methods.

Cellular fractionation and immunoblotting analyses

Following harvesting, cells were lysed in a NP-40 lysis buffer [20 mmol/L HEPES, pH 7.0, 10 mmol/L KCl, 2 mmol/L MgCl₂, 0.5% NP-40, 1 mmol/L NaF, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL aprotinin]. Thereafter, the cells were homogenized by a tightly fitting Dounce homogenizer followed by centrifugation at 1,500 × g for 5 minutes to sediment the nuclei. The supernatant was then centrifuged at 16,100 × g for 20 minutes, and the resulting supranatant formed the non-nuclear fraction. To extract nuclear proteins, the isolated nuclei were resuspended in NETN buffer (20 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP-40, 1 mmol/L NaF, 10 mmol/L NaF, 1 mmol/L PMSF, and 2 mg/mL aprotinin) followed by sonication. Nuclear lysates were then collected after centrifugation at 16,100 × g for 20 minutes. To obtain whole-cell lysates for western blot analysis, cells were lysed with Tween-20 lysis buffer and sonicated. Following quantification by Bradford analysis, equal protein amounts were loaded and analyzed by SDS-PAGE as described previously (17).

Radiation survival

Survival following radiation exposure was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies as described previously (17).

Immunofluorescent staining of γH2AX foci

Cells were plated on chamber slides and exposed to 10 μg/mL of drugs for 1.5 hours before irradiation. Twenty-four hours following 2 Gy radiation, cells were fixed in 2% paraformaldehyde and permeabilized in 0.2% Triton X-100. The cells were then probed with anti-γH2AX antibody (Upstate) followed by Alexa Fluor 488–conjugated secondary antibody (Invitrogen). Fluorescent γH2AX foci were then captured using a Zeiss Axiosplan fluorescent microscope. To quantitate γH2AX 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.

**Cell-cycle analysis**

Cell-cycle phase of tumor cell was determined by flow cytometry using propidium iodide (PI) staining as described previously (17).

**Apoptosis assessment**

Apoptosis was detected by flow cytometry using an Annexin V/PI dual staining kit from BD Biosciences Pharmingen as described previously (17).

**Determination of radiation response in human tumor xenografts**

Athymic nude mice were obtained from Harlan Bio-products for Science and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals was in accordance with institutional guidelines. H226 or SCC1483 cells were injected subcutaneously into the dorsal flank area of the mice. Tumor volume was monitored by direct measurement with calipers and calculated by the formula: \( \frac{\text{large diameter} \times \text{small diameter}^2}{6} \). Following the establishment of tumor, mice were randomly selected to receive control human IgG, radiation alone, Sym004 alone, or radiation in combination with Sym004. Sym004 was administered via intraperitoneal injection twice per week 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 jigs that exposed the dorsal flank (harboring tumor xenografts) to irradiation without exposing non–tumor-bearing normal tissues.

**Statistical analysis**

The Student t test was used to evaluate the significance of difference between Sym004 and control group. Differences between treatments were considered statistically significant when \( P < 0.05 \).

**Results**

**Sym004 induces EGFR degradation and inhibits proliferation**

Sym004 is a mixture of two anti-EGFR antibodies that has been shown to induce a rapid and efficient degradation of EGFR (16). To confirm this observation, we first examined the level of EGFR by immunoblotting following Sym004 treatment for 0.5, 2, and 24 hours in NSCLCs and HNSCCs. As shown in Fig. 1A, treatment with 10 \( \mu \)g/mL of Sym004 significantly reduces the total EGFR level within 24 hours in all tested cell lines. The reduction in EGFR after Sym004 treatment appears to reflect posttranslational downregulation of EGFR, as no significant change of EGFR mRNA level was observed in cells treated with Sym004 up to 48 hours (Fig. 1B). As shown in Fig. 1A, a loading control. B, effect of Sym004 on EGFR gene expression was examined by RT-qPCR as described in Materials and Methods. C, cells were exposed to serial concentrations of Sym004 for 72 hours. Thereafter, growth of tumor cells was determined by cell proliferation analysis.

**Figure 1.** Effect of Sym004 on EGFR expression and cell proliferation. A, cells were treated with 10 \( \mu \)g/mL Sym004 for 0, 0.5, 2, or 24 hours. Thereafter, total and phosphorylated EGFR level was determined by immunoblotting analysis. α-Tubulin (α-Tu) serves as a loading control. B, effect of Sym004 on EGFR gene expression was examined by RT-qPCR as described in Materials and Methods. C, cells were exposed to serial concentrations of Sym004 for 72 hours. Thereafter, growth of tumor cells was determined by cell proliferation analysis.
previous report (18). Although the precise mechanism for this biphasic inhibition of Sym004 is not clear, a pronounced inhibition of cell proliferation was observed after treatment with Sym004 (Fig. 1C). Sym004 significantly inhibits the proliferation of all tested cells in a dose-dependent manner.

**Sym004 enhances radiosensitivity**

We next investigated whether the superior downregulation of EGFR induced by Sym004 could be translated into augmentation of radiation response in tumor cells. We examined radiation response of tumor cells following Sym004 treatment using clonogenic survival analysis. As shown in Fig. 2, treatment with Sym004 before radiation significantly reduced clonogenic survival when compared with controls in all cell lines tested. The radiation dose enhancement ratios of survival at 10% (ER10) induced by Sym004 are 1.45 (H226), 1.21 (H292), 1.12 (SCC1), and 1.21 (SCC1483), respectively.

**Sym004 inhibits radiation-induced EGFR signaling and cell-cycle progression**

To further investigate underlying mechanisms for the effect of Sym004 on radiation response, immunoblotting analysis was conducted to examine the effect of Sym004 on radiation-induced EGFR signaling and cell-cycle progression. Consistent with previous findings (19), we observed radiation-induced activation of EGFR (T1173) and downstream MAPK signaling in the control H226 cells 1 to 4 hours after 6 Gy radiation as shown in Fig. 3A. This radiation-induced EGFR signaling correlated well with an increase of p-RB that serves as a key regulatory factor to stimulate G1–S progression. However, pre-treatment with Sym004 inhibited radiation-induced EGFR survival signaling and the expression level of p-RB. A significant increase of cleaved PARP (ΔPARP) and BAD that resulted from the activation of apoptosis was also found in Sym004-treated cells 24 hours after radiation.

To validate the effect of Sym004 on radiation-induced cell-cycle progression, we examined the cell-cycle phase distribution 1 and 2 days following exposure to radiation treatment. As shown in Fig. 3B, the S-phase cell-cycle populations were enhanced 1 day (D1) after radiation in the control H226 cells. This result correlates well with previous observation of an increase of EGFR signaling and p-Rb following radiation treatment in Fig. 3A. However, treatment with Sym004 significantly inhibited radiation-induced cell-cycle G1–S progression and resulted in cell-cycle arrest in the G0–G1 phase. In addition, augmentation of radiation-induced G2–M arrest was observed on Sym004-treated cells when compared with controls.
Similar results were found in three additional tumor cell lines (data not shown).

**Sym004 inhibits DNA damage repair**

We then characterized the DNA damage profile following radiation by examining the numbers of phosphorylated histone 2AX (γH2AX) foci in the nucleus that resulted from radiation-induced DNA double-strand breaks (DSBs). As shown in Fig. 4A, a significant increase of γH2AX foci was observed in Sym004-pretreated H226 and SCC1 cells by a factor of 1.4 to 1.6 at 2 hours after radiation when compared with control cells. As radiation response is initiated with the recognition of DNA damage and often results in cell-cycle arrest for repair, we next examined the effect of Sym004 on several checkpoint proteins for initiation of radiation-induced DNA damage repair such as BRCA1, ATM, and CHK1. As shown in Fig. 4B, all these checkpoint molecules were significantly activated in a dose-dependent response following radiation in control cells without Sym004 pretreatment. Following 2 or 24 hours of pretreatment with Sym004, the activation of these molecules was significantly inhibited, especially in the 10 Gy–treated cells. Notably, the inhibitory effect of Sym004 was more profound in cells exposed to a high dose of radiation at 10 Gy than 2 Gy. This finding also validates the more profound cell killing observed in Sym004-treated cells following a high-dose 9 Gy radiation in Fig. 2.

We further examined the capacity of Sym004 to inhibit DNA damage repair by examining several key proteins involved in the repair of lethal DSB in the nucleus such as DNAPK, NBS1, and Mre11. Among them, the block of nuclear DNAPK translocation is known to play an important role in regulating cetuximab-induced radiosensitivity (20, 21). Consistently, we found a significant inhibition of nuclear translocation of DNAPK following 6 Gy radiation in both Sym004-treated H226 and SCC1 cells as shown in Fig. 4C. In addition, the levels of Mre11, Rad50, and pNBS1 that form a complex in DSB sites to promote repair processes in the nucleus were reduced by Sym004 treatment. Consistent with the inhibition of pBRCA1 in Fig. 4B, the level of nuclear BRCA1 was also decreased in Sym004-treated cells. As inhibition of all these repair molecules is known to sensitize tumor cells to radiation, these results suggest that Sym004 appears to augment the radiation effect via inhibiting DNA repair machinery in tumor cells.
arrested in G0–G1 and G2–M. Notably, we found that a higher level of EGFR was present in the nuclear fraction of Sym004-pretreated SCC1 cells, but not in H226 cells as shown in Fig. 4C. As previous studies have shown a negative impact of nuclear EGFR on the sensitivity and survival following radiation treatment (22, 23), the finding of high nuclear EGFR in SCC1 cells compared with H226 cells correlates well with the reduced radiation response in SCC1 cells than that of H226 cells following Sym004 treatment at a dose ≤ 6 Gy.

Sym004 enhances radiation-induced apoptosis

As unrepaired tumor cells are prone to apoptotic cell death, we examined the capacity of Sym004 to induce apoptosis following radiation using Annexin V/PI flow cytometric analysis. Figure 5 depicts representative FACS cytograms of dual stained cells from control or Sym004-pretreated cells in all cell lines tested. Following 6 Gy radiation exposure, the percentage of early apoptotic cells (Annexin V-positive/PI-negative) was slightly increased in the control cells. The fold increase of the early apoptotic population was 2.6, 1.5, 1.3, and 1.3 for H226, H292, SCC1, and SCC1483, respectively. However, treatment with Sym004 before radiation induced a more profound induction of apoptosis with fold increases of 6.7 (H226), 2.7 (SCC1), 2.5 (SCC1), or 2.4 (SCC1483), respectively. Interestingly, the highest enhancement of apoptosis was observed in H226 cells. This result also correlated with a more profound cell killing of H226 cells in the clonogenic survival analysis. In addition, an increase of cells in the late apoptotic/necrotic phase (italic number) in the top right quadrants of Fig. 5 was found in Sym004-treated cells when compared with control. These results strongly suggest that Sym004 hinders the EGFR-mediated radiation damage repair response and results in a stronger induction of apoptosis.

Sym004 augments radiation response in human tumor xenografts

To expand upon the in vitro findings, we investigated the capacity of Sym004 to augment radiation in both NSCLC and HNSCC human xenografts using a variety of treatment dose and schedule schemes. We first examined the response of NSCLC xenografts (H226) following combination treatment of radiation with two different doses of Sym004 at 1.6 (S1.6) or 4.8 (S4.8) mg/kg. As expected, Sym004 alone inhibited tumor growth in a dose-dependent manner as shown in Fig. 6A. Interestingly, the combination of radiation (1.5 Gy) and either dose of Sym004 induced a dramatic inhibition of tumor growth, whereas single treatments only caused a modest effect when compared with the control group. The combined radiation and high-dose Sym004 (S4.8) achieved a more pronounced antitumor effect than low-dose Sym004 (S1.6), reflecting its capacity to delay tumor regrowth.
To further evaluate the impact of combined Sym004 and radiation treatment, we examined radiation response in a HNSCC xenograft (SCC1483) following 2 different treatment schemes as shown in Fig. 6B. The left shows the tumor response following a single treatment of high dose Sym004 (80 mg/kg, S80) and/or radiation (18 Gy) at day 10. As expected, high-dose radiation resulted in significant tumor shrinkage during the first few weeks following treatment. However, surviving tumor cells commenced regrowth within 2 weeks after radiation. The combination of radiation and Sym004 was very potent to regress established tumors and thereafter induced significant tumor regrowth delay over 9 to 10 weeks after treatment. Similarly, the combination treatment of fractionated radiation and Sym004 also caused a significant growth delay as shown in the right of Fig. 6B, but the capacity of the fractionated treatment to inhibit tumor regrowth was more modest than that of the single treatment. This likely reflects more modest cell killing in the fractionated treatments with low-dose radiation and drug exposure. Although there are slightly different response profiles between the single and fractionated treatment regimens, these results confirm and extend the previous in vitro findings and indicate a profound capacity of Sym004 to augment radiation response in vivo.

Discussion
Sym004 is a recently developed novel EGFR targeting approach comprising a mixture of 2 distinct anti-EGFR mAbs. With a potent capacity to inhibit EGFR via the induction of rapid and efficient internalization and degradation of EGFR, Sym004 exhibits a more pronounced growth inhibition than current EGFR targeting agents across a variety of tumor cells (16). In the present study, we provide evidence that the powerful downregulation of EGFR induced by Sym004 can be translated into a profound augmentation of radiation response in tumor cells. In tumor xenografts, we observed a superior antitumor capacity of Sym004 when combined with either single or fractionated radiation (Fig. 6). In addition, we found potent cell killing in Sym004-treated cells, especially when cells were exposed to high-dose radiation in clonogenic survival analysis (Fig. 2). These results are consistent with the more profound inhibition of checkpoint proteins for DNA damage by Sym004 after 10 Gy radiation when
compared with 2 Gy as shown in Fig. 4B. These data suggest that the effect of Sym004-induced EGFR down-regulation can be amplified when cells are exposed to high-dose radiation.

The potent enhancement of radiosensitivity we observed with Sym004 was seldom observed with other EGFR targeting agents, including cetuximab, from our previous studies over the last 14 years (5, 20, 24). To establish a direct comparison, we conducted a side-by-side analysis of clonogenic survival in Sym004- and cetuximab-treated cells following a 9-Gy radiation exposure. As shown in Supplementary Fig. S1A, we observed a 2.5- to 7.2-fold greater reduction in clonogenic survival in Sym004-treated cells when compared with cetuximab-treated cells in all of the cell lines tested. Furthermore, Western blot analysis showed that Sym004 is more potent than cetuximab to inhibit radiation-induced phosphorylation of EGFR, AKT, and CDC2 24 hours after radiation that regulate survival signaling and cell-cycle progression (Supplementary Fig. S1B). Most importantly, Sym004 showed a stronger impact than cetuximab to inhibit DNA damage repair signaling and prompt the induction of apoptosis. A significant inhibition of radiation-induced p-NBS1 and p-p53 as well as induction of γH2AX was observed in Sym004-treated cells (Supplementary Fig. S1B). With the observed loss of EGFR in Sym004-treated cells, these results suggest that Sym004 is more potent than cetuximab to enhance radiosensitivity likely reflecting its profound capacity to downregulate EGFR.

Consistently, we observed a correlation between Sym004-induced radiation response and EGFR downregulation across several lines of study in the current report. As shown in Fig. 1A, EGFR and pEGFR levels were robustly decreased at 24 hours. In turn, a more significant inhibition of radiation-induced pBRCA1, pATM, and pCHK1 was observed in cells exposed to Sym004 for 24 hours than at 2 hours as shown in Fig. 4B. In addition, Sym004 was more potent at enhancing radiosensitivity in H226 and H292 cells than in SCC1 and SCC1483 cells, especially at a dose ≤ 6 Gy (Fig. 2). The enhanced radiosensitivity in the H226 and H292 cell lines correlated with a more rapid and efficient EGFR downregulation in H226 and H292 compared with SCC1 and SCC1483 cell lines (Fig. 1A). These consistent results suggest EGFR
downregulation as a key contributor to Sym004-induced radiosensitivity. However, a more detailed comparison to include additional cell lines and radiation doses will be valuable to expand these observations from the currently tested four cell lines.

Surprisingly, we found that a significant proportion of EGFR was present in the nuclear but not cytosolic fraction of SCC1 after 24 hours of Sym004 pretreatment, whereas EGFR was present in the cytosolic fraction of untreated control cells as shown in Fig. 4C. Further studies showed that Sym004 caused a significant nuclear accumulation within 30 minutes in all cell lines tested (Supplementary Fig. S2A). Although nuclear EGFR was downregulated in all cell lines at 24 hours, SCC1 and SCC1483 showed a lower rate and intensity of EGFR downregulation when compared with H226 and H292 cells. This finding is consistent with a lower radiation response in SCC1 cells than that of H226 cells following 24 hours of Sym004 treatment. Using immunoprecipitation, we further identified that the association of EGFR and Importin 9/10 or Sce61β that are involved in EGFR nuclear import (25, 26) was higher in SCC1 than those in H226 cells 24 hours after Sym004 treatment (Supplementary Fig. S2B). These findings suggest that Sym004 can induce EGFR nuclear translocation and that nuclear EGFR may be involved in regulating radiation response.

In addition to Sym004, cetuximab has been previously shown to induce nuclear translocation of EGFR in tumor cells, including SCC1 and SCC1483 (27, 28). However, different response profiles of nuclear EGFR translocation were observed when cetuximab-treated and Sym004-treated SCC1 and SCC1483 cells were compared. Cetuximab-induced nuclear EGFR was maintained up to 50 to 96 hours in both SCC cells (28). In contrast, Sym004-induced nuclear EGFR was maintained less than 24 hours as shown in Supplementary Fig. 4. The rapid reduction of nuclear EGFR translocation could result from the degradation of EGFR induced by Sym004. The lower level of nuclear EGFR correlated with a higher radiation response in Sym004-treated SCC cells when compared with cetuximab-treated cells as shown in Supplementary Fig. S1.

A more detailed investigation of Sym004- and cetuximab-induced EGFR nuclear translocation and the resultant impact on radiosensitivity is highly desired. Specifically, anti-EGFR mAb can induce nuclear EGFR accumulation that may enhance resistance to radiation (28, 29). Several molecular image tracking experiments are underway to examine EGFR trafficking following Sym004 or cetuximab treatment. Surprisingly, our preliminary results from 3-dimensional (3D) imaging suggest that EGFR is located on the nuclear surface, as opposed to inside the nucleus, following treatment with both anti-EGFR agents (data not shown). Side views of the intact nucleus show the accumulation of EGFR on the external (surface) of nucleus. As most studies of EGFR trafficking have been conducted using cellular fractionation and 2D imaging, the nuclear translocation of EGFR induced by cetuximab or Sym004 may reflect limitations of cellular fractionation and 2D imaging. More detailed molecular imaging studies are in progress to further clarify the effect of anti-EGFR agents on EGFR nuclear trafficking.

In conclusion, Sym004 shows the capacity to augment radiation response in NSCLC and HNSCC tumors both in vitro and in vivo. With a potent ability to induce rapid and efficient EGFR internalization and degradation, Sym004 may afford a more powerful anti-EGFR therapy approach than the currently used single EGFR mAbs. Preclinical pharmacokinetic studies in primates and early clinical trial safety and feasibility studies in humans indicate that Sym004 is well-tolerated and does not induce unexpected toxicities (30). Several clinical trials are in progress to evaluate the clinical potential of Sym004 for patients with HNSCCs and metastatic colorectal cancer (31, 32). With mature clinical trial data showing a modest survival advantage (~10%) for patients with HNSCCs treated with cetuximab with radiation (7), the more powerful impact of Sym004 compared with cetuximab in the current report provides a rationale to design clinical studies to test the impact of Sym004 with radiation for patients with HNSCCs in an effort to further improve overall outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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