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Combined EGFR1 and PARP1 Inhibition Enhances the Effect of Radiation in Head and Neck Squamous Cell Carcinoma Models

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a challenging cancer with little change in five-year overall survival rate of 50–60% over the last two decades. Radiation with or without platinum-based drugs remains the standard of care despite limited benefit and high toxicity. HNSCCs often overexpress epidermal growth factor receptor (EGFR) and inhibition of EGFR signaling enhances radiation sensitivity by interfering with repair of radiation-induced DNA breaks. Poly (adenosine diphosphate-ribose) polymerase-1 (PARP1) also participates in DNA damage repair, but its inhibition provides benefit in cancers that lack DNA repair by homologous recombination (HR) such as BRCA-mutant breast cancer. HNSCCs in contrast are typically BRCA wild-type and proficient in HR repair, making it challenging to apply anti-PARP1 therapy in this model. A recently published study showed that a combination of EGFR and PARP1 inhibition induced more DNA damage and greater growth control than each single agent in HNSCC cells. This led us to hypothesize that a combination of EGFR and PARP1 inhibition would enhance the efficacy of radiation to a greater extent than each single agent, providing a rationale for paradigm-shifting combinatorial approaches to improve the standard of care in HNSCC. Here, we report a proof-of-concept study using Detroit562 HNSCC cells, which are proficient for DNA repair by both HR and non-homologous end joining (NHEJ) mechanisms. We tested the effect of adding cetuximab and/or olaparib (inhibitors of EGFR and PARP1, respectively) to radiation and compared it to that of cisplatin and radiation combination, which is the standard of care. Our results demonstrate that the combination of cetuximab and olaparib with radiation was superior to the combination of any single drug with radiation in terms of induction of unrepaired DNA damage, induction of senescence, apoptosis and clonogenic death, and tumor growth control in mouse xenografts. Combined with our recently published phase I safety data on cetuximab/olaparib/radiation triple combination, the data reported here demonstrate a potential for combining biologically-based therapies that might optimize radiosensitization in HNSCC.

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INTRODUCTION

Radiotherapy remains an important treatment modality for head and neck squamous cell cancer (HNSCC). Numerous studies have combined radiation with chemotherapies or targeted agents in an effort to enhance tumor control. Current standard of care is radiation plus a platinum drug.

Yet, a compilation of data from a multitude of clinical trials that evaluated the benefits of cisplatin combined with radiation suggests only about a 5% benefit in survival (1). RTOG 91-11, a landmark phase III study in laryngeal cancers, also failed to show a long-term survival benefit of cisplatin concurrent with radiation treatment, which was attributed to toxicity and deaths not associated with cancer recurrence (2). Treatment intensification using accelerated radiation or induction chemotherapy followed by radiation has also failed to deliver improved results (3). In addition to toxicity issues associated with cisplatin-based combination therapy, HNSCC patients may develop resistance to cisplatin through a variety of mechanisms (4–6). The overall five-year survival rate of 50–60% for HNSCC has changed little in the last two decades. Better treatment strategies are clearly needed for HNSCC.

The therapeutic effect of radiation relies on its ability to induce DNA single- and double-strand breaks (SSBs and DSBs). Cells counter the effect of radiation by activating DNA repair. The two major pathways that repair DNA DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ). Successful repair negates the effect of radiation and therefore inhibitors of DNA repair are expected to enhance the therapeutic effect of radiation. Many HNSCCs show elevated EGFR expression (7, 8), which is associated with intrinsic radioresistance (9), enhanced DNA repair (11) and poor patient survival (7, 10). It has been reported that EGFR inhibition in HNSCC cells blocked cell proliferation and increased apoptosis (11). The combination of the anti-EGFR antibody cetuximab with radiation has been studied extensively in preclinical and clinical settings. Cetuximab improved locoregional control and survival compared to radiation alone and was well tolerated (5, 6, 12). A randomized phase III trial compared radiation and cetuximab treatment to radiation alone in unselected HNSCC patients, demonstrating a superior five-year overall survival (45.6%) in combined therapy versus radiation alone (36.4%) (13). The most detrimental DNA damages after radiation treatment are DSBs, which are preferentially repaired in mammalian cells by NHEJ (14). A key component of the NHEJ repair process is the nuclear protein DNA-dependent protein kinase catalytic subunit (DNA-PKcs), encoded by PRKDC in humans. In combination with two DNA binding factors, Ku70 and Ku80, DNA-PKcs serves as a scaffold for recruiting DNA repair proteins to DNA strand breaks. EGFR physically interacts and activates DNA-PK as the result of EGFR translocation to the nucleus (15). The addition of cetuximab prior to irradiation inhibits EGFR transport to the nucleus and prevents autophosphorylation of DNA-PK at Thr2609, with a subsequent

decrease in DNA-PK activity. Consequently, DNA damage repair is inhibited and contributes to the radiosensitization by cetuximab (16).

The application of anti-EGFR therapies in HNSCC, however, remains challenging for a number of reasons. First, the level of EGFR expression does not correlate with response to EGFR inhibitors (17). In addition, certain EGFR mutations, which confer sensitivity to EGFR inhibition in lung cancer, occur less frequently in HNSCC (18). Also, patients who respond to EGFR inhibitors frequently develop resistance (8). To further improve on the radiation-cetuximab treatment combination, the addition of anti-EGFR agents to a cisplatin-radiotherapy backbone was explored as the logical next step; however, several trials failed to show improved outcomes while toxicity increased (12, 19). These results have motivated the search for another agent that may be safely combined with the radiation/anti-EGFR treatment combination. One promising strategy incorporates the use of inhibitors of poly(ADP-ribose) polymerase (PARP).

PARP1, and its related family member PARP2, are nuclear proteins that transfer the ADP moiety from β -nicotinamide adenine dinucleotide (NAD^+) to target molecules, mostly proteins. PARylation is a post-translational modification involved in several cellular processes, including sensing and relaying the presence of DNA SSBs (20). Cleavage of PARP1 by caspase also serves as a marker of apoptosis (21). Radiation-induced DNA SSBs activate PARP, leading to increased NAD^+ consumption, and direct enzyme interactions that activate XRCC1 repair enzyme, chromatin restructuring and cell-cycle checkpoints (22). In addition to autophosphorylation mentioned above, DNA-PK is primarily phosphorylated at Thr2609 by ATM, which also leads to activation of DNA-PK for NHEJ. PARP1 inhibition blocks PARylation of ATM, which prevents translocation of ATM to the nucleus and subsequent DNA-PK activation (23). More recently, PARP1 has been shown to contribute to DNA-PKcs activation by PARylation (24).

Cancer cells with HR repair defects such as BRCA1 or BRCA2 dysfunction are hypersensitive to PARP inhibition because the loss of PARP1 activity leads to the persistence of DNA lesions normally repaired by HR (22). Suppression of PARP has resulted in cancer cell death when BRCA1 or BRCA2 is deficient (25, 26) and demonstrated impressive clinical activity as a monotherapy (27, 28) with recent FDA approval in advanced ovarian cancer. Marked activity has been reported with combinations of olaparib, an orally bioavailable PARP1 inhibitor, with radiation in breast cell lines that harbor BRCA mutations. HNSCC cells typically lack BRCA mutations and are proficient for HR repair, raising questions about the utility of PARP1 inhibitors as monotherapy in this cancer type and leading to studies that explore the use of PARP1 inhibitors in combination with other agents.

In a study to assess PARP1 inhibitors in combination therapy, olaparib was found to enhance the effect of radiation in HNSCC cell lines that are BRCA wild-type, and did so at lower doses than were required to show single-agent activity without radiation (29). In another study exploring the use of PARP inhibitors in HNSCC, simultaneous inhibition of EGFR using cetuximab and PARP using ABT-888 (without radiation) produced persistent DNA damage and apoptosis (30). It was hypothesized that cetuximab inhibited both HR and

NHEJ, rendering the cells sensitive to DNA damage that results from proliferation without PARP. The finding that simultaneous inhibition of EGFR and PARP led to greater defects in DNA repair than each agent alone led to the prediction that the combination would show greater enhancement of radiation than each agent alone. In contrast, in another published study it was found that PARP1 became phosphorylated after irradiation of HNSCC cells and that this modification was prevented when EGFR was inhibited with erlotinib, suggesting that PARP1 functions downstream of EGFR (31). More important, HNSCC cells treated with a combination of erlotinib and olaparib showed a similar number of p53BP1 DNA repair foci as cells treated with each single agent. Based on these data, we would predict that simultaneous inhibition of EGFR and PARP1 would not enhance the effect of radiation more than each single agent. Here, we tested these opposing predictions. Our data support the hypothesis that simultaneous inhibition of EGFR and PARP in the context of irradiation will have greater effect on DNA repair, cell growth and tumor growth than inhibiting EGFR or PARP alone in a HNSCC model.

MATERIALS AND METHODS

Ethics Statement

Investigation has been in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Gene Expression

Cells were plated and allowed to adhere overnight. RNA was extracted using RNeasy extraction (QIAGEN®, Valencia, CA). Gene expression profiling was performed using Affymetrix® U133 Plus 2.0 microarrays (Santa Clara, CA) and expressed as robust multi-array average (RMA) by the University of Colorado Cancer Center Genomics shared resource (Aurora, CO).

Cell Culture

Detroit562 (Det562) and FaDu were obtained from the American Type Culture Collection (ATCC®, Gaithersburg, MD) and maintained in Dulbecco's Modified Eagle's media supplemented with 10% fetal bovine serum (FBS). Cell line identity was confirmed by STR fingerprinting at the University of Colorado Cancer Center sequencing facility.

Inhibitors and Irradiation

Olaparib was purchased from LC Laboratories (Woburn, MA) and dissolved in dimethyl sulfoxide to a concentration of 10 mM. Cetuximab was obtained from ImClone Systems (New York, NY). For *in vitro* studies, radiation was delivered using a Faxitron® X-ray irradiator (Tucson, AZ) at a dose rate of 0.32 Gy/min. For animal experiments, radiation was delivered using a RS2000 X-ray irradiator (Rad Source Technologies Inc., Buford, GA) at a dose rate of 1.39 Gy/min. The Faxitron irradiator (Model RX-650) uses a beryllium window and was operated at 115 kV; quality assessment is performed annually using the NanoDot® Dosimeter (Landauer® Inc., Glenwood, IL). For single-agent experiments, cells were treated with radiation (2 Gy), cisplatin, cetuximab, or olaparib, and harvested for immunoblotting 2

h postirradiation. For all other *in vitro* studies, cetuximab was added 24 h prior to irradiation and olaparib was applied 4 h before irradiation. Cisplatin (cis-diammineplatinum chloride (II) dichloride) was purchased from Sigma-Aldrich® (St. Louis, MO), dissolved in DMSO to a concentration of 10 mM just before use and added 1 h before irradiation. The sequence of inhibitor-irradiation was designed to mimic how these treatments are administered in the clinic.

Immunoblotting

The following antibodies were used: phospho-p44/p42^{Thr202/Tyr204}, total p44/p42 (MAPK1/2), DNA-PK (PRKDC), PARP1, γ -H2AX and p21 (CDKN1A) were acquired from Cell Signaling Technology® (Beverly, MA), phospho-DNA-PKcs^{Thr2609} from Novus Biologicals (Littleton, CO) and γ -actin (ACTB) from Sigma-Aldrich.

Clonogenic Survival

Detroit562 and FaDu originated from p16-negative carcinomas with *TP53* missense mutations that result in overexpression of p53 (45). Detroit562 was derived from a metastatic site of pharyngeal carcinoma, and FaDu was derived from a primary hypopharyngeal carcinoma. Cells were stably transfected with NucLight™ Red Lentivirus (Essen Bioscience Inc., Ann Arbor, MI) to enable automated cell counting using the IncuCyte® S3 live cell imaging system (Sartorius, Goettingen, Germany). The IncuCyte system is capable of capturing phase contrast, and red and green fluorescent images of live cells. The phase contrast channel was set to count colonies with an area that corresponded to at least 50 cells, based on the number of red fluorescent cells. Clonogenic survival curves were generated using standard procedures. Briefly, cells were trypsinized, counted with trypan blue, plated in six-well plates and allowed to adhere overnight. Cells were treated with cetuximab (50 nM), olaparib (1 μ M), or cisplatin (1 μ M) and radiation (0, 2, 4 or 6 Gy) and incubated for 10 days. Drug doses were selected to represent relevant and achievable levels in the clinic. The dose of 2 Gy was chosen to approximate the dose per fraction that a patient undergoing standard-of-care fractionated radiation would receive. Images were captured using the IncuCyte S3 every 24 h, and survival curves at the 10-day end point were modeled in Excel using the linear-quadratic equation: Survival fraction = $e^{-(\alpha D + \beta D^2)}$, where D is the dose in Gy.

Homologous Recombination Assay

The promoter-less plasmids pGL4.14[*luc2*/hygro] and pGL4.20[*luc2*/puro] vectors were purchased from Promega, Inc. (Madison, WI). The SV40 promoter sequence was cloned into the XhoI/BglII MCS of pGL4.14[*luc2*/hygro] to create the constitutive *luc2* expression vector pGL4.14SV40[*luc2*/hygro]. pGL4.14SV40[*luc2*/hygro] was cut with DraIII/FseI to remove 521 bp of the *luc2* gene, and pGL4.20[*luc2*/puro] was digested with ApaI/BsrGI to excise 461 bp within the *luc2* gene. After treatment with the Klenow fragment, cut plasmids were re-ligated and deletions in the resulting plasmids were confirmed by sequencing. These vectors were designated pGL4.14SV40[*luc2*/hygro]-luc and pGL4.20[*luc2*/puro]-luc. They share a region of 655 bp such that when vectors are co-transfected into recipient cells that are HR-competent, luciferase activity is reconstituted.

Non-Homologous End Joining Assay

Autophosphorylation of DNA-PKcs (Thr2609) was determined by Western blots of cellular extracts (at 2 and 24 h postirradiation). A NHEJ reporter plasmid was constructed using pGL4.20 (*luc2*) as a backbone. Translation of luciferin was prevented by the insertion of a sequence (SceCas) containing an out-of-frame ATG start site flanked by two I-SceI cut sites between the SV40 promoter and the ORF (Fig. 4C). The I-SceI gene was amplified from *Saccharomyces cerevisiae* (a kind gift from Robert Scalfani, University of Colorado Anschutz Medical Campus) and cloned into pLXSN. For the NHEJ assay, pLXSN-I-SceI was transfected in Det562 cells containing the stably integrated pGL4.20 (*luc2*)-SV40SceCas and pZSGreen-C1 using SuperFect (QIAGEN) to mimic radiation-induced strand breaks. At 48 h post-transfection, cisplatin, olaparib, cetuximab or olaparib with cetuximab were added in triplicate wells at concentrations described above. At 48 h after drug addition, cells were counted in each well using the IncuCyte S3 imaging system and assayed for expression of luciferase. Luciferase activity was standardized to cell counts for each sample. Baseline luciferase activity in cells with only pGL4.20SV40SceCas (no pLXSN-I-SceI) was subtracted from all treatments with pLXSN-I-SceI.

DNA damage was determined by Western blotting and immunofluorescence with Alex-Fluor® 488-conjugated antibodies to γ -H2AX at 2 and 24 h postirradiation. Briefly, cells were allowed to attach overnight in plates with glass coverslips, then treated as detailed above. For imaging, cells were fixed, permeabilized, blocked and immunostained as recommended by the antibody manufacturer. Images were captured using a Leica fluorescence microscope at 40 $\times$ magnification. To quantitate γ -H2AX expression, cells were collected and immunoblotted at the same time from plates without coverslips.

Senescence-Associated β -Galactosidase Assay

Quantitative β -galactosidase activity was monitored by the hydrolysis of fluorescein di(β -D-galactopyranoside) as described by Yang and Hu (46) using the same treatment conditions 72 h after irradiation.

Animal Studies

Detroit562 HNSCC xenografts were established in the right hind flank of nude mice (nu/nu; 5–8 weeks old females; Charles River Laboratories, Wilmington, MA) by subcutaneous injection of 5×10^6 cells/mouse mixed 1:1 with Matrigel® (BD Biosciences, Franklin Lakes, NJ) in a 100- μ l total volume. When tumors reached an average volume of 150–200 mm³, mice were randomized into six groups of 10 animals/group. Treatment groups were: Control (vehicle only); radiation only; cetuximab with radiation; olaparib with radiation; cisplatin with radiation; and cetuximab and olaparib with radiation. Cetuximab was given by intraperitoneal (I.P.) injection at a 1 mg/kg dose on days 1 and 4. X-ray irradiation (2 Gy) was administered on days 2 and 5 to mice positioned under a lead shield such that only the tumor-bearing flank was exposed. Olaparib (25 mg/kg) was given by I.P. injection 4 h before irradiation. Animals receiving cisplatin were given 1 mg/kg by I.P. injection 1 h prior to irradiation. The RS2000 (Rad Source Technologies) used for animal studies delivered X rays at a dose rate of 1.39 Gy/min and has a 0.3-mm copper filter irradiating at 160 kV. Dose delivery of the RS2000 was assessed annually by the radiation safety office at the University

of Colorado Anschutz Medical Campus. Treatments were given for three weeks. Experimental end points were tumors greater than 2 cm in any direction or significant tumor-associated ulceration. Growth curves were plotted until the first tumor in each group reached the specified end point. All experiments were carried out in strict compliance with the University of Colorado Institutional Animal Care and Use program.

Immunohistochemistry and In Vivo Senescence-Associated β -Galactosidase

Detroit562-naïve tumor xenografts that were approximately 100 mm³ in average volume were treated with drug/radiation as described in the text, harvested 96 h after treatment and either fixed with 10% neutral-buffered formalin and embedded in paraffin, or frozen in Tissue-Tek® optimum cutting temperature (OCT; Sakura Finetek USA, Torrance, CA) and stored at -80°C. Staining for p21 and cleaved caspase-3 were conducted on 4- μ m sections of fixed tissue according to antibody manufacturer recommendations (Cell Signaling Technology). Frozen sections (4 μ m) were fixed and processed for β -galactosidase activity using the senescence beta-galactosidase staining kit from Cell Signaling Technology (Danvers, MA).

RESULTS

PARP1 and EGFR Expression in HNSCC Cell Lines

We compared the relative gene expression of EGFR and PARP1 in a panel of 10 HNSCC cell lines and found similar mRNA levels in the cell lines with the exception of CCL30, which showed lower EGFR1 expression (Fig. 1A). We chose two of the cell lines with PARP1 and EGFR expression, Det562 and FaDu, for further study based on their known radiation responses and amenability to xenografting. We used immunoblotting to confirm that both cell lines express PARP1 and EGFR proteins (Fig. 1B).

Cetuximab/Olaparib Combination Induces Clonogenic Death

Clonogenic assays measure the ability of single cells to repair damaged DNA and proliferate to form a colony. This process approximates closely the regrowth and recurrence of tumors after treatment with radiation or chemotherapy. We used clonogenic assays to determine the effect of combined EGFR1 and PARP1 inhibition on irradiated Det562 and FaDu cells. Biologically relevant doses of 1 μ M olaparib and 50 nM cetuximab decreased clonogenic survival in both cell lines (Fig. 1C). While FaDu was more sensitive to radiation ($\alpha/\beta = 7.5$ Gy) compared to Det562 ($\alpha/\beta = 11.6$ Gy), Det562 had a greater enhanced cell killing in the quadratic region of the survival curve ($\alpha/\beta = 2.9$ Gy) than FaDu ($\alpha/\beta = 5.4$ Gy) with combined EGFR/PARP1 inhibition. Cetuximab alone produced modest or no improvement of the α/β ratios of the two lines, while the addition of either cisplatin or olaparib alone showed little or modest benefit.

Cetuximab/Olaparib Combination Results in Persistent DNA Damage after Irradiation

We used low-level radiation (2 Gy) which, on its own, appeared to modestly increase the level of γ -H2AX, a marker for DNA DSBs, detected by immunoblotting at 2 or 24 h postirradiation (Fig. 2A and C, Supplementary Fig. S1; <https://doi.org/10.1667/RR15480.1.S1>). For a more quantitative measure of DSBs, we used Western blotting to

monitor γ -H2AX (Fig. 2B and D). This method also showed that cetuximab, olaparib or cisplatin (used as standard-of-care comparison) on its own produced very little increase in γ -H2AX (Fig. 2E, 2 h time point). The addition of cetuximab, olaparib or cisplatin to radiation, however, increased the γ -H2AX signal relative to radiation alone at both 2 and 24 h postirradiation for Det562 and at 2 h postirradiation for FaDu. The increase was more pronounced for Det562 in the triple combination (Fig. 2B), which is consistent with the most pronounced effect of the triple combination for Det562 in clonogenic assays (Fig. 1C). For FaDu, the effect of the triple combination on γ -H2AX at 24 h postirradiation was similar to radiation treatment alone, also consistent with the clonogenic survival data.

Cetuximab/Olaparib Combination Inhibits DNA-PK Phosphorylation after Irradiation

To address the mechanistic basis for persistent DNA damage in cells treated with cetuximab, olaparib and radiation, we assayed for the level and phosphorylation of DNA-dependent protein kinase (DNA-PK). DNA-PK plays a critical role in NHEJ. It is recruited to broken DNA ends where its catalytic subunit (cs) is phosphorylated by DNA-PK itself or by the ATM kinase (34). Nuclear import of EGFR is linked to activation of DNA-PK, and cetuximab abolishes the import of EGFR to the nucleus (16).

Activating phosphorylation on Thr2609 of DNA-PKcs, as detected by a phospho-specific antibody on Western blots, increased at 2 h postirradiation for both Det562 and FaDu (Fig. 3A). This increase was also seen with olaparib alone in Det562. Cisplatin and cetuximab on their own made little difference. DNA-PK phosphorylation decreased to undetectable levels by 24 h postirradiation (Fig. 3B and C, radiation only). Cisplatin initially repressed DNA-PK phosphorylation after irradiation in both Det562 and FaDu cells (Fig. 3B and C, 2 h time points). But at 24 h postirradiation, all single drug treatments (cetuximab, olaparib, cisplatin) combined with radiation showed increased phosphorylated DNA-PKcs compared to radiation alone at the same time point in Det562 cells (Fig. 3B, 24 h time point). Most importantly, however, is the finding that the combination of cetuximab/olaparib was most effective at inhibiting radiation-induced DNA-PK phosphorylation at both time points. A similar observation was made for the triple combination of cetuximab/olaparib/radiation in FaDu cells at both time points (Fig. 3C), although cetuximab alone was also able to suppress radiation-induced DNA-PK phosphorylation at the 24 h time point in this cell line. Because DNA-PK phosphorylation is a surrogate marker for NHEJ, the results described above suggest that the cetuximab/olaparib combination inhibited NHEJ in irradiated cells.

Cetuximab/Olaparib Combination Inhibits NHEJ after Irradiation

The results so far lead to the hypothesis that simultaneous inhibition of EGFR and PARP produces the greatest radiosensitization effect than single agents by compromising DNA repair, especially through reduction of DNA-PK phosphorylation and inhibition of NHEJ. We further hypothesize that although PARP1 inhibitors have shown benefit for cancers that are deficient for DNA repair by HR, simultaneous inhibition of EGFR and PARP1 could be beneficial even in HR-proficient HNSCC. To test these hypotheses, we needed a cell line that is HR-proficient, NHEJ-proficient, EGFR-active (32) and extremely radioresistant. Results described next indicate that Det562 cells fit these criteria (Fig. 4). To monitor HR and NHEJ, we generated plasmid reporters that are modified versions of previously

validated mechanism-specific reporters of DNA repair (33). The HR reporter consists of two plasmids that share a 655 bp region of homology but encode fragments of the luciferase gene (Fig. 4A). HR repair after sequential transfection of the two plasmids restores the luciferase gene, which can be detected as luminescence. Det562 cells show significantly increased luciferase activity from reporter plasmids compared to controls (Fig. 4B), indicating that Det562 cells are proficient for HR and could be used in our proof-of-concept studies. Det 562 cells are HPV-negative and p16-negative, representing the worst cases of HNSCC. Det562 cells also develop tumors rapidly in a mouse flank model, providing another reason for their use in our studies.

The NHEJ reporter is comprised of two plasmids (Fig. 4C and D). A cassette with an out-of-frame start codon flanked by two ISce-I restriction sites is cloned in the MCS of pGL4.20, and expression of ISce-I from a second plasmid removes the out-of-frame start codon. Luciferase expression is restored by NHEJ. Det562 cells show increased luciferase when both the reporter and ISce-I plasmids are present (Fig. 4D, second bar) compared to when only the luciferase plasmid is present (Fig. 4D, first bar), indicating that these cells are proficient also for NHEJ. We used the NHEJ reporter to assay the effect of cetuximab and olaparib on this mode of repair after irradiation (Fig. 4E). Cisplatin or olaparib reduced NHEJ to a significant extent while the combination of cetuximab and olaparib reduced NHEJ to background levels. These results corroborate the data on DNA-PKcs phosphorylation, and support the hypothesis that cetuximab/olaparib/radiation triple combination has the greatest effect on DNA repair.

Cetuximab/Olaparib Combination Induces Apoptosis and Senescence after Irradiation

Radiation produces tumor control because it induces cell death by different mechanisms: apoptosis, senescence and clonogenic death. In the latter two mechanisms, cells remain metabolically alive but are unable to proliferate. The data presented so far showed that the combination of cetuximab and olaparib produced the greatest clonogenic death in irradiated Det562 cells (Fig. 1C). We next addressed the ability of cetuximab and olaparib to modulate the ability of radiation to induce cell death in the same cells by the remaining two mechanisms. We found that radiation-induced apoptosis, detected as cleavage of PARP, was negligible in Det562 cells at both 24 and 48 h postirradiation (Fig. 5A), in agreement with previously reported studies (35) and the fact that these cells are highly radiation resistant (36). Radiation-induced apoptosis was not affected by cetuximab and showed a small increase with olaparib. In contrast, the combination of cetuximab and olaparib produced a significant increase in apoptosis after irradiation. We conclude that targeting both PARP and EGFR induced more apoptosis compared to single drug treatments.

Regulation of DNA DSB repair requires EGFR signaling and PARP1 (31). We measured inhibition of EGFR downstream signaling by immunoblotting for phospho-ERK1/2 at 24 and 48 h postirradiation. We found that the combination of PARP1 and EGFR inhibition decreased ERK1/2 signaling at 24 h (Fig. 5A). At 48 h, phospho-ERK1/2 was greatest in cells treated with radiation only, while all drug combinations reduced ERK1/2 phosphorylation.

Sustained DNA damage induces senescence through the p53-p21 signaling pathway and inhibition of DNA-PK induces accelerated senescence in irradiated cells (37). Therefore, we evaluated the effect of cetuximab/olaparib treatment on p21 levels (Fig. 5B–C) and senescence-associated β -galactosidase activity (Fig. 5D) after irradiation. The combination of olaparib and cetuximab with radiation generated the highest expression of β -galactosidase (Fig. 5D). In keeping with these data, we found that p21 expression was greatest in the triple combination-treated cells (Fig. 5C). Single treatments (Fig. 5C, 2 h time point) or double combinations (Fig. 5B, 24 h time point) showed little change in p21.

Cetuximab/Olaparib Combination Enhances Radiation-Induced Tumor Growth Delay *In Vivo*

The data from clonogenic assays show that the triple combination of cetuximab/olaparib/radiation was better than other combinations and cisplatin/radiation standard of care (Fig. 1). We next investigated whether these findings apply *in vivo* using Detroit562 xenografts in nude mice. In this model, neither cisplatin nor olaparib administered before irradiation had a significant effect on tumor growth delay compared to radiation alone while cetuximab enhanced the effect of radiation (Fig. 6A, quantified in Fig. 6B). Most importantly, the triple combination of cetuximab and olaparib with radiation significantly delayed tumor growth compared to cetuximab/radiation, olaparib/radiation and cisplatin/radiation, in agreement with the clonogenic survival data.

To investigate potential mechanisms of tumor growth delay, in a separate experiment of mice bearing tumor xenografts, the animals were treated with a single dose of drugs followed by 2 Gy exposure, as described for the tumor growth delay experiment, and tumors were harvested 96 h later. IHC staining for p21 showed minimal expression in untreated tumors, with the maximum staining observed in tumors treated with cetuximab, olaparib and radiation (Fig. 6C, brown stain). In keeping with these observations, β -galactosidase activity, a senescence marker, was also greatest in tumors treated with the two drugs and radiation (Fig. 6C, blue stain). We also detected increased staining for cleaved caspase-3, an apoptosis marker, in tumors treated with the triple combination (Fig. 6C, red stain). These data are in agreement with the *in vitro* data on PARP cleavage, p21 and senescence-associated β -galactosidase (Fig. 5). Taken together, these findings suggest that co-targeting EGFR and PARP1 activities before irradiation decreases tumor growth, at least in part, by accelerated senescence and apoptosis.

DISCUSSION

In the current study, we evaluated the effects of adding an olaparib-cetuximab combination to radiation treatment in HNSCC models. Both *in vitro* and *in vivo*, the triple combination outperformed all drug-radiation double combinations in terms of growth inhibition, including a cisplatin-radiation combination. The triple combination also produced the greatest effect compared to double drug-radiation combinations in terms of three different cell death mechanisms: apoptosis, senescence and clonogenic death.

We found that the addition of olaparib and cetuximab to radiation produced greater and more persistent DNA damage than the addition of each agent alone to radiation. Published data

can explain this observation. Ionizing radiation induces DNA SSBs and DSBs. PARP proteins are recruited to and activated by SSB and are needed to promote repair through the c-NHEJ repair pathway. PARP1 participates in activation of a critical factor in the c-NHEJ process, DNA-PKcs, by PARylation (24). In addition, PARP1 is required for DSB repair through the Ku-independent alternative NHEJ or alt-NHEJ pathway (33). EGFR signaling is implicated in DSB repair through γ -NHEJ by translocating to the nucleus after irradiation and is needed for the activation of DNA-PK in the nucleus (30). Once in the nucleus, EGFR also participates in double-stranded DNA damage repair through ERK1/2 signaling-independent alt-NHEJ pathway (38). Olaparib inhibits PARP1 PARylation activity, while cetuximab interferes with EGFR activity and radiation-induced nuclear import (15). EGFR inhibitors also arrest HNSCC cells in G₁ (39) where NHEJ is the only available DSB repair pathway. By targeting DNA-PKcs activation in two ways, these data suggest that a combination of PARP1 and EGFR inhibitors will eliminate more opportunities for DNA repair than each agent alone. Thus, we would expect the combination to enhance the effect of radiation more so than single agents alone. This is exactly what our data show.

We found that the combination of cetuximab and olaparib with radiation resulted in increased apoptosis, increased p21 expression and senescence, and increased clonogenic death in HNSCC cells. These results agree with the published data in other cell types or when using other inhibitors of EGFR and PARP. For example, PARP1 inhibition induces cellular senescence after DNA damage through the p21 pathway in mouse lung tissues (40). Co-targeting DNA-PK (a downstream target of EGFR) and PARP1 in non-small cell lung cancer cell lines promotes senescence (41). We propose that combined EGFR/PARP1 inhibition inhibits DNA repair by NHEJ and elevates senescence through p21 signaling, after irradiation, to tumor growth delay in BRCA1/2 wild-type HNSCC cells.

In summary, our data support the rationale for combining two targeted agents, against PARP1 and EGFR, with radiation to improve the treatment of HNSCC. Emerging data suggest that the efficacy of EGFR inhibition both pre-clinically and clinically may be greater in p16-negative cancers, providing a rationale for the use of EGFR therapy in this subset of patients (42, 43). Det562 cells are p16-negative, representing a patient population that could benefit from anti-EGFR therapy. The use of PARP inhibitors in the clinic is currently limited to BRCA mutant breast cancer patients. Most HNSCC patients harbor wild-type BRCA, decreasing their chances of benefiting from anti-PARP1 therapy. Det562 cells are BRCA wild-type. Therefore, our data suggest a way for BRCA wild-type HNSCC patients to benefit from PARP1 therapy by combining it with anti-EGFR therapy in a radiation setting. One key question is whether the triple combination of PARP inhibitor, EGFR inhibitor and radiation, potential benefits notwithstanding, may prove to be too toxic to be clinically relevant. In this regard, our completed phase I studies provide encouraging data; we found that olaparib could be safely combined with cetuximab and radiation (44). Thus, paradigm-shifting combinatorial approaches like concurrent perturbation of EGFR and PARP signaling pathways may provide an alternative and perhaps more effective cytotoxic approach to traditional cisplatin-radiation combinations in poor-prognosis head and neck cancer. In conclusion, the data reported herein provide a scientific rationale for future studies of the triple combination in clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

PARP1 and EGFR expression in HNSCC cell lines and clonogenic death in response to their inhibition. Panel A: Expression of EGFR and PARP1 mRNA in a panel of HNSCC cell lines. RMA = robust multi-array average. Panel B: Immunoblot showing EGFR and PARP1 expression of Det562 and FaDu cells. Panels C and D: Clonogenic survival of Det562 and FaDu cells, respectively, after irradiation with and without cetuximab (50 nM), olaparib (1 μ M) or cisplatin (1 μ M) treatment. Cell nuclei were fluorescently labeled, counted and plated. After drug treatment and irradiation, plates were placed in a live cell imager and colony formation captured every 24 h for 10 days. Data are representative of three independent replicate wells, error bars are $\pm$ S.E. Numbers in parenthesis are calculated α/β ratios.

FIG. 2.
Combined PARP1 and EGFR1 inhibition corresponds to increased DNA damage.
Immunofluorescent (panels A and C) and Western blots (panels B and D) for detection of γ -H2AX at 2 and 24 h postirradiation with or without cetuximab (50 nM), olaparib (1 μ M), irradiation (2 Gy) and cisplatin (1 μ M). Panels A and B show Det562 cells and panels C and D show FaDu cells. Panel E: The effect of single treatments at the 2 h time point. Beta-actin was used as a loading standard.

FIG. 3.

Combined PARP1 and EGFR1 inhibition downregulates DNA-PKcs autophosphorylation. Panel A: Western blots of phospho(Thr2609)- and total DNA-PKcs 2 h after single treatments: cetuximab (50 nM), olaparib (1 μ M), irradiation (2 Gy) and cisplatin (1 μ M) at 2 and 24 h postirradiation. Beta-actin was used as a loading standard (same images as in Fig. 2E are shown because experimental samples were the same). Panels B and C: Western blots of phospho(Thr2609)- and total DNA-PKcs in Det562 (panel B) and FaDu (panel C) cells

with or without cetuximab (50 nM), olaparib (1 μ M), irradiation (2 Gy) and cisplatin (1 μ M) at 2 and 24 h postirradiation. Beta-actin was used as a loading standard.

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FIG. 4.

Combined PARP1 and EGFR1 inhibition inhibits NHEJ. Panels A and B: Homologous recombination assay based on reconstitution of luciferase expression. Plasmid no. 1 is pGL4.14[*luc2*/hygro] with SV40 promoter inserted and part of the luciferase gene removed. Plasmid no. 2 is pGL4.20[*luc2*/puro] with part of the luciferase gene removed. The new plasmids share a region of 655 bp of the *luc2* gene such that when they are co-transfected into recipient cells that are homologous recombination competent, luciferase activity is reconstituted. Panel B: Detroit562 cells were transiently transfected with pGL4.20[*luc2*/

puro]-luc (plasmid no. 2), pGL4.14[*luc2*/hygro]-luc (plasmid no. 1 without the SV40 promoter), pGL4.14[*luc2*/hygro]-luc + pGL4.20[*luc2*/puro]-luc (plasmid no. 2), pGL4.14SV40[*luc2*/hygro]-luc (plasmid no. 1), pGL4.14SV40[*luc2*/hygro]-luc + pGL4.20[*luc2*/puro]-luc (experimental condition) or pGL4.14SV40[*luc2*/hygro] (positive control), then assayed for luciferase expression (Promega Luciferase assay system) 24 h after transfection. Error bars are $\pm$ S.E. of independent replicates. Panel C: NHEJ reporter based on ISceI-cleavage of an out-of-frame start codon. The sequence was cloned into pGL4.20 (*luc2*) between the SV40 promoter and *Luc2*. Panel D: Luciferase activity of cells stably transfected in Det562 cells with pGL4.20SV40SceCas alone or with pLXSN-ISceI. In the presence of both plasmids, ISceI cleaves the sites and NHEJ produces luciferase activity above the background of pGL4.20SV40SceCas alone. pGL4.20SV40 is the positive control with constitutive luciferase expression. Panel E: Luciferase activity 24 h after transfection with pLXSN-ISceI. Cells were counted by live cell imaging before luciferase assay and luciferase expression was normalized to cell counts. Baseline expression (no ISceI) was subtracted from pLXSN-ISceI transfected cells. Differences in luciferase expression from independent triplicates were determined using Tukey's range test of all means. * $P < 0.05$, ** $P < 0.001$.

FIG. 5.

Combined PARP1 and EGFR1 inhibition downregulates ERK1/2 phosphorylation and promotes PARP1 cleavage and senescence. Panel A: Det562 cells treated with or without cetuximab (50 nM), olaparib (1 μ M), radiation (2 Gy) and cisplatin (1 μ M) and harvested 24 or 48 h postirradiation were immunoblotted for total and phospho-ERK1/2 (Thr202/Tyr204), and PARP1. Beta-actin was used as a loading standard. Panel B: Western blot of p21 at 24 h postirradiation. The effect of single treatments at the 2 h time point are shown in panel C. Beta-actin was used as a loading standard. (Same images as in Fig. 2E are shown because

experimental samples were the same). Panel D: Combined cetuximab and olaparib induces senescence after irradiation. Cells were assayed for β -galactosidase activity by cleavage of fluorescein from fluorescein di- β -D-galactopyranoside 72 h postirradiation. Fluorescein concentrations in cell supernates were determined using a fluorescein standard and fluorescent plate reader at 485 nm excitation and 525 nm emission. To calculate average fluorescein per cell, the total number of cells were counted in independent triplicate wells treated in parallel to cells used for the senescence assay. Data are means of three independent samples $\pm$ S.E.

FIG. 6.

The combination of cetuximab and olaparib with radiation significantly delays tumor growth, and induces p21 expression, accelerated senescence and increased apoptosis. Panel A: Detroit562 tumor growth after treatment. Treatments were vehicle, radiation only (2 Gy), and radiation with cisplatin (1 mg/kg), olaparib (25 mg/kg), cetuximab (1 mg/kg) or cetuximab/olaparib (1 mg/kg and 25 mg/kg, respectively). Arrows indicate treatment time points and data points are mean tumor volume ($n = 10$) $\pm$ S.E. Curves are shown until any animal within a group reached maximum allowable tumor volume. Panel B: Number of days

that tumors in each group took to reach 1,000 mm³ in tumor volume (DTV4; mean $\pm$ S.E.). Panel C: Tumors were harvested 96 h after single-fraction irradiation (2 Gy), or cisplatin (1 mg/kg), olaparib (25 mg/kg), cetuximab (1 mg/kg) or olaparib/cetuximab with irradiation (2 Gy). Tumors were divided and fixed either in PBS-buffered formalin or frozen in OCT and stored at -80°C . p21 expression and cleaved caspase-3 were determined by IHC of sections of formalin-fixed tissue, and sections from frozen tissue were stained for β -galactosidase activity to detect senescence.