Targeting Aberrant DNA Double-Strand Break Repair in Triple-Negative Breast Cancer with Alpha-Particle Emitter Radiolabeled Anti-EGFR Antibody

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Abstract

The higher potential efficacy of alpha-particle radiopharmaceutical therapy lies in the 3- to 8-fold greater relative biological effectiveness (RBE) of alpha particles relative to photon or beta-particle radiation. This greater RBE, however, also applies to normal tissue, thereby reducing the potential advantage of high RBE. As alpha particles typically cause DNA double-strand breaks (DSB), targeting tumors that are defective in DSB repair effectively increases the RBE, yielding a secondary, RBE-based differentiation between tumor and normal tissue that is complementary to conventional, receptor-mediated tumor targeting. In some triple-negative breast cancers (TNBC; ER−/PR−/HER-2−), germline mutation in BRCA-1, a key gene in homologous recombination DSB repair, predisposes patients to early onset of breast cancer. These patients have few treatment options once the cancer has metastasized. In this study, we investigated the efficacy of alpha-particle emitter, 213Bi-labeled anti-EGF receptor antibody, cetuximab, in BRCA-1−defective TNBC. 213Bi-cetuximab was found to be significantly more effective in the BRCA-1−mutated TNBC cell line HCC1937 than BRCA-1−competent TNBC cell MDA-MB-231. siRNA knockdown of BRCA-1 or DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), a key gene in non–homologous end-joining DSB repair pathway, also sensitized TNBC cells to 213Bi-cetuximab. Furthermore, the small-molecule inhibitor of DNA-PKcs, NU7441, sensitized BRCA-1−competent TNBC cells to alpha-particle radiation. Immunofluorescent staining of γ-H2AX foci and comet assay confirmed that enhanced RBE is caused by impaired DSB repair. These data offer a novel strategy for enhancing conventional receptor-mediated targeting with an additional, potentially synergistic radiobiological targeting that could be applied to TNBC. Mol Cancer Ther; 12(10); 2043–54. ©2013 AACR.

Introduction

Radioimmunotherapy of established large solid tumors has not achieved clinical success (1, 2), partly because radiolabeled antibodies are not able to penetrate and deliver sufficient doses (typically less than 30 Gy) to elicit responses (3, 4). Accordingly, radioimmunotherapy of solid tumors is best implemented when the tumor size is small or, ideally, at a very early stage when the tumors are still microscopic clusters of malignant cells (5, 6). In contrast, in patients with non–Hodgkin lymphoma, equivalent or even lower tumor doses are able to elicit objective responses (7). Several factors contribute to this differential response; these include the unique biologic efficacy of anti-CD20 antibody, uniform and high expression of the CD20 antigen, and the easy accessibility of lymphoma cells to radiolabeled antibodies (8). More importantly, compared with solid tumors, non–Hodgkin lymphoma cells are exquisitely sensitive to radiation with typical D0 values in the range of 1.3 to 1.8 Gy without an appreciable shoulder on the survival curves (9). Genetic analysis has revealed that the increased radiosensitivity of non–Hodgkin lymphoma cells can be attributed to impaired DNA repair due to inactivation of ataxia-telangiectasia mutated kinase (ATM), p53 and DNA-PKcs genes (10).

Solid tumors are often associated with defects in the DNA damage response (DDR) pathways and loss-of-function in DDR. Germline mutations in these genes cause genomic instability and predispose patients to the development of cancer (11). For example, 5% to 10% of hereditary breast cancer (12), 10% to 15% of ovarian cancer (13), and 5% to 10% of pancreatic cancers (14) are caused by mutations in BRCA-1/2, key genes involved in DSB repair responses. Familial form of colorectal cancer (about 3%–4%), hereditary non–polyposis colorectal cancer (HNPPC), is associated with defective mutations in DNA mismatch

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repair genes, such as $MSH2$ and $MLH1$ (15). In glioblastoma, promoter methylation on $O^6$-methylguanine-DNA methyltransferase ($MGMT$) gene in base alkylation reversal repair was found in 40% of patients and is a reliable predictor for clinical outcome (16).

The differential DDR between normal tissue cells with intact DNA repair and repair-defective tumors cells can be used to further enhance the efficacy of highly potent alpha-particle radiotherapeutical therapy. Alpha particles travel a short distance (<100 μm) and deposit highly focused energy along their tracks (80 keV/μm) enabling a single track to generate DSB (17). Eukaryotic cells can repair DSBs through two main pathways, homologous recombination (HR) and non–homologous end joining (NHEJ; ref. 18). Here, we hypothesized that targeted alpha-particle radiation is more effective against solid tumors that contain somatic loss-of-function mutations in genes involved in HR and NHEJ pathways and examined the feasibility of radiobiological targeting that may complement or synergize with conventional receptor-mediated targeting. The $BRCA-1$–defective triple-negative breast cancer (TNBC) model was used to evaluate this approach.

Breast cancer is a heterogeneous disease where tumors display highly varied histopathologic features, gene expression profiles, response to therapy, and prognosis even though they arise from the same organ. Studies in genome-wide gene expression profiles have established four breast cancer types: luminal (type A and B), HER-2–positive, normal breast–like, and basal-like (19). Among the four types of breast cancer, basal-like breast cancers constitute approximately 15% of all breast cancers and are typically $ER^-$, $PR^-$, and HER-2$. TNBC is poorly differentiated and highly aggressive; patients with TNBC are almost twice as likely as other patients with breast cancer to develop distant metastasis and, therefore, suffer shorter survival (20). $BRCA-1$–defective tumors often belong to TNBC and share many clinical and pathologic features (21). Importantly, most TNBCs have high expression of EGFR receptor (EGFR; ref. 22) making it an ideal target for alpha radioimmunotherapy.

Our previous studies have shown that alpha-particle emitter, $^{212}$Bi-labeled, anti-rat HER-2/neu monoclonal antibody $7.16.4$ prolongs the survival of HER-2/neu transgenic mice (neuN) bearing syngeneic breast cancer bone metastasis and lung metastasis but did not lead to cure (23,24). In this study, we first evaluated the efficacy of alpha emitter $^{212}$Bi-labeled anti-EGFR monoclonal antibody ( cetuximab) in TNBC cells with mutated $BRCA-1$. Then, we tested inhibition of DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), a key enzyme in the NHEJ pathway, to sensitize $^{212}$Bi-cetuximab.

**Materials and Methods**

**Breast cancer cell lines and reagents**

Four human TNBC cell lines, MDA-MB-231, MDA-MB-436, MDA-MB-468, HCC1937 ($BRCA-1$–defective), and a control cell line MCF-7 ($ER^+, PR^+, HER-2^-$) were obtained from the American Type Culture Collection (ATCC). The cell lines were authenticated and tested by ATCC using short tandem repeat profiling and karyotyping tests. The cell lines were grown in RPMI-1640 media containing 10% FBS, 0.5% penicillin/streptomycin (Invitrogen), 1% l-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 0.02% gentamicin, and 0.2% insulin (Sigma) and maintained at 37°C in 5% CO$_2$.

Anti-human EGFR monoclonal antibody, cetuximab, was obtained from Eli Lilly & Co. under a Material Transfer Agreement. Anti-human phospho-Histone H2AX (Ser139) monoclonal antibody was purchased from Millipore. Alexa Fluor 488–conjugated goat anti-mouse antibody was purchased from Tocris Bioscience.

**Antibody radiolabeling with $^{212}$Bi and $^{111}$In**

Cetuximab was conjugated to SCN-CHX-A$^2$-DTPA as described earlier (23). $^{225}$Ac was provided by the Institute for Transuranium Elements (Karlsruhe, Germany; refs. 25, 26) and $^{212}$Bi was eluted from an $^{225}$Ac/$^{212}$Bi generator built-in house (27). Cetuximab conjugated to the chelate was incubated with $^{212}$Bi (10 mCi/mg) for 8 minutes in a reaction buffer (pH 4.5) containing 3 Mol/L ammonium acetate (Fisher Scientific) and 150 mg/ml l-ascorbic acid (Sigma) preheated to 37°C. The radiolabeling reaction was quenched with 1 μL of 100 mmol/L EDTA and radiolabeled cetuximab was purified by size-exclusion Microspin G-25 column (GE Healthcare). Cetuximab was also radiolabeled with $^{111}$In (PerkinElmer) according to a published procedure (28). The reaction efficiency and purity of the radioimmunoconjugates was determined with instant thin layer chromatography (ITLC) using silica gel impregnated paper (Agilent Technologies). The immunoreactivity of $^{212}$Bi-cetuximab was evaluated by incubating 5 ng of $^{212}$Bi-cetuximab with excess antigen binding sites (1 × 10$^7$ MDA-MB-231 cells) twice on ice for 30 minutes each time. Antibody immunoreactivity was calculated as the percentage of $^{212}$Bi-cetuximab bound to the cells.

**EGFR expression measured by flow cytometry and Scatchard analysis**

Expression of EGFR on the four TNBC cells was determined by fluorescein isothiocyanate (FITC)-labeled cetuximab using FACS Calibur (Becton Dickinson Biosciences). The EGFR expression level was also quantified by Scatchard analysis. Briefly, 1 × 10$^6$ cells were incubated with serial dilutions of $^{111}$In-cetuximab (0.01 to 4.0 μg/mL) for 45 minutes at 4°C. Cells were washed with PBS three times before both cell pellets and supernatants were collected and counted with a gamma counter (CompuGamma CS, Pharmacia). The equilibrium-binding curve of bound/free antibody versus bound antibody concentration was fitted and the number of binding sites, $B_{max}$, and antibody dissociation constant, $K_D$, were calculated.
siRNA knockdown of BRCA-1, DNA-PKcs, and RT-PCR

The siRNA knockdown studies are carried out mainly to account for impact of the receptor number and cell sizes on cell survival after DSBI induction by radiolabeled antibody. siRNAs targeting BRCA-1 and DNA-PKcs as well as nontargeting scrambled siRNA were obtained from Ambion. Twenty-four hours before transfection, TNBC cells were plated into 24-well plates at a density of 5 x 10⁴ cells per well in cell culture media without antibiotics. Cells were transfected with Lipofectamine (Invitrogen) in OPTI-MEM media (Invitrogen) at a siRNA concentration of 20 pmol/well (add 100 µL of siRNA/Lipofectamine 2000 complexes to 0.5 mL medium). Forty-eight hours after transfection, breast cancer cells were examined for knockdown of gene expression by real time reverse transcription (RT) PCR. Total cellular RNA was isolated with PerfectPure RNA cultured Cell Kit (5 Prime) according to the manufacturer’s instruction. For cDNA synthesis, 1 µg of RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Bioscience). BRCA-1 and DNA-PKcs mRNA level were measured by quantitative real-time RT-PCR using SYBR Green PCR Master Mix on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The primers for BRCA-1 are: forward GGCTATCCTCTCGAGTGACATTTTA, reverse GCTTTACAGGGTTATGGCA TGGT; for DNA-PKcs are: forward CCACCTACAGGACATAGCGACGAATGC, reverse CGTGCGGGTAGTTATGGCA ATAGCGACGAATGC. β-Actin mRNA level was used as control and the primers are: forward ACCAAGTCGGACAGCATGGAG, reverse GTGAGGATCTTCATGAGGTAGTC.

Specific kill of TNBC cells by ²¹³Bi-cetuximab

Survival curves of TNBC cells after treatment with ²¹³Bi-cetuximab were measured by colony formation assay. The four TNBC cells and MCF-7 cells were plated in 24-well plates. Two days after cell plating, they were incubated with ²¹³Bi-cetuximab (concentration from 1.0–8.0 µCi/mL) overnight in duplicates and transferred to Petri dishes for colony growth. All survival curves studies were repeated several times to confirm the findings. To investigate the effects of BRCA-1 and DNA-PKcs on cell kill by alpha radiation, TNBC cells were transfected with siRNAs targeting BRCA-1 and DNA-PKcs. Forty-eight hours after transfection, cells were treated with ²¹³Bi-cetuximab overnight and transferred to Petri dishes for colony growth. To compare the effect of small-molecule inhibitor of DNA-PKcs on cell kill by alpha radiation, TNBC cells were treated with NU7441 (1.0 µM) before ²¹³Bi-cetuximab treatment and cells were continuously incubated with NU7441 for 16 hours before they were transferred for colony formation (29).

Immunofluorescent staining of γ-H2AX foci as a biomarker for DSBs

DSBs induced by ²¹³Bi-cetuximab were measured by immunofluorescent staining of phosphorylated histone H2AX (γ-H2AX). TNBC and MCF-7 cells were treated with 8 µCi ²¹³Bi-cetuximab. At various time points (20 minutes, 1, 2, 4, 6, and 24 hours) after initiation of ²¹³Bi-cetuximab treatment, cells were washed with PBS and fixed with 2% paraformaldehyde. After incubation with 0.5% NP-40 to permeabilize cell membrane, γ-H2AX was detected by mouse anti-human phosphorylated histone H2AX (Ser139) and Alexa Fluor 488-conjugated goat anti-mouse IgG. Cell nuclei were stained with Hoechst 33342 (Invitrogen). The fluorescent images were examined and captured by a Nikon E800 fluorescence microscope equipped with NIS-Element software (Nikon). The number of γ-H2AX foci per cell was counted (>50 cells per data point).

For TNBC cells transfected with siRNA targeting either DNA-PKcs or BRCA-1, the number of γ-H2AX foci was counted at 1 and 24 hours after treatment with ²¹³Bi-cetuximab. In addition, to compare the effect of small-molecule DNA-PKcs inhibitor NU7441 on DSB repair after alpha radiation, TNBC cells were treated with NU7441 as described before with ²¹³Bi-cetuximab and the number of γ-H2AX foci was quantified at 1 and 24 hours after treatment.

Comet assay and cell-cycle analysis

MDA-MB-231 and HCC1937 cells were incubated with ²¹³Bi-cetuximab (8µCi/mL) in cell culture media containing 2% FBS at 37°C. Cells were harvested for comet assays under neutral condition at 1 or 24 hours after initiation of incubation using the Trevigen CometAssay kit (Trevigen). Briefly, cells were mixed with low melting point agarose and plated on microscope slides and allowed to gel. Cells were then lysed for 1 hour at 4°C followed by rinse in TBE buffer [10.8% (w/v) Tris base, 5.5% (w/v) boric acid, and 0.93% (w/v) EDTA]. After electrophoresis in TBE buffer for 30 minutes at 1 V/cm, slides were washed in water and dehydrated with ethanol, air dried overnight, and then treated with SYBR Green for DNA staining. Comets were imaged using a Zeiss Imager.Z1 fluorescent microscopy (Carl Zeiss AG) and analyzed using the software CometScore (Auto comet.com). The Olive Tail Moment (OTM) was calculated as the average of at least 70 comets.

For cell-cycle assay, at 1 or 24 hours after ²¹³Bi-cetuximab treatment, cells were harvested and fixed in 70% ethanol overnight. After PBS wash, cells were stained with 0.02 mg/mL propidium iodide (Sigma) in 0.1% (v/v) Triton X-100/PBS containing 0.2 mg RNase A (Sigma). Flow cytometry was conducted on a FACS-Calibur and data were analyzed with Cell Quest Pro (BD Bioscience).

Biodistribution of radiolabeled cetuximab in EGFR-positive TNBC

Nude mice (5 mice/group) were injected with 1 x 10⁶ MDA-MB-231 cells subcutaneously in the mammary fat pad region. Ten weeks after tumor inoculation (tumor size 0.5–1.0 cm in diameter), mice were injected intravenously with approximately 20 µCi ¹¹¹In-cetuximab and were...
then sacrificed at 1, 3, 6, 24, 48, and 72 hours postinjection. Major organs, including blood, heart, lungs, liver, spleen, kidney, stomach, intestine, muscle, femur, and tumor were collected and counted in the gamma counter. The uptake of $^{111}$In-cetuximab in the tumor and organs was decay corrected to the time of injection and calculated as %ID/g ± SD.

**Dosimetry**

Biodistribution data from $^{111}$In-cetuximab were converted to $^{213}$Bi-cetuximab biodistribution based on our previous findings that radioisotope properties of $^{111}$In is a good surrogate for $^{213}$Bi antibody kinetics (23). An exponential expression was fitted to the time activity data, and the cumulative activity in each normal organ was calculated by analytic integration of the fitted expression. Absorbed doses were calculated as described in the study conducted by Sgouros and colleagues (30). Absorbed dose is thus calculated as $D = (A \times \Delta t + \bar{A} \times \Delta t_0 - \Delta t) / M$, wherein $D$ is absorbed dose (no RBE adjustment), $\bar{A}$ is the total number of disintegrations in an organ or tumor, $M$ is the weight of the organ, and the mean energy emitted per nuclear transition for alpha particles, $\Delta t_0$ and electrons, $\Delta t_e$ is $1.33 \times 10^{-12}$ and $1.05 \times 10^{-13}$ Gy kg/Bq-s, respectively. For cell level dosimetry, the absorbed dose to the TNBC cells treated with nonbinding antibody was calculated as the absorbed dose to the irradiated media volume assuming all alpha-particle energies are absorbed locally: $D = \Delta E_{Bi213} \times l_1 / V_0$ where $\Delta E_{Bi213}$ is the mean alpha-particle and electron energy per decay (Gy-kg/Bq-s), $l_1$ is the time of treatment, $A_0$ is the initial activity, $\rho$ is the density of the cell (assuming water equivalent at 1.0 g/cm$^3$), $V_0$ is the volume of the treatment, and $\lambda$ is the decay constant for $^{213}$Bi. The absorbed dose was divided by two, as the cells are attached to the bottom of the tissue culture plates and are assumed to receive half of the radiation from above them. The absorbed dose to EGFR-positive TNBC cells targeted by $^{213}$Bi-cetuximab was calculated using a cellular S factor (31, 32) for $^{213}$Bi, the absorbed dose to the TNBC cells treated with alpha-particle emitter–labeled $^{213}$Bi-cetuximab. BRCA-1–defective HCC1937 cells are more sensitive to $^{213}$Bi-cetuximab compared with MDA-MB-231 and MDA-MB-436 cells. MDA-MB-468 cells are the most sensitive among the TNBC cells treated. D. DNA damage caused by $^{213}$Bi-cetuximab at 1 hour after treatment and DNA damage repair at 24 hours after treatment in MDA-MB-231 and HCC937 cells as assessed by neutral comet assay. Representative images showing head and tail fluorescent intensities of MDA-MB-231 and HCC1937 cells. E. Quantification of comet tail Olive moments at 1 and 24 hours after treatments.
(Nikon 80i) and analyzed with NIS-Element imaging analysis software (Nikon) after cells were stained with Hoechst 33342 (Invitrogen). Cell and nucleus radius of MDA-MB-231 cell were measured as 9.2 \pm 0.8 and 6.4 \pm 0.8 \mu m, respectively.

**Statistical analysis**

The statistical significance of differences between two groups was analyzed using MedCalc (MedCalc. Software). Differences with \( P \) values < 0.05 were considered statistically significant.

**Results**

**EGFR expression, radiolabeling, and antibody immunoreactivity**

Flow cytometry with cetuximab-FITC found EGFR expression on all four TNBC cell lines, but not on MCF-7 cells (Fig. 1A). MDA-MB-468 had the highest expression level. The results of Scatchard analysis using \(^{111}\)In-cetuximab are shown in tabular form in Table 1, with increased EGFR expression on MDA-MB-436, MDA-MB-231, HCC1937, and MDA-MB-468 cells. Also shown on Table 1 are the \( K_D \) values of radiolabeled cetuximab for these cell lines, which are similar to values obtained with unlabeled antibody. Reaction efficiency and purity after size exclusion purification of \(^{213}\)Bi-labeled cetuximab was 93.5\% (\( n = 7 \)) and 97.2\% \pm 0.4\% (\( n = 4 \)) as determined by ITLC. Both reaction efficiency and purity of \(^{111}\)In-labeled Cetuximab were routinely more than 98\%. The fraction of \(^{213}\)Bi-cetuximab that is able to bind MDA-MB-231 cells in the immunoreactivity assay was 89.7\%.

**In vitro cytotoxicity of \(^{213}\)Bi-cetuximab to TNBC cells and immunofluorescent staining of \( \gamma \)-H2AX**

\(^{213}\)Bi-cetuximab kills EGFR-expressing TNBC cells effectively (Fig. 1C). The activity concentrations that can kill 50\% (ED\(_{50}\)) of MDA-MB-231 and MDA-MB-436 cells are 3.2 and 3.5 \( \mu Ci/\mu L \), compared with 7.8 \( \mu Ci/\mu L \) in EGFR-negative MCF-7 cells. Noticeably, the radiosensitivity of BRCA-1-defective HCC1937 cells to \(^{213}\)Bi-cetuximab is significantly enhanced with an ED\(_{50}\) of 0.63 \( \mu Ci/\mu L \). MDA-MB-468 cells are the most sensitive to \(^{213}\)Bi-cetuximab treatment with an ED\(_{50}\) of 0.50 \( \mu Ci/\mu L \). Neutral Comet assay showed that DSBs are repaired in MDA-MB-231 cells but not in BRCA-1-defective HCC1937 cells at 24 hours after treatment (Fig. 1D). Two-way ANOVA test showed significant decrease in Olive moment in MDA-MB-231 cells from 1 to 24 hours (\( P < 0.0001 \)) but not in HCC1937 cells (\( P = 0.88 \)). The difference in Olive moment between MDA-MB-231 and HCC1937 cells is also statistically significant at 1 hour (\( P = 0.02 \)) and 24 hour (\( P < 0.001 \); Fig. 1E). Pretreating TNBC cells with the DNA-PKcs inhibitor NU7441 (1.0 \( \mu mol/L \)) further improved the efficacy of \(^{213}\)Bi-cetuximab for TNBC cells (Fig. 2A–C), with ED\(_{50}\) of 0.61, 0.77, 0.58 \( \mu Ci/\mu L \) for MDA-MB-231, MDA-MB-436, and HCC1937 cells, respectively.

Immunofluorescent staining of \( \gamma \)-H2AX at 1 and 24 hours after treatment with \(^{213}\)Bi-cetuximab is shown

### Table 1. Dissociation constant (\( K_D \)) and receptor density for the cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( K_D ) (nmol/L)</th>
<th>EGFR/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>0.8</td>
<td>2.8 \times 10^5</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>0.2</td>
<td>0.8 \times 10^5</td>
</tr>
<tr>
<td>HCC1937</td>
<td>0.8</td>
<td>4.5 \times 10^5</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>2.0</td>
<td>2.7 \times 10^6</td>
</tr>
</tbody>
</table>

Figure 2. DNA-PKcs inhibitor NU7441 enhances cytotoxicity of \(^{213}\)Bi-cetuximab in TNBC cells. Cell survival curves of MDA-MB-231 (A), MDA-MB-436 (B), and HCC1937 (C) after treatment by \(^{213}\)Bi-cetuximab or \(^{213}\)Bi-cetuximab plus NU7441.
in Fig. 3. Unlike random γ-H2AX foci that are usually formed after the cells are irradiated with gamma rays (data not shown), the γ-H2AX foci typically formed noticeable straight-line tracks within cell nuclei after treatment by 213Bi-cetuximab, indicating DSBs caused by alpha-particle tracks (e.g., Fig. 3A). The time course of γ-H2AX foci formation after alpha radiation treatment was evaluated at 20 minutes, 1, 2, 4, 6, and 24 hours after

Two-way ANOVA test showed that there was a statistically significant decrease (\(^{\text{a}}\)) of DSBs foci in MDA-MB-231 \((P < 0.001)\), MDA-MB-436 \((P < 0.001)\), and MCF-7 \((P < 0.001)\) cells from 1 to 24 hours after treatment with 213Bi-cetuximab alone, whereas such decrease was not observed in HCC1937 \((P = 0.13)\) and MDA-MB-468 \((P = 0.63)\) cells. NU7441 treatment significantly inhibited the decrease of γ-H2AX foci from 1 to 24 hours in MDA-MB-231 \((P = 0.43)\) and MDA-MB-436 \((P = 0.68)\) cells, but did not affect HCC1937 and MDA-MB-468 cells.

Figure 3. A, representative images showing immunofluorescently stained γ-H2AX foci at 1 and 24 hours after treatment with 213Bi-cetuximab or 213Bi-cetuximab plus NU7441 in MCF-7 and the four TNBC cell lines studied. B, quantification of number of γ-H2AX foci at 1 and 24 hours after treatment by 213Bi-cetuximab or 213Bi-cetuximab plus NU7441.
initiation of treatment. The number of γ-H2AX foci peaked at 1 hour and decreased gradually thereafter (data not shown). The reduction in γ-H2AX foci from 1 to 24 hours was therefore used as a measure of DSB repair capacity after 213Bi-cetuximab treatment. In MCF-7, MDA-MB-231, and MDA-MB-436 cells, the number of γ-H2AX foci per cell all decreased significantly from 15.5 ± 4.8 (1 hour) to 5.2 ± 2.4 (24 hours), 18.7 ± 4.7 (1 hour) to 10.0 ± 5.1 (24 hours) and 16.0 ± 3.8 (1 hour) to 4.9 ± 2.3 (24 hours), respectively. In contrast, in BRCA-1–defective HCC1937 cells and MDA-MB-468 cells, there was no significant change in the number of γ-H2AX foci at 1 hour (22.5 ± 6.6 and 31.3 ± 8.0) and 24 hours (20.3 ± 6.5 and 33.8 ± 10.3; Fig. 3B), strongly suggesting compromised, if not complete lack of DSB repair in these two cell lines.

Treatment of MCF-7, MDA-MB-231, and MDA-MB-436 cells by the DNA-PKcs inhibitor NU7441 and 213Bi-cetuximab abolished the significant decrease of γ-H2AX foci from 1 hour to the 24-hour period, likely a result of DSB repair inhibition (Fig. 3). For the two cell lines already sensitive to 213Bi-cetuximab, HCC1937, and MDA-MB-468, adding NU7441 to 213Bi-cetuximab did not affect the number of γ-H2AX foci from 1 hour to 24 hours. (Fig. 3B).

**siRNA knockdown of BRCA-1 or DNA-PKcs enhanced radiosensitivity of TNBC cells**

Gene expression of either BRCA-1 or DNA-PKcs was downregulated in the four TNBC cells, MCF-7, MDA-MB-231, MDA-MB-436, and HCC1937 after transfection of siRNA targeting either BRCA-1 or DNA-PKcs as confirmed by real-time RT-PCR (Fig. 4A). Knockdown of either DNA-PKcs or BRCA-1 improved the radiosensitivity of TNBC cells to 213Bi-cetuximab. In MCF-7 cells, the ED50 was reduced from 6.5 μCi/mL from control siRNA to 3.4 and 1.9 μCi/mL in cells knocked down with BRCA-1 or DNA-PKcs siRNA, respectively (Fig. 4B). In MDA-MB-231 cells treated with 213Bi-cetuximab, the ED50 improved significantly from 1.8 μCi/mL in cells transfected by negative control scrambled siRNA to 0.56 μCi/mL in cells transfected by siRNA targeting BRCA-1 and to 0.78 μCi/mL in cells transfected by siRNA targeting DNA-PKcs (Fig. 4C). Likewise, siRNA knockdown of
BRCA-1 in MDA-MB-436 cells enhanced its radiosensitivity to 213Bi-cetuximab with an ED50 change from 5.6 to 1.4 mCi/mL (Fig. 4D). In the BRCA-1–mutated HCC1937 cells, knockdown of DNA-PKcs slightly reduced the ED50 from 0.60 to 0.53 mCi/mL, consistent with that observed in the parental cells treated with DNA-PKcs inhibitor NU7441 and 213Bi-cetuximab.

Immunofluorescent staining of γ-H2AX foci confirmed the inhibition of DSB repair in MDA-MB-231 cells transfected with siRNA targeting DNA-PKcs or BRCA-1 (Fig. 5). Cell-cycle analysis showed that both TNBC cells, MDA-MB-231 and HCC1937, were arrested in G2–M phase after treatment with 213Bi-cetuximab. Inhibiting DNA-PKcs in MDA-MB-231 cells further enhanced the G2–M arrest after 213Bi-cetuximab treatment (Fig. 6).

**Biodistribution of 111In-cetuximab EGFR-positive TNBC tumors**

The biodistribution of 111In-cetuximab in nude mice bearing a subcutaneous MDA-MB-231 tumor is shown in Fig. 7. 111In-cetuximab was cleared slowly from blood with an effective half-life of 40.8 hours and accumulated in the MDA-MB-231 tumors; %ID/g in the tumors increased from 2.9 ± 0.5% at 1 hour to its peak of 40.6 ± 8.9% at 48 hours after injection. Tumor uptake persisted at 72 hours at 38.0 ± 7.9%ID/g. Like most antibodies, liver is the normal organ after blood with the highest uptake of 21.1 ± 9.5%ID/g at 1 hour. Consistent with the finding that cetuximab does not cross react with mouse EGFR, no significant uptake in other normal organs was observed. Finally, 111In-cetuximab cleared slowly from major organs with half-life ranging from 40.8 to 173.3 hours.

**Dosimetry**

Absorbed doses from 120 μCi 213Bi-cetuximab to normal organs are shown in Table 2. The blood-absorbed dose from alpha particles is 5.23 Gy and liver has the highest normal organ absorbed dose with 4.28 and 0.22 Gy from alpha- and beta-particle emissions, respectively. These results are consistent with our observations in other dosimetric studies of alpha-particle emitter–labeled intact antibodies (23). The RBE for MDA-MB-231 cells based on calculations using nonspecific and specific antibody showed that nonspecific 213Bi-rituximab gives an RBE of 3.8 using 37% cell survival as biologic endpoint and 137Cs gamma rays as reference, whereas a similar RBE of 3.7 was found for 213Bi-cetuximab. When DSB repair genes DNA-PKcs and BRCA-1 are knocked down by siRNA, the RBES increased significantly to 8.6 and 15.6, respectively (Table 3).

**Discussion**

In this study, we showed that targeting alpha-particle radiation (213Bi) with anti-EGFR antibody cetuximab is highly effective in killing BRCA-1–defective TNBC cells where DSB repair is compromised. We further showed that inhibiting DSB repair pathways, either the HR pathway via BRCA-1 or NHEJ pathway via DNA-PKcs, preferentially sensitizes breast cancer cells with intact DSB repair function to alpha particles. Thus, similar to the concept of prescreening HER-2–positive breast cancer before treatment with trastuzumab, one could envision, in the era of cancer genomics, prescreening breast tumors with loss-of-function mutations in DSB repair genes would increase the efficacy of targeted alpha radioimmunotherapy.

Targeted alpha-particle emitters are currently under clinical investigations for leukemia (33), ovarian cancer (34), malignant melanoma (35), and glioblastoma (36) because of their high potency. The recent clinical success of Radium-223 (Alpharadin) in castration-resistant prostate cancer with bone metastases (37) supports further development of alpha-particle emitters and highlights the importance of a unique biologic mechanism in the highly specific accumulation of targeted radioisotopes. EGFR is highly expressed on a variety of epithelial cancers, 99mTc, 64Cu, 89Y, 89Zr anti-EGFR antibodies (cetuximab, panitumumab) have been extensively investigated as imaging agents in colorectal cancer, head and neck squamous cell
carcinoma (HNSCC), and lung cancer (38–40). As radiotherapeutics, anti-EGFR antibodies have been labeled with both beta emitters $^{90}$Y and $^{177}$Lu, and alpha emitter, $^{213}$Bi, and were found effective in several preclinical models of HNSCC, bladder, and colorectal cancer (41, 42). Few clinical trials have been launched partially due to the prevalent expression of EGFR on normal epithelial cells that lead to skin and gastrointestinal toxicity observed in cetuximab trials (43). A clinical study of intravenous $^{125}$I-labeled anti-EGFR antibody 425 with radiotherapy in patients with glioblastoma found no survival benefit compared with radiotherapy alone (44). Targeting...
produced DSBs by alpha-particle radiation, determines compromised repair of DSB lesions, rather than initially heterozygous DNA repair genes.

Tumors that have homologous loss-of-function defects at DSB repair pathway genes often arise from the normal tissues that are heterozygous and preserve DNA repair function. Nieuwenhuis and colleagues measured the rejoining of DNA breaks in normal fibroblast and lymphocytes cells with heterozygous BRCA-1/BRCA-2 mutations after X-ray radiation and found no defect in their ability to repair DNA breaks (45). In external beam radiotherapy, multiple studies were not able to link pathogenic heterozygous mutations in DDR genes such as ATM, BRCA-1, BRCA-2, and RAD50 to normal tissue radiosensitivity (46). These findings suggest that high LET alpha-particle radiation could be targeted to treat tumors with homozygous loss-of-function in DNA repair while sparing normal tissues with heterozygous DNA repair genes.

In cells deficient in NHEJ and HR, we found that compromised repair of DSB lesions, rather than initially produced DSBs by alpha-particle radiation, determines cell survival. This is consistent with heavy ion-beam studies. Irradiation of glioblastoma cells MOSJ9 deficient in DNA-PKcs with heavy ion beam found no reduction of γ-H2AX foci after 21 hours (47). In Ku80-deficient (NHEJ-deficient) Chinese hamster ovary (CHO) cells treated with alpha particles, significantly more γ-H2AX foci were present (58.4%–69.5%) 2 hours after radiation compared with normal CHO cells (36.5%–42.8%; ref. 48).

Cell survival curves showed that 213Bi-cetuximab is most efficacious in BRCA-1–defective HCC1937 cells and BRCA-1 knockdown MDA-MB-231 cells. In patients with TNBC that are not BRCA-1–defective, agents that lead to BRCA-1 inhibition combined with targeted alpha radiotherapy would be useful. Until recently, only a handful of BRCA-1 inhibitors, however, are under early stage evaluation primarily due to low specificity, as many of these protein–protein interactions, unlike enzyme active sites, also have large, multiple contact points and poorly defined topology (49). Many other DSB repair inhibitors are currently available for combination with targeted alpha therapy for TNBC including the DNA-PKcs inhibitor used in this study (29), ATM inhibitor and Rad51 inhibitors (50).

PARP-1 inhibitors are also under clinical investigation for patients with breast and ovarian cancer with BRCA-1 mutations under the synthetic lethality hypothesis (21). A phase II trial showed that PARP-1 inhibitor iniparib in combination with gemcitabine and carboplatin significantly improved progression-free survival and overall survival in patients with TNBC (51). The subsequent phase III trial, however, failed to confirm its survival benefit (52). As alpha-particle emitter radiolabeled antibody is targeting the same DNA repair deficiency as PARP-1 inhibitors, these clinical results suggested the potential of alpha radioimmunotherapy in patients with TNBC, but at the same time warrant more careful preclinical studies of, for example, the correlation of EGFR expression level, mutation rate with their deficiency in BRCA-1, and response to alpha radiation.

An interesting observation in this study is that MDA-MB-468 cells were exquisitely sensitive to the treatment of 213Bi-cetuximab, even more so than the BRCA-1–defective HCC1937 cells. One of the main reasons probably is its high expression of EGFR receptors (almost 10 times higher) compared with other TNBC cells lines, potentially delivering significantly higher alpha doses to the tumor cells. In addition, the cell survival curve with gamma rays showed that the MDA-MB-468 cells are sensitive to radiation and exhibit a diminished shoulder region, consistent with its radiosensitivity to UV radiation attributed to the deficient Rb gene and deficiency in recovery of mRNA synthesis (53). The possible dysregulation of DSB repair in these cells remains to be studied.

We have conducted initial in vivo evaluation of 213Bi-cetuximab in a mouse model of TNBC. Because of poor tumorigenesis of the HCC1937 cells, however, we cannot interpret the data reliably. We are currently developing a more robust mouse model to evaluate the efficacy of alpha radioimmunotherapy in tumors with homozygous

### Table 2. Tissue-absorbed doses

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α-Particle</th>
<th>Electron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5.23</td>
<td>0.27</td>
</tr>
<tr>
<td>Heart</td>
<td>1.34</td>
<td>0.07</td>
</tr>
<tr>
<td>Lung</td>
<td>2.14</td>
<td>0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>4.28</td>
<td>0.22</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.21</td>
<td>0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.15</td>
<td>0.06</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.37</td>
<td>0.02</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.66</td>
<td>0.03</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.19</td>
<td>0.01</td>
</tr>
<tr>
<td>Femur</td>
<td>0.40</td>
<td>0.02</td>
</tr>
</tbody>
</table>

### Table 3. Radiosensitivity (D0) and RBE of the MDA-MB-231 cell line under different exposure and DNA repair pathway inhibition conditions

<table>
<thead>
<tr>
<th>Agent, manipulation</th>
<th>D0 (Gy)</th>
<th>RBE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>213Bi-Rituximab (irrelevant Ab)</td>
<td>0.84</td>
<td>3.8</td>
</tr>
<tr>
<td>213Bi-Cetuximab</td>
<td>0.87</td>
<td>3.7</td>
</tr>
<tr>
<td>213Bi-Cetuximab, siRNA scrambled control</td>
<td>0.69</td>
<td>4.7</td>
</tr>
<tr>
<td>213Bi-Cetuximab, siRNA DNA-PKcs-/DNA-PKcs-</td>
<td>0.37</td>
<td>8.6</td>
</tr>
<tr>
<td>213Bi-Cetuximab, siRNA BRCA1-/BRCA1-</td>
<td>0.21</td>
<td>15.6</td>
</tr>
</tbody>
</table>

*RBE is reported using 37% cell survival as the biologic endpoint and Cs-137 gamma rays as the reference radiation.
loss-of-function and toxicity in normal tissues with heterozygous at DSB repair genes.

In conclusion, we have shown that TNBC can be targeted by alpha-particle-emitter–labeled anti-EGFR antibody. The defective DSB repair machinery in a subset of TNBC can be uniquely suitable for alpha radiation treatment. New approaches combining inhibitors of DSB repair pathways and internal alpha-particle emitters targeting remain to be explored. Finally, the safety of such an approach in patients with heterozygous expression of DSB repair genes needs to be evaluated.

Disclosure of Potential Conflicts of Interest

G. Sgouros has ownership interest in a patent licensed to Actinium Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: H. Song, G. Sgouros
Development of methodology: H. Song, M. Hedayati

References

Targeting Aberrant DNA Double-Strand Break Repair in Triple-Negative Breast Cancer with Alpha-Particle Emitter Radiolabeled Anti-EGFR Antibody

Hong Song, Mohammad Hedayati, Robert F. Hobbs, et al.


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