B7-H3-targeted ^{212}Pb radioimmunotherapy of ovarian cancer in preclinical models

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ABSTRACT

Introduction. Novel therapies that effectively kill both differentiated cancer cells and cancer initiating cells (CICs), which are implicated in causing chemotherapy-resistance and disease recurrence, are needed to reduce the morbidity and mortality of ovarian cancer. These studies used monoclonal antibody (mAb) 376.96, which recognizes a B7-H3 epitope expressed on ovarian cancer cells and CICs, as a carrier molecule for targeted α -particle radioimmunotherapy (RIT) in preclinical models of human ovarian cancer.

Methods. mAb 376.96 was conjugated to the chelate 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1,4,7,10-tetra-(2-carbamoylmethyl)-cyclododecane (TCMC) and radiolabeled with ^{212}Pb , a source of α -particles. *In vitro* Scatchard assays determined the specific binding of ^{212}Pb -376.96 to adherent differentiated or non-adherent CIC-enriched ES-2 and A2780cp20 ovarian cancer cells. Adherent ovarian cancer cells and non-adherent CIC-enriched tumorspheres treated *in vitro* with ^{212}Pb -376.96 or the irrelevant isotype-matched ^{212}Pb -F3-C25 were assessed for clonogenic survival. Mice bearing *i.p.* ES-2 or A2780cp20 xenografts were injected *i.p.* with 0.17-0.70 MBq ^{212}Pb -376.96 or ^{212}Pb -F3-C25 and were used for *in vivo* imaging, *ex vivo* biodistribution, and therapeutic survival studies.

Results. ^{212}Pb -376.96 was obtained in high yield and purity (>98%); K_d values ranged from 10.6-26.6 nM for ovarian cancer cells, with 10^4 - 10^5 binding sites/cell. ^{212}Pb -376.96 inhibited the clonogenic survival of ovarian cancer cells up to 40 times more effectively than isotype-matched control ^{212}Pb -F3-C25; combining ^{212}Pb -376.96 with carboplatin significantly decreased clonogenic survival compared to either agent alone. *In vivo* imaging and biodistribution analysis 24 h after *i.p.* injection of ^{212}Pb -376.96 showed high peritoneal retention and tumor tissue accumulation (28.7% ID/g in ES-2 ascites, 73.1% ID/g in A2780cp20 tumors); normal tissues

showed lower and comparable uptake for ^{212}Pb -376.96 and ^{212}Pb -F3-C25. Tumor-bearing mice treated with ^{212}Pb -376.96 alone or combined with carboplatin survived 2-3 times longer than mice treated with ^{212}Pb -F3-C25 or non-treated controls.

Conclusion. These results support additional RIT studies with ^{212}Pb -376.96 for future evaluation in patients with ovarian cancer.

INTRODUCTION

Ovarian cancer is the most lethal among all gynecological cancers. Although many patients initially respond to standard treatment (surgery followed by platinum-based chemotherapy), the majority succumb to chemotherapy-resistant and metastatic disease, contributing to an overall 5-year survival of 46% in the United States [1, 2]. Additional therapies that are effective against recurrent and chemotherapy-resistant ovarian cancer are needed to improve patient outcomes.

Growing evidence indicates that ovarian cancer is initiated and propagated by cancer initiating cells (CICs) [3, 4], a subpopulation of tumor cells that are capable of unlimited self-renewal, resistance to standard chemotherapy, and high tumorigenicity in immunodeficient mice [5, 6]. An effective therapy regimen to prevent metastatic and recurrent ovarian cancer must eliminate both differentiated cancer cells and CICs and must counteract the multiple mechanisms cancer cells utilize to avoid destruction [7-10].

Clinical studies have investigated intraperitoneal (*i.p.*) targeted radioimmunotherapy (RIT) with β -particles to enhance therapeutic efficacy against ovarian cancer [11-13]. These applications have failed to produce significant clinical responses in patients, possibly due to inadequate absorbed radiation doses from low linear energy transfer (LET) β -particles (0.1-1

keV/ μm) [14, 15] and survival of ovarian CICs at doses that do not cause normal organ toxicities. Replacement of β -particle emitters with α -particle emitting radionuclides for targeted RIT offers greater potential for effective cell death because of the short path length (50-80 μm), high LET (100 keV/ μm), and high relative biological effectiveness of α -particles [14-16]. α -particles are widely accepted to cause cell death regardless of the cell's oxygen levels or sensitivity to chemotherapy or alternative radiotherapy regimens (external beam or β -particle RIT) [14, 17]. The efficacy of targeted RIT using the monoclonal antibody (mAb) trastuzumab conjugated with ^{212}Pb ($t_{1/2} = 10.64$ h), which decays to the α -particle emitter ^{212}Bi ($t_{1/2} = 60.5$ min), has been demonstrated in preclinical models of HER2⁺ human cancer [17]. The success of these studies and lack of toxicity in non-human primates [18] led to a phase I clinical trial with ^{212}Pb -trastuzumab in patients with HER2⁺ malignancies (ovarian and colon cancer) mainly confined to the peritoneal cavity [19, 20]. ^{211}At -MX35 F(ab')₂ is another α -particle RIT agent used in clinical trials with ovarian cancer patients [21]. The clinical studies with ^{212}Pb and ^{211}At have shown α -particle radioimmunoconjugates (RICs) are well tolerated after *i.p.* administration in ovarian cancer patients. In this report, mAb 376.96, which binds to an extracellular epitope of the co-stimulatory protein B7-H3 (CD276), was used as a carrier for ^{212}Pb to prove the concept of *i.p.* α -particle RIT targeted to this epitope in preclinical models of human ovarian cancer. B7-H3 is an attractive target for RIT of ovarian cancer due to the expression of this protein in 93% of ovarian cancer lesions [22].

MATERIALS AND METHODS

Reagents and instrumentation

All reagents were prepared from commercially available materials (Thermo Fisher, Sigma) unless specified otherwise. ^{212}Pb was isolated as previously described [23] from a $^{224}\text{Ra}/^{212}\text{Pb}$ generator obtained from Oak Ridge National Laboratory (Oak Ridge, TN). Murine mAb 376.96 [24] and isotype-matched control murine mAb F3-C25 that binds to murine anti-HLA Class II mAbs [25] were produced and characterized as previously described. A calibrated high-purity germanium (HPGe) detector (model GMX10P4-70; Ortec, Oak Ridge, TN) operated at -4000 V housed in a lead shield (Ortec) was used to determine the radionuclidic purity and radioactivity of ^{212}Pb for all radiolabeling procedures. Spectra were processed using Gamma Vision-32 software (version 6.09; Ortec). Radioactivity measurements of samples from the *in vitro* and *in vivo* experiments were performed on calibrated Cobra II (Packard, Meriden, CT) or Wizard² (Perkin Elmer, Shelton, CT) gamma counters using an energy window centered on the main gamma peak from ^{212}Pb (238.6 keV, 43.6%) after cross-calibrating the instruments with the HPGe detector. Unless stated otherwise, radioactivity analyses were corrected for radioactive decay.

Radiolabeling studies

mAbs 376.96 and F3-C25 were conjugated with the bifunctional chelator 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1,4,7,10-tetra-(2-carbamoylmethyl)-cyclododecane (TCMC; Macrocyclics, Plano, TX) following previously described procedures [26] to generate TCMC-mAb 376.96 and TCMC-mAb F3-C25. The average chelate/mAb ratios were determined by a spectrophotometric assay [27]. Radiolabeling and purification of the ^{212}Pb -TCMC-mAb RICs (^{212}Pb -376.96 or ^{212}Pb -F3-C25) were performed as previously described [23] using 22-44 GBq of ^{212}Pb per μmol TCMC-mAb conjugate (0.15-0.3 MBq/ μg). Immediately after collecting

the purified RICs, an aliquot was removed for additional characterization and 5 μ L 0.1 mol/L EDTA and 50 μ L 30% human serum albumin (Sigma-Aldrich, St. Louis, MO) were added to the RICs. The protein content of the removed aliquot was determined by Lowry analysis [28]. The radiochemical conversions and purities of the crude preparations and isolated RICs were determined as previously described [23].

Human ovarian cancer cell lines

ES-2 cells obtained from the American Type Culture Collection (Manassas, VA) and carboplatin-resistant A2780cp20 cells obtained as frozen stocks from Dr. Anil Sood (M.D. Anderson Cancer Center, Houston, TX) were used as previously described [29]. Cells grown as monolayers (“Adherent” conditions) were cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) in tissue culture treated plates (Corning Costar, Corning, NY). Cells grown as non-adherent tumorspheres (“CIC” conditions) were cultured with serum-free X-VIVO 10 medium (Lonza, Walkersville, MD) supplemented with 5 μ g/mL bovine insulin and 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ) in ultra low attachment plates (Corning Costar). Cell culture and *in vitro* experiments were performed at 37 °C in a 5% CO₂ humidified atmosphere unless specified otherwise.

***In vitro* binding assays**

Adherent cell binding assays: Binding assays with ²¹²Pb-376.96 and adherent ES-2 or A2780cp20 cells were performed as previously described for an alternative RIC labeled with ^{99m}Tc to calculate the binding affinity (K_d), binding sites per cell, and internalized fraction [30].

CIC binding assays: ES-2 or A2780cp20 cells were cultured for three days at 100,000 cells/mL

under CIC conditions to form tumorspheres. Cells and tumorspheres were collected, dissociated into a single-cell suspension with Accutase (Innovative Cell Technologies, San Diego, CA), rinsed in PBS, and counted. Cells were seeded into microcentrifuge tubes at 500,000 live cells/tube and incubated with serial dilutions of ^{212}Pb -376.96 with or without unlabeled mAb 376.96 as a blocking agent to determine binding specificity as in the Adherent assay [30], with centrifugation to pellet cells after each step. The K_d , binding sites per cell, and internalized fraction were calculated as above.

***In vitro* clonogenic survival assays**

Adherent clonogenic assays: ES-2 or A2780cp20 cells were plated at 100,000 cells/well in 24-well plates (Corning Costar) two days before treatment with RICs, defined as day 0 of the study. One day after plating (day -1), designated wells were treated with vehicle (PBS) or carboplatin (5 $\mu\text{mol/L}$) for one day. On day 0, cells were rinsed with PBS and incubated with serial dilutions of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 in 0.4 mL assay medium (DMEM pH 7.4 with 30 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol sodium pyruvate, 1% bovine serum albumin) for 2 h at 37 °C with gentle swirling. Cells were rinsed twice with PBS and incubated in fresh medium containing vehicle or carboplatin as on day -1. Two days later (day +2), cells were trypsinized, counted, serially diluted in normal (Adherent) medium, and plated at 100 live cells/well in six replicate wells of a tissue culture treated 6-well plate (Corning Costar) for clonogenic survival. Following 7 (day +9) and 10 (day +12) days for A2780cp20 and ES-2 cells, respectively, the percent clonogenic survival relative to vehicle-treated controls was determined as previously described [31].

CIC clonogenic assays: ES-2 or A2780cp20 cells were plated at 100,000 cells/well in 24-well ultra low attachment plates in CIC conditions to form tumorspheres three days before treatment with RICs, defined as day 0 of the study. Two days after plating (day -1), designated wells were treated with vehicle (PBS) or carboplatin (5 $\mu\text{mol/L}$) for one day. On day 0, intact tumorspheres were collected, rinsed with PBS, and incubated with serial dilutions of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 in 0.4 mL assay medium for 2 h at 37 °C with gentle swirling to keep tumorspheres in suspension. Tumorspheres were rinsed twice with PBS and incubated in new ultra low attachment 24-well plates with fresh CIC medium containing vehicle or carboplatin as on day -1. Two days later (day +2), tumorspheres were collected and dissociated into single cells with Accutase, counted, and serially diluted in normal (Adherent) medium. Cells were plated and analyzed for clonogenic survival as in the Adherent clonogenic assays.

Animal subjects and husbandry

Animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and were performed in compliance with guidelines from the Public Health Service Policy and Animal Welfare Act of the United States. For imaging studies, ES-2 *i.p.* xenografts were generated in female C.B-17 SCID mice (6-8 weeks old; Charles River, Wilmington, MA). For biodistribution and therapy studies, ES-2 and A2780cp20 *i.p.* xenografts were generated in female athymic nude mice (4-6 weeks old) from Harlan (Indianapolis, IN) and Charles River, respectively. Mice were given Nutra-Gel Diet (Bio-Serv, Flemington, NJ) in addition to standard chow for two weeks after administering the RICs. Mice were weighed twice per week and euthanized if >10% weight loss occurred or when disease progression (abdominal swelling) became apparent.

***In vivo* imaging and biodistribution studies**

For imaging studies, SCID mice were implanted *i.p.* with 600,000 ES-2 cells. Fifteen days later, mice were injected *i.p.* with ^{212}Pb -376.96 (0.84 MBq, $\sim 7\ \mu\text{g}$) and imaged for 15 min on a dual-head planar gamma camera (X-SPECT, Gamma Medica-Ideas, Northridge, CA) with a 1 mm medium-energy pinhole collimator 20-22 h post injection of the RIC. For biodistribution studies, groups of athymic nude mice were implanted *i.p.* with 600,000 ES-2 cells or 2 million A2780cp20 cells. Fourteen days post-implantation of ES-2 cells or sixteen days post-implantation of A2780cp20 cells, groups of mice were injected *i.p.* with $\sim 0.74\ \text{MBq}$ ($\sim 4\ \mu\text{g}$) ^{212}Pb -376.96 (n=5 mice/group for ES-2 and A2780cp20 models) or ^{212}Pb -F3-C25 (n=4 mice/group for ES-2, n=5 mice/group for A2780cp20) in 0.2 mL PBS. Mice were euthanized $\sim 24\ \text{h}$ post injection and selected tissues were removed and counted in a gamma counter. Percent uptake of the injected dose per gram (% ID/g) was calculated by comparing the tissue activity to solutions with known ^{212}Pb activity.

***In vivo* therapy studies**

Groups of athymic nude mice (n=10/group) were implanted *i.p.* with 600,000 ES-2 cells or 2 million A2780cp20 cells. Designated groups were given a single *i.p.* dose of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 (0.17-0.70 MBq) four days after implantation of ES-2 cells (day 0 for ES-2 groups) or ten days after implantation of A2780cp20 cells (day 0 for A2780cp20 groups). Designated groups of mice bearing A2780cp20 xenografts were given carboplatin (50 mg/kg *i.p.*) once per week starting one day before treatment with ^{212}Pb -376.96; weekly carboplatin

treatment was continued until death or euthanasia. Mouse survival was recorded as the elapsed time (after day 0) until death or euthanasia due to advanced disease.

Statistical analyses

Data were analyzed using Microsoft Excel or GraphPad Prism (Version 5.02). When comparing multiple groups, one-way or two-way ANOVA tests were performed, followed by Bonferroni tests to compare differences between individual groups. Differences in median survival were determined by log-rank (Mantel-Cox) tests. All p values correspond to two-tailed tests.

RESULTS

Characterization of ^{212}Pb -376.96 and ^{212}Pb -F3-C25

Daily elution of the $^{224}\text{Ra}/^{212}\text{Pb}$ generator yielded 90-95% of the theoretical ^{212}Pb radioactivity in high radionuclidic purity; breakthrough of ^{224}Ra was not detected until after 9-10 days of continual use, after which breakthrough gradually increased (~0.5% per day). mAb 376.96 containing 12.6 TCMC chelates per mAb was radiolabeled with ^{212}Pb to produce ^{212}Pb -376.96 in high yield (98.5%, n=6) as indicated by ITLC analysis. After purification, ^{212}Pb -376.96 was obtained with an average radiochemical purity >98% and an average decay-corrected recovery of 71.5% (n=6). The control mAb F3-C25 containing 11.2 TCMC chelates per mAb molecule was also radiolabeled with ^{212}Pb to produce ^{212}Pb -F3-C25 with an average radiochemical purity >97% and an average decay-corrected recovery of 74.6% (n=4) following purification. Specific activities of 24.8-30 GBq/ μmol (0.17-0.2 MBq/ μg) for the RICs were used in the *in vitro* and *in vivo* studies described below.

²¹²Pb-376.96 binds to differentiated ovarian cancer cells and ovarian CICs

In vitro binding assays with ES-2 and A2780cp20 human ovarian cancer cell lines determined the affinity and internalization of ²¹²Pb-376.96 with adherent cells and with dissociated cells from tumorspheres cultured in CIC conditions. Both cell lines demonstrated recognized characteristics of CIC proliferation [32-34] due to their formation of large (120-200 μm), dense tumorspheres with high viability (>95%) in the CIC conditions used here. The binding specificity of ²¹²Pb-376.96 ranged from 71-88%. The average K_d, percent internalized activity, and binding sites per cell are shown in Table 1. Representative Scatchard plots are shown in Supplementary Figure S1. While the K_d values for ²¹²Pb-376.96 were comparable across the cell lines and growth conditions, A2780cp20 cells showed more binding sites and internalized radioactivity compared to ES-2 cells.

²¹²Pb-376.96 inhibits the clonogenic survival of differentiated ovarian cancer cells and CICs

In vitro clonogenic survival assays determined the targeted cytotoxicity of ²¹²Pb-376.96 against differentiated adherent cells and non-adherent CICs. Non-specific cytotoxicity was determined utilizing identical conditions with the isotype-matched control ²¹²Pb-F3-C25. Table 2 lists the IC₅₀ values from these clonogenic survival experiments. ²¹²Pb-376.96 was significantly more effective at inhibiting adherent cell and CIC clonogenic survival compared to ²¹²Pb-F3-C25 (p<0.05). ²¹²Pb-376.96 more effectively inhibited the clonogenic survival of A2780cp20 cells compared to ES-2 cells as indicated by the lower IC₅₀ values for the adherent and CIC A2780cp20 cells.

Additional clonogenic survival assays with A2780cp20 cells used different doses of the RICs combined with carboplatin. ²¹²Pb-376.96 or ²¹²Pb-F3-C25 was applied to the cells one day

after treatment with carboplatin, since carboplatin treatment followed by RIT with ^{212}Pb has been shown to be more effective than alternative treatment schedules in other cancer models [35, 36]. Combining 5 $\mu\text{mol/L}$ carboplatin with 2, 7, or 21 kBq/mL ^{212}Pb -376.96 decreased clonogenic survival of A2780cp20 adherent cells and CICs to a greater extent than either agent alone (Figure 1). Clonogenic survival under these conditions was significantly lower compared to combination treatment with carboplatin and control ^{212}Pb -F3-C25 ($p < 0.001$).

^{212}Pb -376.96 targets *i.p.* xenografts of ovarian cancer *in vivo*

In vivo imaging studies were performed to determine the peritoneal retention of ^{212}Pb -376.96 in SCID mice bearing *i.p.* ES-2 ovarian cancer xenografts that grew as peritoneal ascites. Planar gamma camera imaging performed 22-23 h post-injection of ^{212}Pb -376.96 indicated that an average of 66.5% of the decay-corrected ^{212}Pb activity was located in the peritoneal cavity of the mice. Supplementary Figure S2 shows a representative image from these studies.

Subsequent studies determined the relative tissue distributions of ^{212}Pb -376.96 and ^{212}Pb -F3-C25 in athymic nude mice bearing *i.p.* ovarian cancer xenografts. The biodistribution results from mice bearing fourteen-day ES-2 xenografts are shown in Figure 2A and Supplementary Table S1. The highest tissue uptake of ^{212}Pb -376.96 at 24 h after *i.p.* injection was observed in the ascites cells ($28.7 \pm 5.9\%$ ID/g). Relatively high uptake was also observed in the spleen ($17.6 \pm 8.6\%$ ID/g), reproductive organs ($15.5 \pm 3.8\%$ ID/g), and liver ($10.3 \pm 3.4\%$ ID/g). The uptake of the control RIC ^{212}Pb -F3-C25 in the ascites ($16.6 \pm 13.1\%$ ID/g) was lower than that observed in mice injected with ^{212}Pb -376.96; however, the difference was not significant ($p = 0.10$). The targeted and control RICs showed similar uptake in normal tissues of the mice.

Biodistribution analysis was also performed with mice bearing sixteen-day A2780cp20 *i.p.* xenografts, which grew as tumor nodules with slower disease progression than the ES-2 model. The tumor uptake of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 at 24 h after *i.p.* injection in the animals that presented with resectable, macroscopic tumors at the time of the study (2 of 5 mice per group, see discussion below) was $73.1 \pm 33.4\%$ ID/g and $22.3 \pm 7.1\%$ ID/g, respectively (Supplementary Table S2). Despite the notably higher tumor uptake of ^{212}Pb -376.96 relative to ^{212}Pb -F3-C25, the difference was not significant ($p=0.17$) due to the limited sample size of mice with tumors. As observed in the ES-2 studies, the rest of the tissues analyzed ($n=5$ per group) showed similar uptake between the two RICs, with relatively high concentrations of ^{212}Pb activity in the spleen and liver (Figure 2B).

^{212}Pb -376.96 significantly prolongs the survival of mice bearing *i.p.* xenografts of ovarian cancer

Athymic nude mice bearing four-day ES-2 *i.p.* xenografts were administered ^{212}Pb -376.96 or ^{212}Pb -F3-C25 to determine the effects on survival after RIT treatment at an early stage of disease development. The median survival of non-treated control mice was 15 days, while median survival was significantly prolonged to 32, 32, and 42.5 days in groups treated with a single *i.p.* dose of 0.17 MBq, 0.35 MBq, and 0.51 MBq ^{212}Pb -376.96, respectively (Figure 3A). The median survival of mice treated with 0.33 MBq ^{212}Pb -F3-C25 (28 days) was significantly shorter than that of the groups treated with 0.35 or 0.51 MBq ^{212}Pb -376.96 ($p<0.05$). All groups of mice showed a transient drop in body weight during the first week after injection of the RICs (Supplementary Figure S3). Recovery of initial weight occurred in 2-3 weeks for all groups except the group treated with 0.51 MBq ^{212}Pb -376.96, which took longer to recover (~4 weeks).

The *in vivo* therapeutic efficacy of targeted RIT alone or combined with carboplatin was examined in athymic nude mice bearing ten-day A2780cp20 *i.p.* xenografts. Carboplatin was selected because it is clinically used to treat patients with ovarian cancer and is a radiosensitizer [37]. Carboplatin treatment began one day before RIT based on previous studies that showed this treatment schedule with ^{212}Pb -trastuzumab produced the greatest therapeutic effect in mice bearing *i.p.* xenografts of an alternative cancer model [35]. Carboplatin treatment alone slightly prolonged the median survival of mice relative to the non-treated control group (27.5 days and 22 days, respectively; $p>0.05$). Treatment with 0.35 MBq, 0.53 MBq, or 0.70 MBq ^{212}Pb -376.96 significantly prolonged median survival of mice in a dose-dependent manner (43, 46, and 51 days, respectively) compared to non-treated control mice ($p<0.001$) (Figure 3B). Survival of the mice treated with 0.53 MBq ^{212}Pb -F3-C25 (41 days) was significantly shorter than that of the mice treated with 0.53 or 0.70 MBq ^{212}Pb -376.96 (46 and 51 days, respectively) ($p<0.05$). There was not a significant difference in median survival of mice by combining carboplatin treatment with 0.70 MBq ^{212}Pb -376.96 (53.5 days) relative to 0.70 MBq ^{212}Pb -376.96 alone (51 days) ($p>0.05$). The weight of mice in the A2780cp20 study was not as severely affected by treatment compared to mice in the ES-2 study (Supplementary Figure S4). Rapid weight loss occurred in half the mice in the 0.70 MBq ^{212}Pb -376.96 treatment group; this was attributed to a lack of drinking water in the cage of these mice soon after treatment, as the affected animals quickly recovered after water was made available. The group of mice treated with carboplatin and 0.70 MBq ^{212}Pb -376.96 lost ~6% of their initial body weight the first week after RIT treatment and recovered to their original weight by week two of the study.

DISCUSSION

The primary goal of debulking surgery for ovarian cancer is to achieve no residual disease (NRD) status or to “optimally debulk” a patient to <1 cm gross residual disease. However, microscopic disease remains after surgery and causes eventual recurrence in almost all ovarian cancer patients. Giving adjuvant chemotherapy prolongs patients’ progression free and overall survival with ovarian cancer but does not eliminate all microscopic disease. Finding strategies that help eliminate microscopic tumors should enhance the survival of patients with ovarian cancer. Due to the limited range of α -particles in tissues, targeted α -particle RIT strategies are particularly suitable for treating microscopic neoplasms that remain after debulking surgery. B7-H3 epitopes are expressed in several malignancies including ovarian cancer [22, 38, 39], and a B7-H3-specific mAb has been used for RIT in human patients with neuroblastoma [40]. mAb 376.96 used here binds to a specific extracellular epitope of B7-H3 that is expressed on differentiated ovarian cancer cells, CICs, ascites cells, and all tested lesions resected from ten patients with ovarian cancer, while the epitope is not significantly expressed in normal tissues [24, 29, 41]. Characterizing the mAb 376.96-defined epitope’s expression could enhance the selection of individuals that would benefit from targeted RIT with ^{212}Pb -376.96.

The expression (10^4 - 10^5 binding sites/cell) of the B7-H3 epitope on the ES-2 and A2780cp20 ovarian cancer lines grown in adherent or non-adherent CIC conditions warranted the use of these cell lines as relevant models for targeted RIT with ^{212}Pb -376.96. Cells in the *in vitro* clonogenic assays were exposed to the targeted or control RICs for a relatively short time (2 h) before removing non-bound radioactivity in order to maximize the differences in cytotoxicity due to cell-associated radioactivity relative to non-bound radioactivity in the solution. The cells’ relative sensitivities to ^{212}Pb -376.96 correlated to their expression of the B7-H3 epitope, as would be anticipated for targeted rather than non-specific binding of the RIC. The significantly

greater sensitivity of the cells (three- to forty-fold) to a targeted RIC relative to a control RIC in these studies is equivalent to or greater than those reported for other cancer cells and radionuclides [16, 42-45]. The *in vitro* IC₅₀ concentrations of ²¹²Pb-376.96 against adherent ovarian cancer cells and CICs (1.6-33 kBq/mL) were lower than the maximum concentrations of alternative α -particle RICs calculated to be present in the peritoneum of ovarian cancer patients (42-100 kBq/mL) during clinical trials [15, 19, 21]. This demonstrates the utility of ²¹²Pb-376.96 at clinically achievable concentrations to kill both differentiated cells and CICs that promote metastases, chemoresistance, and recurrent disease. The favorable cytotoxicity results of combining carboplatin with ²¹²Pb-376.96 against A2780cp20 adherent cells and CICs warrant future combinatorial therapy studies against carboplatin-resistant ovarian cancer models.

²¹²Pb is not ideal for imaging due to the multiple high-energy γ -rays emitted by this radionuclide; however, the ability to simultaneously use this radionuclide for imaging and therapy is beneficial for characterizing the pharmacokinetics of the therapeutic agent in real time. ²⁰³Pb (t_{1/2}=51.9 h, 279 keV [81%]) can alternatively be used for SPECT imaging and could serve as a matched-pair diagnostic agent prior to and following RIT with ²¹²Pb. *In vivo* imaging in the studies reported here showed the majority (~67%) of the decay-corrected ²¹²Pb activity remained in the peritoneum of tumor-bearing mice. The successful *in vivo* imaging in mice demonstrated here is notable, considering the scarcity of reports showing the localization of ²¹²Pb in living subjects. Analyses of ²¹²Pb-trastuzumab in healthy macaques [18] and in human patients with HER2⁺ peritoneal malignancies [19, 20] showed 83-95% of the decay-corrected activity was retained in the peritoneal cavity up to 48 h after administration. These differences are likely due to disparities in the RICs and metabolic activities of the subjects from the various studies. While metabolite and excretion analyses were not performed in the present study, previous work with

^{212}Pb -RICs has shown the majority of the non-retained radioactivity to be excreted through the kidneys [19, 20]. Dosimetry analyses of ^{212}Pb and ^{212}Bi were beyond the scope of the present investigation and will be performed in future experiments. The high peritoneal retention of ^{212}Pb -376.96 observed here is anticipated to cause low dosimetry to normal organs as previously shown for alternative α -particle RICs [18-21]. The relatively high radioactivity observed to be associated with the spleen and liver in the biodistribution studies may be due to clearance of the RICs through the reticuloendothelial system due to the high chelate/mAb ratios. Alternatively, this may be due to the specific catabolic pathway of the IgG_{2a} isotype mAbs used here as previously shown in rats [46].

Biodistribution analyses in mice bearing *i.p.* ovarian cancer xenografts with differing phenotypes (*i.p.* ascites or tumor nodules) indicated a trend toward specific targeting of the *in vivo* xenografts with ^{212}Pb -376.96 relative to the ^{212}Pb -F3-C25 control mAb. Mice in the ^{212}Pb -F3-C25 group presented with differing amounts of *i.p.* ES-2 ascites at the time of the study, which resulted in the variable ^{212}Pb accumulation in this group. α -particle RICs can kill significant proportions of malignant cells in the peritoneal compartment [47]. Therefore, it is possible that the removed ES-2 ascites in these studies contained primarily dead cells or cell debris while a significant proportion of live cells remained attached to peritoneal tissues in the mice. This sampling error and likelihood of non-specific binding in dead cells may have biased the results towards artificially high radioactivity in the ascites of the ^{212}Pb -F3-C25 group. An early time point of disease progression was selected for biodistribution in the A2780cp20 model to determine the uptake of the RICs in small tumor nodules that would approximate the size of non-resected neoplasms in human patients following debulking surgery. While the *in vivo* therapy studies demonstrated that all mice develop macroscopic tumors and succumb to disease

progression, 40% of the mice presented with removable macroscopic tumor nodules (totaling 10-50 mg per mouse) at the early stage of tumor development during the biodistribution studies. Favorable accumulation of ^{212}Pb -376.96 relative to ^{212}Pb -F3-C25 was observed in these limited tumor samples, although additional studies utilizing more mice with measurable tumors would be needed to validate the observed difference in tumor uptake between the targeted and control RICs.

In vivo RIT with ^{212}Pb -376.96 was effective against the *i.p.* ovarian cancer xenograft models used here, as indicated by the two- to three-fold longer survival of treated groups compared to non-treated control groups. The doses selected for these studies were comparable to doses of alternative ^{212}Pb -RICs that produced significant therapeutic effects (two- to ten-fold greater than non-treated control groups) in mice with *i.p.* xenografts of human malignancies [35, 36, 48]. The trend of prolonged mouse survival with higher doses of ^{212}Pb -376.96, coupled with the finding that all mice eventually succumbed to disease burden, suggests that *in vivo* saturation of the target epitope had not occurred with the doses used here [47]. Microdosimetry calculations and experimental results indicate that administering α -particle RICs in high specific activity at doses that saturate the target epitope *in vivo* is a viable strategy to maximize the absorbed dose to tumors without significantly increasing the dose to normal tissues [15, 49]. The significantly prolonged survival of mice that received ^{212}Pb -376.96 relative to a comparable dose of ^{212}Pb -F3-C25 demonstrates the advantage of targeting the mAb-defined B7-H3 epitope. In concordance with previous studies using other RICs, mouse survival was significantly prolonged by α -particle RIT compared to chemotherapy in the A2780cp20 model used here. The dose of carboplatin used here was selected to match that used in a previous study that showed significant therapeutic efficacy in combination with ^{212}Pb RIT against an alternative colon cancer model [35]. Although

higher doses of chemotherapy in the present study may have resulted in prolonged survival of mice compared to the observed results, a moderate dose was elected to maximize the potential effects of combinatorial treatment in a carboplatin-resistant model of ovarian cancer while minimizing the likelihood of undesirable toxicity. The similar therapeutic effects resulting from treatment with the targeted RIC alone or in combination with carboplatin suggests that the therapeutic enhancement was primarily due to ^{212}Pb RIT as opposed to chemotherapy under the conditions employed. The transient losses in body weights for mice in these studies suggested the treatment regimens remained below the maximum tolerated dose. The focus of this preliminary report was to establish the proof-of-concept for B7-H3 targeted *i.p.* ^{212}Pb -RIT in ovarian cancer models; as such, further toxicological data was not collected in these studies. Future experiments will monitor additional parameters (e.g., blood leukocyte levels, liver and renal function, tissue pathology) to identify acute and long-term toxicological effects from treatment with ^{212}Pb -376.96. It is worth noting that targeted *i.p.* ^{212}Pb -RIT is tolerated in human patients as demonstrated by the minimal toxicity observed in the clinical trial with ^{212}Pb -trastuzumab [19, 20].

The collective results from these studies indicate the success of targeted RIT with ^{212}Pb -376.96 against *i.p.* models of human ovarian cancer. In agreement with previous studies using alternative cancer models, the findings discussed here demonstrate the *in vitro* and *in vivo* benefits of targeted relative to non-targeted α -particle RIT in ovarian cancer models. Future work with ^{212}Pb -376.96 will be required to determine if alternative timing of RIT administration after cancer cell implantation, additional RIT doses, or other combinations of RIT with chemotherapy or inhibitors of the Wnt/ β -catenin signaling pathway [50, 51] will effectively eliminate disease in preclinical models of ovarian cancer.

CONCLUSION

^{212}Pb -376.96 specifically targeted differentiated and CIC populations of human ovarian cancer cell lines. This RIC alone and combined with carboplatin inhibited the clonogenic survival of ovarian cancer cells and significantly prolonged the survival of mice bearing aggressive ovarian cancer xenografts. B7-H3-targeted ^{212}Pb -RIT combined with chemotherapy is worthy of additional preclinical studies and future clinical evaluations in patients with ovarian cancer.

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TABLES

Table 1. *In vitro* binding analysis of ^{212}Pb -376.96 to ovarian cancer cells.

	$K_d \pm \text{S.E.M.}$ (nmol/L)	Binding sites/cell $\pm$ S.E.M. ($\times 10^4$)	Percent internalized
Adherent ES-2	10.6 ± 2.5	7.5 ± 0.7	54
CIC ES-2	26.6 ± 11.6	6.4 ± 1.7	31
Adherent A2780cp20	15.2 ± 11.2	79 ± 6.9	95
CIC A2780cp20	19.3 ± 13.9	66 ± 42	93

Table 2. *In vitro* inhibition of adherent cell or CIC clonogenic survival by ^{212}Pb -376.96 or ^{212}Pb -F3-C25.

Cell line	<u>$\text{IC}_{50} \pm \text{S.E.M. (kBq/mL)}$</u>	
	^{212}Pb-376.96	^{212}Pb-F3-C25
Adherent ES-2	33 ± 3.0	112 ± 3.7
CIC ES-2	30 ± 13	71 ± 21
Adherent A2780cp20	1.6 ± 0.13	69 ± 12
CIC A2780cp20	5.2 ± 0.25	32 ± 2.8

LEGENDS FOR FIGURES

Figure 1. *In vitro* clonogenic survival of A2780cp20 adherent cells (A) or CICs (B) after treatment with 2, 7, or 21 kBq/mL ^{212}Pb -376.96 or ^{212}Pb -F3-C25 alone or combined with carboplatin. Bars correspond to cells exposed to vehicle (white), 5 $\mu\text{mol/L}$ carboplatin alone (checkered), ^{212}Pb -376.96 alone (diagonal lines), ^{212}Pb -376.96 with 5 $\mu\text{mol/L}$ carboplatin (black), or ^{212}Pb -F3-C25 with 5 $\mu\text{mol/L}$ carboplatin (horizontal lines). Data are presented as mean ($\pm$ standard deviation) % clonogenic survival relative to vehicle-treated controls. ** $p < 0.01$; *** $p < 0.001$; ns, not significant

Figure 2. Biodistribution analysis at 24 h after *i.p.* injection of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 in athymic nude mice bearing 14-day ES-2 (A) or 16-day A2780cp20 (B) *i.p.* xenografts. After euthanization of mice, tissues were collected, weighed, and counted to determine the percent of the injected dose per gram (% ID/g) of tissue. Data are presented as mean $\pm$ standard deviation.

Figure 3. Kaplan-Meier curves showing the effects of ^{212}Pb -376.96 or ^{212}Pb -F3-C25 on survival

of athymic nude mice bearing human ovarian cancer xenografts. (A) Groups of mice bearing 4-day ES-2 *i.p.* xenografts either were left untreated (filled circles) or injected *i.p.* with 0.33 MBq ^{212}Pb -F3-C25 (filled diamonds), 0.17 MBq ^{212}Pb -376.96 (filled squares), 0.35 MBq ^{212}Pb -376.96 (filled triangles), or 0.51 MBq ^{212}Pb -376.96 (open triangles). (B) Groups of mice bearing 10-day A2780cp20 *i.p.* xenografts either were left untreated (filled circles) or injected *i.p.* with carboplatin (50 mg/kg weekly, filled diamonds), 0.53 MBq ^{212}Pb -F3-C25 (open squares), 0.35 MBq ^{212}Pb -376.96 (filled squares), 0.53 MBq ^{212}Pb -376.96 (upright triangles), 0.70 MBq ^{212}Pb -376.96 (downward triangles), or 0.70 MBq ^{212}Pb -376.96 with carboplatin (50 mg/kg weekly, open circles). Treatment with carboplatin began one day before injection with ^{212}Pb -376.96.

A. Adherent A2780cp20

B. CIC A2780cp20

Figure 1.

Figure 2.

Figure 3.