Introduction

Targeted radiation therapy with monoclonal antibodies (mAb) using β-emitting radionuclides has been shown to be an efficacious strategy for the treatment and management of cancer patients (1, 2). Due to the combination of shorter path length (50–80 μm) and higher linear energy transfer (100 KeV/μm), targeted α-particle radioimmunotherapy (RIT) offers the potential for more specific tumor cell killing, with less damage to surrounding normal tissue than β-emitters. Although this strategy has been successfully applied toward the eradication of leukemia (3, 4), the same physical properties suggest that targeted α-therapy would be suitable for the elimination of minimal residual or micrometastatic disease in other types of human malignancies (5–7).

212Pb-emitters. Although this strategy has been successfully applied toward the eradication of leukemia (3, 4), the same physical properties suggest that targeted α-therapy would be suitable for the elimination of minimal residual or micrometastatic disease in other types of human malignancies (5–7).

Targeted and pretargeted RIT using α-emitters such as 212Bi (T1/2 = 1.01 hours) and 212Pb (T1/2 = 10.6 hours) have shown significant therapeutic efficacy in both in vitro and in vivo model systems (8–10). 212Pb is the longer lived parental radionuclide of 212Bi and, as such, it serves as an in vivo generator of 212Bi. The 212Pb/212Bi system, therefore, is a promising α-particle emitting source that provides an alternative option for the treatment and management of cancer (8, 11).

Trastuzumab (Herceptin) is a humanized mAb that targets HER2 and has been well documented to have antitumor activity for the management of breast cancer (12, 13). Previously, this laboratory showed the efficacy of α-particle RIT using the CHX-A" diethylenetriaminepentaacetic acid linker with 212Bi in intraperitoneal models for pancreatic and ovarian cancer using trastuzumab as the targeting moiety (6). Complementary to those results, suitable chelation chemistry for the retention of 212Pb with the protein was also designed and synthesized, that is, 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1,4,7,10-tetra-(2-carbamoymethyl)-cyclododecane (4-NCS-Bz-TCMC; TCMC) to overcome the limitations associated with the direct use of the shorter half-life bismuth radioisotopes, 211Bi or 212Bi, and to obviate a source of toxicity originating from intracellular dissociation of 212Pb from 1, 4, 7, 10-tetraazacyclododecane-N,N',N"N"'-tetraacetic acid (8, 14). Studies using 212Pb showed the feasibility of this isotope in RIT for the treatment of disseminated intraperitoneal disease; 212Pb-TCMC-trastuzumab given as a
single injection showed therapeutic efficacy, which increased with multiple injections given at approximately monthly intervals (8).

The mechanism by which α-particle RIT induces cell death is not completely understood (15–18). A recent report indicated that upon exposure to α-particles, cell survival was not impacted by traversals of the cytoplasm, but by traversal of nuclei (19). Seidl and colleagues reported that RIT with an α-emitting radionuclide induced nonapoptotic cell death at 72-hour posttreatment (20). It is noteworthy that there have been few studies related to the determination of the actual mechanisms involved in the α-particle RIT cytotoxicity. The literature to date has been effectively restricted to in vitro studies, which, by their very nature, are self-limiting and not reflective of RIT treatment of tumors in a complex environment (21, 22).

The studies reported herein were designed to gain an understanding of the underlying mechanism(s) of action of 212Pb-TCMC-trastuzumab therapy in a systematic fashion using the murine model currently under investigation in our laboratory. The ultimate objective will be to incorporate the knowledge gained into the design of future therapy studies and to improve the therapeutic benefit of targeting HER2 with α-particle emitting radionuclides. The studies reported herein describe the apoptotic response, cell-cycle distribution, DNA repair, and changes in chromatin remodeling in LS-174T intraperitoneal xenograft tumors following RIT with 212Pb. The studies suggested that 212Pb-TCMC-trastuzumab therapy-induced cell killing in the LS-174T intraperitoneal xenograft model occurred principally by G2/M arrest, accompanied by a delay in DNA damage repair.

Materials and Methods

Cell line

The human colon carcinoma cell line (LS-174T) was used for all in vivo studies. LS-174T was grown in a supplemented Dulbecco’s Modified Eagle’s Medium (DMEM) as previously described (23). All media and supplements were obtained from Lonza. The cell line has been screened for Mycoplasma and other pathogens before in vivo use according to National Cancer Institute (NCI) Laboratory Animal Sciences Program policy. No authentication of the cell line was conducted by the authors.

Chelate synthesis, mAb conjugation, and radiolabeling

The synthesis, characterization, and purification of the bifunctional ligand TCMC have been previously described (8, 14). Trastuzumab (Herceptin; Genentech) was conjugated with TCMC by established methods using a 10-fold molar excess of ligand to mAb as previously reported (8). The final concentration of trastuzumab was quantified by the method of Lowry (24). The number of TCMC molecules linked to the mAb was determined using a spectrophotometric based assay (25). A 10 mCi 224Ra/212Pb generator was supplied by AlphaMed, Inc. The preparation of the generator and radiolabeling procedure has been previously described (8).

Tumor model, treatment, and tumor harvesting

Studies were carried out with 19 to 21 g female athymic mice (NCI-Frederick) bearing 3-day intraperitoneal LS-174T xenografts as previously reported (8). The viability of the LS-174T cells (>95%) was determined using trypan blue. Mice were injected intraperitoneally with 1 × 106 LS-174T cells in 1 mL of DMEM. The inoculum size for this cell line represented the minimum amount of cells required for tumor growth in 100% of the mice (6). 212Pb-TCMC-trastuzumab (10 μCi in 0.5 mL PBS) was administered to the mice 3 days postimplantation of tumor (n = 10–15). This treatment group was compared with sets of mice that received 212Pb-TCMC-HulG, unlabeled trastuzumab, or HulG, or no treatment. Mice receiving trastuzumab or HulG were injected 3 days after tumor implantation with 10 μg of the respective material. The mice used for the cell-cycle and proliferation studies were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdUrd; 1.5 mg in 0.5 mL PBS; Sigma) 4 hours before euthanasia. Tumors were harvested from mice bearing intraperitoneal LS-174T xenografts at 6, 24, 48, 72, 96, and 120 hours. The amount of tumor collected was not measured; however, on the basis of previous studies, the tumor burden at 7 days is typically 128.5 ± 205.6 mg (26). The tumors at each time point were pooled together, macroscopically inspected, and adherent tissues were removed. The tumor tissues were then thoroughly rinsed in ice-cold PBS 3 times, divided, and processed accordingly for each assay. The tumor tissues were either stored at −80°C until use or paraffin embedded after fixing in 1% formalin. All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee.

Flow cytometry

Cell-cycle distribution and DNA synthesis was determined by flow cytometry as previously described with some modifications (27). The tumors were fixed in cold 70% ethanol at 4°C, washed in PBS twice, minced finely, and incubated in 1 mL 0.04% pepsin (Sigma) w/v in 0.1 N HCl (Mallinckrodt, Inc.) for 1 hour at 37°C with shaking. The digest was filtered through a 70-μm nylon mesh after passage through a 25-gauge needle. Following centrifugation at 10,000 × g for 10 minutes, the resulting pellet was resuspended in 1 mL of 2 N HCl (Mallinckrodt, Inc.) and incubated at 37°C for 20 minutes with shaking. The nuclear suspension was neutralized with 0.1 mol/L sodium tetraborate (Sigma), washed twice in PBS containing 0.5% bovine serum albumin (BSA) and 0.5% Tween-20 (PBSTB), and resuspended in PBSTB. The nuclei (100 μL) were incubated with 20 μL of fluorescein isothiocyanate (FITC)-labeled anti-BrdU mAb (BD Biosciences-Pharmin-gen) for 1 hour at 4°C, followed by 2 washes in cold PBS.
The samples were then resuspended in 2 mL of propidium iodide (50 μg/mL in PBTB; Sigma) containing RNase A (50 μg; Sigma) and incubated for 30 minutes at 4°C. Flow cytometry was done using a FACSCalibur (BD Biosciences), collecting 15,000 events with cell debris excluded from data collection. DNA content (propidium iodide) and DNA synthesis (BrdU/Lrd content) were analyzed using 2 parameter data collection with CellQuest (BD Biosciences) software, whereas single parameter DNA distribution was done and analyzed using Modfit LT ver. 3.0 (Verity Software House, Inc).

**Determination of apoptosis**

Apoptotic bodies were scored using hematoxylin and eosin (H&E) staining as described previously (28). Five fields were analyzed per tumor section, and the number of apoptotic bodies per 100 nuclei scored expressed as a percentage. The following criteria were used to distinguish apoptotic bodies: (a) isolated distribution of apoptotic bodies; (b) shrunken cells usually with empty space between neighboring cells; (c) eosinophilic cytoplasm; (d) condensation of nuclei into dense particles; (e) fragmentation of the nuclei into several bodies; and (f) the absence of inflammatory reaction surrounding the apoptotic cells (nonnecrotic cells).

The presence of apoptotic bodies on tumor sections was also determined using the Dead End Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) System (Promega). The sections were incubated in 100 μL of a 20 μg/mL proteinase solution at room temperature for 10 minutes, washed twice in PBS, and fixed in 4% formaldehyde. The sections were incubated in 100 μL of TdT reaction mixture for 60 minutes at 37°C, and washed 3 times in cold PBS. The sections were mounted using Vectashield with DAPI (641.5x839.6)
Detection Kit (GE Healthcare) and the images acquired by Fuji LAS 4000.

Statistics
At least 3 independent experiments were done for each point described. All values were expressed as mean ± SD. Student t test was used for paired data, and multiple comparisons were done with the ANOVA. A P value less than 0.05 was considered statistically significant.

Results

\( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) induces apoptosis in intraperitoneal human colon carcinoma–treated xenografts

Anticancer drugs and radiation have been shown to activate apoptosis pathways in solid tumors (29, 30). To determine whether \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) induces apoptosis in the human colon carcinoma LS-174T xenografts, the TUNEL assay was done using paraffin-embedded tumor sections. The TUNEL assay results obtained at 24 hours after exposure to \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) indicated a clear induction of apoptosis (Fig. 1A) that was greater than the other treatments. Next, the apoptotic bodies were quantitated using IHC and H&E staining. Apoptosis was noted on the basis of morphologic criteria described in the Methods. Quantitation of the apoptotic bodies showed that \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) treatment significantly increased in apoptotic rates (Fig. 1B) compared with the \( ^{212}\text{Pb}-\text{TCMC-HulgG} \) nonspecific control treatment at 24 to 72 hours (\( P < 0.05 \)), suggesting greater effective targeted cell killing by \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) in the tumor tissues.

\( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) interferes with DNA repair

Radiation causes DNA damage, the most harmful in terms of cellular cytotoxicity being double-strand breaks (DSB). An increase in DNA DSBs and impaired DNA damage repair has been invoked to explain the synergy between drugs and ionizing radiation (31). To investigate DNA damage by \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \), IHC was done using paraffin-embedded tumor sections. Induction of DNA DSB damage was evident as measured by phosphorylated H2AX, a marker for DNA double-strand damage (Fig. 2A). The neutral comet assay was also used to show physical DNA strand damage. Enhancement of the fluorescence intensity of tail was observed in both \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \)- and \( ^{212}\text{Pb}-\text{TCMC-HulgG} \)-treated tumors. Next, to determine the effect of \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) on DNA damage repair, DNA content in the tails were compared at different time points up to 96 hours after the various treatments. After 24 hours, \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) showed significantly higher percent DNA in the tail than any other treatment, including \( ^{212}\text{Pb}-\text{TCMC-HulgG} \). At 24 hours, 18% of the tumor sample presented with DNA strand breaks. There was a steady decrease in the percentage of DNA damage and by 96 hours the percentage was 10. Thus, by 96 hours, inhibition of DNA damage repair elicited by \( ^{212}\text{Pb}-\text{TCMC-trastuzumab} \) was not yet been completely reversed. In comparison, the \( ^{212}\text{Pb}-\text{TCMC-HulgG} \) resulted in 13% DNA strand breaks at 24 hours. By the 96-hour time point, DNA in the

![Figure 1](image-url)
tail for $^{212}$Pb-TCMC-HuIgG was similar to that of unla-
beled HuIgG, indicating that DNA damage repair had
returned to normal levels following treatment. This
showed that DNA damage repair was compromised to
greater extent by the treatment with $^{212}$Pb-TCMC-tras-
tuzumab ($P < 0.001$).

DSBs can be repaired by different pathways, the most
important of which are homologous repair (HR) and
nonhomologous end joining (NHEJ; ref. 32). To identify
the DNA repair pathways involved in the study presented
herein, Rad51 and DNA-PKcs, which play important
roles in HR and NHEJ, respectively, were investigated.
The densitometric analysis of the Western blots illustrated
in Fig. 2C shows that at the protein expression level, Rad51
was significantly downregulated by $^{212}$Pb-TCMC-trastu-
zumab ($P < 0.05$) treatment. However, Rad51 was not
affected in the presence of the unlabeled trastuzumab,
HuIgG, or $^{212}$Pb-TCMC-HuIgG at 24 hours. DNA-PKcs
was not affected following any of the treatments, suggest-
ing that differences in induction of the HR pathway
protein Rad51 may, at least in part, be responsible for the
delayed DNA damage repair following the $^{212}$Pb-TCMC-
trastuzumab treatment.

To examine the involvemnt of caspase-3, which plays
an important role in the condensation and degradation of
chromatin of apoptotic cells in $^{212}$Pb-TCMC-trastuzu-
mb-induced apoptosis, cleaved caspase-3 was analyzed
using immunoblot techniques. Cleaved caspase-3 was
observed in the tumor tissues 24 hours after treatment
with $^{212}$Pb-TCMC-HuIgG (Fig. 2D). In contrast to this
result, it seemed that $^{212}$Pb-TCMC-trastuzumab treat-
ment significantly reduced the level of cleaved caspase-
3 ($P < 0.001$), suggesting that $^{212}$Pb-TCMC-trastuzumab-
induced apoptosis occurred via a caspase-3-independent
mechanism.
molecular Cancer Therapeutics

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Table 1. Analysis of DNA synthesis in LS-174T tumor xenografts following treatment with $^{212}$Pb-TCMC-trastuzumab

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>0</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
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<tr>
<td>None</td>
<td>23.8 ± 1.3</td>
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<td></td>
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<tr>
<td>$^{212}$Pb-Trastuzumab</td>
<td>13.7 ± 1.2</td>
<td>7.3 ± 1.4</td>
<td>2.6 ± 0.7</td>
<td>7.5 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>$^{212}$Pb-HuIgG</td>
<td>16.4 ± 1.2</td>
<td>8.1 ± 0.9</td>
<td>2.4 ± 0.1</td>
<td>2.8 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td>13.2 ± 0.4</td>
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<tr>
<td>Trastuzumab</td>
<td>15.1 ± 1.0</td>
<td>21.4 ± 1.1</td>
<td>14.8 ± 12.9</td>
<td>27.5 ± 1.5</td>
<td>20.6 ± 1.8</td>
<td>18.2 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>HuIgG</td>
<td>15.9 ± 0.8</td>
<td>19.0 ± 0.4</td>
<td>20.7 ± 1.3</td>
<td>23.4 ± 1.1</td>
<td>22.1 ± 0.6</td>
<td>16.7 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Results represent the average of a minimum of 3 replications (± SD).

$^{212}$Pb-TCMC-trastuzumab attenuates proliferation in S-phase and induces G2 cell-cycle arrest

In response to radiation, cells typically require cell-cycle arrest at G1 and a slow S-phase to allow DNA repair. As a consequence of reduced intracellular signaling, trastuzumab as a sole agent induces early escape from this cycle arrest and thereby promotes an accumulation of DNA damage (33). The effect of $^{212}$Pb-TCMC-trastuzumab treatment on cell-cycle distribution was examined. In the same study, mice were injected with BrdUrd 4 hours prior to tumor collection to pulse label the tumor xenografts to evaluate DNA synthesis. DNA content and BrdUrd incorporation was then determined using a 2-parameter analysis by flow cytometry. As shown in Table 1, tumor cells harvested from untreated mice were found to have the expected uptake of BrdUrd (23.8 ± 1.3%); the resulting cell-cycle distribution was also in the expected range (Table 2). On the other hand, 6 hours after $^{212}$Pb-TCMC-trastuzumab treatment, there was a noticeable decrease in BrdUrd incorporation (13.7 ± 1.2%). BrdUrd incorporation decreased further to 2.6 ± 0.7% at 48 hours and remained at this low level throughout the remainder of the 120-hour study period. In contrast to $^{212}$Pb-TCMC-trastuzumab treatment, however, DNA synthesis after treatment with $^{212}$Pb-TCMC-HuIgG did reinitiate by 120 hours after decreasing at the early time points. The continuation of DNA synthesis by the tumors that were treated with unlabeled trastuzumab, or HuIgG alone, provided evidence that the cessation of DNA synthesis is specific to the α-radiation and that continued depression of DNA synthesis after 120 hours was specific to the targeted $^{212}$Pb-TCMC-trastuzumab treatment. In

Table 2. Cell-cycle distribution analysis in intraperitoneal tumor xenografts of LS-174T following treatment with $^{212}$Pb-TCMC-trastuzumab

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase</th>
<th>0</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
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</thead>
<tbody>
<tr>
<td>$^{212}$Pb-Trastuzumab</td>
<td>G1</td>
<td>67.5 ± 2.7</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>S</td>
<td>17.7 ± 2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2-M</td>
<td>14.8 ± 0.7</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>$^{212}$Pb-HuIgG</td>
<td>G1</td>
<td>68.6 ± 3.8</td>
<td>66.9 ± 1.3</td>
<td>67.7 ± 4.0</td>
<td>68.1 ± 1.3</td>
<td>66.6 ± 0.7</td>
<td>73.2 ± 0.1</td>
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<td></td>
<td>S</td>
<td>14.3 ± 5.4</td>
<td>5.9 ± 0.1</td>
<td>6.3 ± 1.8</td>
<td>7.9 ± 0.7</td>
<td>8.6 ± 0.0</td>
<td>4.6 ± 1.9</td>
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<tr>
<td></td>
<td>G2-M</td>
<td>17.1 ± 1.6</td>
<td>27.3 ± 1.2</td>
<td>26.1 ± 21</td>
<td>22.9 ± 0.6</td>
<td>29.7 ± 0.7</td>
<td>22.2 ± 2.0</td>
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<tr>
<td>Trastuzumab</td>
<td>G1</td>
<td>63.9 ± 5.2</td>
<td>65.1 ± 2.3</td>
<td>64.6 ± 0</td>
<td>67.1 ± 0.5</td>
<td>63.1 ± 0.3</td>
<td>65.5 ± 2.6</td>
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<tr>
<td></td>
<td>G2-M</td>
<td>20.0 ± 5.3</td>
<td>7.4 ± 1.3</td>
<td>5.8 ± 0</td>
<td>5.5 ± 0.1</td>
<td>7.8 ± 0.7</td>
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<tr>
<td>HuIgG</td>
<td>G1</td>
<td>16.0 ± 0.1</td>
<td>27.5 ± 1.0</td>
<td>29.6 ± 0</td>
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<td>17.5 ± 0.9</td>
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<tr>
<td></td>
<td>S</td>
<td>69.5 ± 2.6</td>
<td>63.0 ± 1.6</td>
<td>68.0 ± 0.1</td>
<td>61.3 ± 1.8</td>
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<td>73.3 ± 4.3</td>
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<td>G2-M</td>
<td>21.7 ± 3.4</td>
<td>26.0 ± 3.2</td>
<td>22.0 ± 1.8</td>
<td>28.1 ± 1.6</td>
<td>23.5 ± 4.3</td>
<td>14.8 ± 2.9</td>
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<tr>
<td></td>
<td>G2-M</td>
<td>8.9 ± 0.9</td>
<td>10.7 ± 1.6</td>
<td>10.1 ± 1.7</td>
<td>10.7 ± 3.4</td>
<td>11.5 ± 1.0</td>
<td>11.9 ± 1.4</td>
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</table>

NOTE: Results represent the average of a minimum of 3 replications (± SD).
addition, there was a decrease in the S-phase fraction with a corresponding increase in the G2-M phase fraction beginning at 24 hours compared with the unlabeled analogs. The lower S-phase and the elevated G2-M phase fractions were maintained throughout the 120-hour study period for the tumors upon $^{212}$Pb-TCMC-trastuzumab treatment. In contrast, the cell-cycle distribution rebounded in those tumors that were collected from mice treated with $^{212}$Pb-TCMC-HuIgG. The initial depression of the S-phase fraction and elevation of G2-M phase fraction seemed to be $\alpha$-radiation related because tumors collected from mice treated with either antibody alone (not labeled with $^{212}$Pb) did not seem to experience any alteration in cell-cycle distribution.

$^{212}$Pb-TCMC-trastuzumab induces modification in chromatin structure of $p21$

Chromatin remodeling is significantly altered in tumors, suggesting a direct role for methylation in cellular transformation. $\gamma$H2AX facilitates the recruitment of damage-responsive proteins and chromatin remodeling complexes to the sites of DNA damage and influences both the efficiency and fidelity of DNA repair (34). To determine whether changes occur in chromatin remodeling following $^{212}$Pb-TCMC-trastuzumab treatment, the ChIP assay was employed using $p21$ promoter–specific primers, one of the known radiation response genes, and immunoprecipitated DNA was analyzed by a qPCR. The ratio between H3K4 methylation and H3K9 methylation was used as a measurement of change (open/close) in chromatin structure (35–37). The abundance of histone modifications identified with transcriptionally activated chromatin states, such as H3 methylated at lysine 4, was observed at 48 hours after treatment with $^{212}$Pb-TCMC-HuIgG and at 72 hours after $^{212}$Pb-TCMC-trastuzumab treatment (Fig. 3). It seems that at earlier time points, histone modifications associated with transcriptionally repressed chromatin states, such as H3 methylated at lysine 9, prevailed on treatment with $^{212}$Pb-TCMC-trastuzumab, indicating that $^{212}$Pb-TCMC-trastuzumab induced the delayed open chromatin structure until 72 hours.

$^{212}$Pb-TCMC-trastuzumab induces reduction of $p21$ at protein level

To assess the induction of $p21$ in response to DNA damage, Western blot analysis of $p21$ was done. Enhanced protein expression of $p21$ was observed at 24 to 48 hours after $^{212}$Pb-TCMC-HuIgG treatment, whereas $^{212}$Pb-TCMC-trastuzumab treatment resulted in enhanced $p21$ expression at 72 hours (Fig. 4A). These results suggested there were correlations between induction of $p21$ protein and modification in chromatin structure in response to $^{212}$Pb-TCMC-trastuzumab and $^{212}$Pb-TCMC-HuIgG treatment. Selecting the 24-hour time point, further analysis was done to compare the effect of all the treatments on the LS-174T xenograft. There was also a significant reduction of $p21$ protein at 24 hours ($P < 0.05$) compared with a nonspecific control, indicating that reduction of $p21$ protein expression was specific to $^{212}$Pb-TCMC-trastuzumab treatment (Fig. 4B).
Discussion

Unlike β-particle or photon irradiation, α-particle radiation is cytotoxic at dose rates as low as 1 Gy/h. The shorter path of α particles may also have the advantage of limiting toxicity to normal tissue adjacent to tumor. Studies from this laboratory have shown the exquisite effectiveness of both 213Bi- and 212Pb-labeled trastuzumab for the treatment of low HER2-expressing intraperitoneal disease (6–8). A unique advantage of RIT versus monotherapy with trastuzumab is that neither high expression nor homogenous expression of HER2 throughout the tumor is required to affect therapy. One strategy to overcome the limitations of the shorter half-lives of the bismuth radioisotopes is to treat with 212Pb (T1/2 = 10.6 hours), which essentially serves as an in vivo generator. The application of 212Pb-labeled trastuzumab in the appropriate setting has shown advantages over 211Bi-labeled trastuzumab (6–8). These studies provided sufficient impetus for the in vivo assessment of the mechanisms of therapeutic efficacy of 212Pb-TCMC-trastuzumab therapy.

The effect of 212Pb-TCMC-trastuzumab treatment on apoptosis seemed to be more pronounced in the targeted cells at 24 hours (P < 0.05) compared with the nonspecific control. 211Pb-TCMC-HuIgG. In prior observations, trastuzumab was reported to enhance radiation-induced apoptosis of cells in an HER2 level–dependent manner (38). Although the results obtained in this study with 212Pb-TCMC-trastuzumab treatment seems to be similar to the prior report with respect to the enhancement of apoptosis, the 2 situations are inherently different. In the experiments presented here, trastuzumab was used as a vector to specifically direct α-particle radiation payload to the tumor. In this instance, trastuzumab was not expected to exert any pharmacologic effects. Irradiated cells release signals and induce responses in cells whose nuclei were not hit by radiation, resulting in genetic damage, genomic instability, or cell death. A high apoptotic rate was also observed for the nonspecific control 212Pb-TCMC-HuIgG–treated group. Consideration must be given to this indirect effect of radiation because transmissible biologic effects resulting from the radiation insult are pronounced following high LET radiation, such as α-particle irradiation.

Direct injury to DNA is generally attributed to radiation therapy. Increased DNA repair has been shown in tumor cells resistant to radiation and anticancer drugs, in comparison with tumor cells sensitive to these modalities. Synergy between drugs and ionizing radiation is attributed to impaired repair of residual DNA DSBs (39). As shown from the percent DNA in the comet tail comparison, DNA damage following 212Pb-HuIgG radiation showed gradual recovery beginning at 24 hours posttreatment. On the other hand, DNA repair inhibition was more pronounced in the mice treated with 212Pb-TCMC-trastuzumab (P < 0.001), as indicated by the persistently higher % DNA in the comet tail at all time points studied. There seemed to be recovery of DNA repair for the 212Pb-TCMC-HuIgG–treated tumors, although recovery was not evident after the 212Pb-TCMC-trastuzumab treatment. These results were further investigated to understand which repair systems were involved. NHEJ and HR are predominant mechanisms in DNA DSB repair. High-level Rad51 expression has been reported in chemoresistant or radioreistant carcinomas (40). Upregulation of Rad51 after irradiation has been previously observed in eukaryotic cells (41). In this study, Rad51 was not affected in the presence of 212Pb-TCMC-HuIgG at 24 hours. In contrast to this result, densitometric analysis of the Western blots clearly indicated that the 212Pb-TCMC-trastuzumab treatment significantly reduced Rad51 at the protein level (P < 0.05). Therefore, inhibition of DNA damage repair induced by 212Pb-TCMC-trastuzumab treatment, evidenced by the reduction of Rad51 protein expression and by the persisted inhibition of DNA damage repair, may be an explanation for the increased cell killing efficacy of 212Pb-TCMC-trastuzumab treatment.

After irradiation with 212Pb, cleaved caspase-3 was observed, albeit at a lower level, after 212Pb-TCMC-trastuzumab treatment (P < 0.001) compared with 212Pb-TCMC-HuIgG treatment, suggesting that apoptosis induced by 212Pb-TCMC-trastuzumab treatment was not dependent on caspase-3. Various studies also reported that high LET α-particle immunoconjugates induced apoptosis and that the mode of cell death triggered by α-particle emitters seemed to be dependent on the type of cells irradiated (42–46).

The lower levels of DNA synthesis that were observed following 212Pb-TCMC-trastuzumab treatment at the early time points persisted beyond 120 hours, but seemed to rebound for the 212Pb-TCMC-HuIgG–treated tumors by the same time point. A similar temporal progression was observed for the phase distribution of cells in which cells were arrested in the G2-phase, with a severely depressed S-phase at the early time points on 212Pb treatment that seemed to rebound for 212Pb-TCMC-HuIgG–treated mice by 120 hours, but not for the group treated with 212Pb-TCMC-trastuzumab. Notably, tumors from mice given 212Pb-TCMC-trastuzumab remained arrested at the G2-M phase with depressed S-phase beyond 120 hours. Synchronization in the G2 phase after 120-hour treatment, together with persistent reduced DNA synthesis, seemed the most prominent difference between the targeted 212Pb-TCMC-trastuzumab and the control 212Pb-TCMC-HuIgG treatment. Such synchronization has been described as the major course of synergy between chemotherapy and external beam irradiation, although radiosensitization may not be achieved in all cell lines or tumors (47). The arrest of cells in the radiosensitive G2-M phase of the cell cycle induced by 212Pb-TCMC-trastuzumab treatment may be another explanation for the increased cell killing efficacy of α-particle RIT. These results are bolstered by the persistently delayed DNA repair on 212Pb-TCMC-trastuzumab treatment indicated by the comet
assay compared with the recovery of DNA repair at later time points on 212Pb-TCMC-HulG treatment.

Epigenetic markers for open and closed chromatin status using H3K4/H3K9 ratio revealed correlations between the induction of p21 protein and modification in chromatin structure of p21 in response to 212Pb-TCMC-trastuzumab and 212Pb-TCMC-HulG treatment. In contrast to the results of 212Pb-TCMC-HulG control, induction of p21 protein and open chromatin structure was delayed until 72 hours by 212Pb-TCMC-trastuzumab, indicating that inhibition of p21 protein at early time points was associated with histone modifications that correlated with repressed transcription. In fact, there was a significant reduction of p21 at the protein level at 24 hours that inhibition of p21 protein at early time points was associated with histone modifications that correlated with repressed transcription. In fact, there was a significant reduction of p21 at the protein level at 24 hours by 212Pb-TCMC-trastuzumab (P < 0.05). These results indicate that the increased killing efficacy of 212Pb-TCMC-trastuzumab treatment was, in part, associated with the delay in open chromatin structure of p21 that correlated to less active transcription at earlier time points. Wendt and colleagues reported that radiation-induced p21 expression and G2 arrest results in resistance to apoptosis and inhibition of p21 displayed enhanced radiation-induced apoptosis (48).

Understanding the mechanisms of cell death and DNA repair is critical to the design of novel strategies that combine chemotherapy with targeted α-particle radiation. Studies of this nature help refine and optimize all of the components to improve efficacy and minimize toxicity. Carefully planned preclinical investigation and improved targeting strategies will facilitate translation into clinical evaluation to move the field forward. Studies are currently underway to evaluate the potential mechanism(s) of chemotherapeutics in combination with α-particle RIT.

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No potential conflicts of interest were disclosed.

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### References

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$^{212}$Pb-Radioimmunotherapy Induces G$_2$ Cell-Cycle Arrest and Delays DNA Damage Repair in Tumor Xenografts in a Model for Disseminated Intraperitoneal Disease

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