Gene expression profiling upon $^{212}$Pb-TCMC-trastuzumab treatment in the LS-174T i.p. xenograft model

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Abstract

Recent studies have demonstrated that therapy with $^{212}$Pb-TCMC-trastuzumab resulted in (1) induction of apoptosis, (2) G2/M arrest, and (3) blockage of double-strand DNA damage repair in LS-174T i.p. (intraperitoneal) xenografts. To further understand the molecular basis of the cell killing efficacy of $^{212}$Pb-TCMC-trastuzumab, gene expression profiling was performed with LS-174T xenografts 24 h after exposure to $^{212}$Pb-TCMC-trastuzumab. DNA damage response genes (84) were screened using a quantitative real-time polymerase chain reaction (qRT-PCR array). Differentially regulated genes were identified following exposure to $^{212}$Pb-TCMC-trastuzumab. These included genes involved in apoptosis ($ABL$, $GADD45\alpha$, $GADD45\gamma$, $PCBP4$, and $p73$), cell cycle ($ATM$, $DDIT3$, $GADD45\alpha$, $GTSE1$, $MKK6$, $PCBP4$, and $SESN1$), and damaged DNA binding (DDB) and repair ($ATM$ and $BTG2$). The stressful growth arrest conditions provoked by $^{212}$Pb-TCMC-trastuzumab were found to induce genes involved in apoptosis and cell cycle arrest in the G2/M phase. The expression of genes involved in DDB and single-strand DNA breaks was also enhanced by $^{212}$Pb-TCMC-trastuzumab while no modulation of genes involved in double-strand break repair was apparent. Furthermore, the $p73$/$GADD45$ signaling pathway mediated by $p38$ kinase signaling may be involved in the cellular response, as evidenced by the enhanced expression of genes and proteins of this pathway. These results further support the previously described cell killing mechanism by $^{212}$Pb-TCMC-trastuzumab in the same LS-174T i.p. xenograft. Insight into these mechanisms could lead to improved strategies for rational application of radioimmunotherapy using $\alpha$-particle emitters.

Introduction

Alpha particles provoke severe tissue damage and induce clusters of DNA strand breaks that lead to cell death by virtue of their high linear energy transfer (LET of $\sim 100$ keV/\(\mu\)m) and short range in tissue. Thus, $\alpha$-emitters such as $^{212}$Bi and $^{212}$Bi are superior with respect to cell killing to $\beta^-$-emitters which are low LET and possess long range in tissue. In a study involving $\alpha$-radiation exposure of cells, the probability of cell death or loss of reproductive capability was 66% per $\alpha$-traversal of the cell [1]. The unique cell killing capabilities of $\alpha$-radiation make application of immunoconjugates labeled with $\alpha$-emitter radionuclides a promising therapeutic option for the treatment of patients with carcinomas that are characterized by disseminated single tumor cells in the peritoneum such as ovarian or gastric cancer [2, 3]. An $\alpha$-particle emitting source regarded as potentially useful for such therapeutic applications is $^{212}$Pb, the longer lived parental radionuclide of $^{212}$Bi for which $^{212}$Pb serves as an in vivo generator [4].

Exposure of cells to ionizing radiation activates multiple signal transduction pathways, which results in complex alterations in gene expression. The transcriptional responses to genotoxic stresses can be monitored on a global scale using microarray. In addition, specific gene expression can be evaluated with greater accuracy and sensitivity by quantitative real-time polymerase chain reaction (qRT-PCR) [5–7]. Such gene expression profiling
may provide a powerful approach toward understanding the molecular basis of tumor biology in response to radiation. Studies of gene expression in this manner may lead to the identification of radiation responsive genes that could potentially provide biomarkers of radiation exposure.

A recent study from this laboratory demonstrated that the reduction of cell proliferation elicited by the α-emitting radioimmunotherapeutic, $^{212}$Pb-TCMC-trastuzumab, is associated with G2/M arrest, blockage of double-strand DNA damage repair and increased apoptosis [5]. To date, gene modulations associated with α-particle radioimmunotherapy (RIT) have not been defined [9, 10]. Therefore, to better understand the molecular basis of α-particle RIT, the changes in gene expression in response to α-particle irradiation were investigated. The aim of this study was to evaluate the gene expression profile associated with the key biological processes of apoptosis, cell cycle arrest, and DNA repair elicited in LS-174T i.p. (intraperitoneal) xenografts after targeted exposure to $^{212}$Pb-TCMC-trastuzumab. Eighty-four genes involved in DNA damage signaling pathways were quantified using qRT-PCR array 24 h after the $^{212}$Pb-RIT. This study provides data which is among the first to describe in detail, the cellular response to α-particle irradiation in vivo.

**Materials and Methods**

**Cell line**

The human colon carcinoma cell line (LS-174T) was used for the in vivo studies and grown in supplemented Dulbecco’s modified Eagle’s medium (DMEM). All media and supplements were obtained from Lonza (Walkersville, MD). The cell line has been screened for mycoplasma and other pathogens before in vivo use according to the 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 [3]. Trastuzumab (Herceptin®; Genentech, South San Francisco, CA) was conjugated with TCMC by established methods using a 10-fold molar excess of ligand to mAb and radiolabeled as detailed elsewhere [3, 11]. A 10 mCi $^{224}$Ra/$^{212}$Pb generator was purchased from AlphaMed (Lakewood, NJ). Purified polyclonal human IgG (HuIgG) fraction (ICN, Irvine, CA) was similarly conjugated with the TCMC ligand and radiolabeled with $^{212}$Pb as described above, providing a nonspecific control antibody for the experiments.

**Tumor model, treatment, and tumor harvesting**

Studies were performed with 19–21 g female athymic mice (NCI-Frederick) bearing 3 day i.p. LS-174T xenografts as previously reported [3]. The viability of the LS-174T cells (>95%) was determined using trypan blue. Mice were injected i.p. with $1 \times 10^8$ LS-174T cells in 1 mL of DMEM. The inoculum size for this cell line represented the minimum number of cells required for tumor growth in 100% of the mice. $^{212}$Pb-TCMC-trastuzumab (10 μCi in 0.5 mL phosphate buffered saline [PBS]) was administered to the mice 3 days postimplantation of tumor ($n = 10–15$). This treatment group was compared with sets of tumor-bearing mice that received $^{212}$Pb-TCMC-HuIgG, unlabeled trastuzumab or HuIgG, or no treatment. Mice receiving trastuzumab or HuIgG were injected 3 day after tumor implantation with 10 μg of the respective material. Tumors were harvested from mice bearing i.p. LS-174T xenografts at 24 h post-treatment. Tumor tissues were stored at −80°C until use. All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee.

**RNA purification**

Total RNA was isolated from tumor tissues using the RNaseasy mini kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s instruction and stored at −80°C until use for gene expression qRT-PCR arrays. Purity of the isolated total RNA was measured using Nano-drop and PCR with β-actin primers. Only total RNA with an A260/A280 ratio >1.9 and no detectable contamination of DNA (PCR) was employed for the gene expression array (qRT-PCR array).

**Human DNA damage PCR array**

The cDNA was reverse transcribed from RNA using the First Strand cDNA Synthesis Kit (SABiosciences, Frederick, MD). Comparison of the relative expression of 84 DNA damage-related genes was characterized with the human DNA damage signaling pathway PCR array (SABiosciences) and the RT² real-time SYBR Green/Rox PCR master mix (SABiosciences) on a 7500 real-time PCR system (Applied Biosystems, Rockville, MD). The array includes genes involved in apoptosis, cell cycle and DDB and repair (Table S3).

**Immunoblotting**

Immunoblot analysis following standard procedures was performed with total protein isolates using tissue protein...
involved in apoptosis is associated with upregulation of genes
change was applied. 
vested untreated tumor and a threshold of a twofold
HuIgG, and unlabeled antibody were compared to har-
212Pb-TCMC-trastuzumab therapy. All experimental
212Pb-TCMC-trastuzumab-induced cell killing
is associated with upregulation of genes involved in apoptosis
Changes in expression of responsive genes involved in
apoptosis upon treatment of the LS-174T tumor xeno-
grafts with 212Pb-TCMC-trastuzumab and 212Pb-TCMC-
HuIgG as well as to unlabeled HuIgG and trastuzumab
and the cell cycle checkpoint (eight genes). Significant dif-
fferences (>twofold, P < 0.05) in expression were found
for seven genes, upregulation of five (ATM, GADD45x,
map kinase kinase 6 [MKK6], PCBP4, and SESN1) and
downregulation of two (DDIT3 and GTEI) after ex-
posure to 212Pb-TCMC-trastuzumab (Table 2). Upregula-
tion of ZAK did not appear to be significant. The nonspecific
control, 212Pb-TCMC-HuIgG, upregulated MKK6 and

Results
PCR array was used for analyzing gene expression in three
independent experiments. The array identified genes that
were significantly up- or downregulated 24 h after the
212Pb-TCMC-trastuzumab therapy. All experimental
group results, that is, labeled antibody, labeled control
HuIgG, and unlabeled antibody were compared to har-
vested untreated tumor and a threshold of a twofold change was applied.

212Pb-TCMC-trastuzumab-induced cell killing
is associated with upregulation of genes involved in apoptosis

Seven of the 13 genes (ABL, CIDEA, GADD45x,
GADD45γ, IP6K3, PCBP4, and p73) appeared to be up-
regulated following the treatment with 212Pb-TCMC-trast-
zuzumab. Five of the gene changes (ABL, GADD45x,
GADD45γ, PCBP4, and p73) were significant (P < 0.05).
No significant downregulation of genes involved in the
apoptotic process was observed. Remarkably, 212Pb-
TCMC-trastuzumab had a dramatic impact (8.3-fold
increase) on the expression of p73, a tumor suppressor
gene belonging to the p53 family of transcription factors,
and on the stress-response family of GADD45 including
GADD45x (4.1-fold increase) and GADD45γ (10.3-fold
increase). In contrast, 212Pb-TCMC-HuIgG, trastuzumab,
and HuIgG resulted in the upregulation of only the
GADD45γ gene with a 3.5-, 2.9-, and 3.2-fold increase, respectively. The p73 gene seemed to be downregulated in
these same groups, though not significantly except trast-
zuzumab treatment alone. Taken together, the increased
stressful growth arrest conditions imposed by 212Pb-
TCMC-trastuzumab induced cell death-associated genes,
evidenced by the upregulated expression of genes, includ-
ing the GADD45 family and p73 involved in regulation of
the apoptotic processes.

212Pb-TCMC-trastuzumab induces genes
involved in G2/M arrest

Gene expression profiling was examined for genes
involved in the regulation of cell cycle arrest (15 genes)
and the cell cycle checkpoint (eight genes). Significant dif-
fferences (>twofold, P < 0.05) in expression were found
for seven genes, upregulation of five (ATM, GADD45x,
map kinase kinase 6 [MKK6], PCBP4, and SESN1) and
downregulation of two (DDIT3 and GTEI) after ex-
posure to 212Pb-TCMC-trastuzumab (Table 2). Upregula-
tion of ZAK did not appear to be significant. The nonspecific
control, 212Pb-TCMC-HuIgG, upregulated MKK6 and

| Symbol | GeneBank ID | 212Pb-trastuzumab | P-value | 212Pb-HuIgG | P-value | Trastuzumab | P-value | HuIgG | P-value |
|--------|-------------|------------------|---------|-------------|---------|-------------|---------|-------|---------|
| ABL    | NM_005157   | 2.7              | 0.040   | 1.1         | 0.040   | 1.1         | 0.119   | -1.6  | 0.226   |
| CIDEA  | NM_001279   | 4.9              | 0.125   | 1.9         | 0.002   | -1.1        | 0.690   | 1.1   | 0.894   |
| GADD45x| NM_001924   | 4.1              | 0.003   | 1.4         | 0.352   | 1.2         | 0.490   | -1.2  | 0.940   |
| GADD45γ| NM_006705   | 10.3             | 0.037   | 3.5         | 0.072   | 2.9         | 0.028   | 3.2   | 0.025   |
| IP6K3  | NM_054111   | 5.8              | 0.327   | 1.5         | 0.102   | -1.9        | 0.035   | -1.0  | 0.883   |
| PCBP4  | NM_020418   | 2.8              | 0.026   | 1.3         | 0.317   | 1.2         | 0.302   | -1.0  | 0.950   |
| p73    | NM_005427   | 8.3              | 0.039   | -1.1        | 0.578   | -2.2        | 0.009   | -1.1  | 0.850   |

Mice-bearing i.p. LS-174T xenografts were treated with 212Pb-TCMC-trastuzumab for 24 h. qRT-PCR array was used for gene expression analysis in three independent experiments. The numbers indicate fold change (>twofold) compared to the untreated control. Additional groups include nonspecifically targeted control, 212Pb-TCMC-HuIgG, and unlabeled HuIgG, as well as trastuzumab alone. Results represent the average of a mini-
mum of three replicates.
Mice-bearing i.p. LS-174T xenografts were treated with $^{212}$Pb-TCMC-trastuzumab for 24 h. qRT-PCR array was used for gene expression analysis.

### Table 2. Genes expression involved in cell cycle in LS-174T i.p. xenografts by $^{212}$Pb-TCMC-trastuzumab therapy.

| Symbol | GeneBank ID | $^{212}$Pb-trastuzumab | P-value | $^{212}$Pb-HuIgG | P-value | Trastuzumab | P-value | HuIgG | P-value |
|--------|-------------|-------------------------|---------|------------------|---------|-------------|---------|-------|---------|
| ATM    | NM_000051   | 2.6                     | 0.049   | −1.5             | 0.213   | −1.4        | 0.318   | −2.2  | 0.127   |
| DDIT3  | NM_004083   | −2.4                    | 0.002   | −3.5             | 0.001   | −1.8        | 0.074   | −1.6  | 0.162   |
| GADD45x| NM_001924   | 4.1                     | 0.003   | 1.4              | 0.252   | 1.2         | 0.493   | −1.2  | 0.940   |
| GTSE1  | NM_016426   | −7.5                    | 0.001   | −1.7             | 0.144   | −1.4        | 0.144   | −1.1  | 0.478   |
| MKK6   | NM_002758   | 2.8                     | 0.016   | 2.6              | 0.003   | 2.3         | 0.005   | 1.9   | 0.077   |
| PCBP4  | NM_020418   | 2.7                     | 0.026   | 1.3              | 0.317   | 1.2         | 0.302   | −1.2  | 0.959   |
| SESN1  | NM_004454   | 2.3                     | 0.001   | 2.6              | 0.002   | 2.0         | 0.125   | 1.3   | 0.227   |
| ZAK    | NM_016653   | 2.1                     | 0.355   | 1.2              | 0.104   | 1.2         | 0.135   | 1.4   | 0.056   |

Mice-bearing i.p. LS-174T xenografts were treated with $^{212}$Pb-TCMC-trastuzumab for 24 h. qRT-PCR array was used for gene expression analysis in three independent experiments. The numbers indicate fold change (≥twofold) compared to the untreated control. Additional groups include nonspecifically targeted control, $^{212}$Pb-TCMC-HuIgG, and unlabeled HuIgG, as well as trastuzumab alone. Results represent the average of a minimum of three replicates.

### Table 3. Genes expression involved in DNA repair in LS-174T i.p. xenografts by $^{212}$Pb-TCMC-trastuzumab therapy.

| Symbol | GeneBank ID | $^{212}$Pb-trastuzumab | P-value | $^{212}$Pb-HuIgG | P-value | Trastuzumab | P-value | HuIgG | P-value |
|--------|-------------|-------------------------|---------|------------------|---------|-------------|---------|-------|---------|
| ATM    | NM_000051   | 2.6                     | 0.049   | −1.5             | 0.803   | −1.4        | 0.818   | −2.1  | 0.127   |
| ATRX   | NM_000489   | 2.6                     | 0.137   | 1.6              | 0.033   | 1.4         | 0.087   | −1.2  | 0.373   |
| BTG2   | NM_006763   | 7.0                     | 0.005   | 3.7              | 0.003   | 1.9         | 0.003   | 1.4   | 0.269   |
| CRY1   | NM_004075   | −3.3                    | 0.941   | −1.0             | 0.573   | 1.2         | 0.157   | 1.1   | 0.426   |
| DDB1   | NM_001923   | 2.0                     | 0.319   | 1.0              | 0.974   | 1.4         | 0.050   | 1.7   | 0.205   |
| ERCC1  | NM_001983   | 3.0                     | 0.157   | −1.2             | 0.605   | −1.0        | 0.270   | 1.4   | 0.156   |
| ERCC2  | NM_000400   | 2.7                     | 0.209   | 1.4              | 0.001   | 1.6         | 0.704   | 1.0   | 0.588   |
| SEMA4A | NM_022367   | 4.7                     | 0.186   | 1.6              | 0.036   | −1.3        | 0.160   | −1.1  | 0.989   |
| XPC    | NM_004628   | 3.3                     | 0.058   | 2.3              | 0.042   | 1.1         | 0.028   | −1.3  | 0.712   |
| XRCC3  | NM_005432   | 2.7                     | 0.378   | −1.5             | 0.380   | 1.4         | 0.339   | −1.2  | 0.709   |

Mice-bearing i.p. LS-174T xenografts were treated with $^{212}$Pb-TCMC-trastuzumab for 24 h. qRT-PCR array was used for gene expression analysis in three independent experiments. The numbers indicate fold change (≥twofold) compared to the untreated control. Additional groups include nonspecifically targeted control, $^{212}$Pb-TCMC-HuIgG, and unlabeled HuIgG, as well as trastuzumab alone. Results represent the average of a minimum of three replicates.

SESNI and downregulated DDIT3 while trastuzumab alone upregulated MKK6 and SESNI but SESNI upregulation was not significant. Among those genes identified, GADD45x was upregulated (4.1-fold increase) and GTSE1 was markedly downregulated (7.5-fold decrease) by $^{212}$Pb-TCMC-trastuzumab. In contrast, genes involved in cell cycle checkpoint were not significantly modulated in any of the treatment groups. These data suggest that the $^{212}$Pb-TCMC-trastuzumab treatment suppresses cell proliferation by inducing genes which are involved in cell cycle arrest in G2/M.

$^{212}$Pb-TCMC-trastuzumab-induced cell killing is associated with genes which are involved in DDB and repair

Twenty-four hours after x-irradiation with $^{212}$Pb-TCMC-trastuzumab qRT-PCR was next utilized to identify genes involved in DDB (26 genes), single-strand break repair (including nucleotide excision repair (NER; 12 genes), base-excision repair (BER; seven genes), mismatch repair (MR; 14 genes), and double-strand break repair (DSB; nine genes). Among those genes involved in DDB, ten genes (ATM, ATRX, BTG2, DDB1, ERCC1, ERCC2, SEMA4, XPC, and XRCC3 [Table 3], p73 [Table 1]) seemed to be upregulated. Of these only three (ATM, BTG2, and p73) were significantly upregulated. Cry1 was the only gene downregulated after $^{212}$Pb-TCMC-trastuzumab therapy. In contrast, $^{212}$Pb-TCMC-HuIgG resulted in the upregulation of BTG2 and XPC and trastuzumab alone borderline upregulated BTG2. p73 and GADD45 are upregulated by MR-dependent ABL. It has been also shown that MR-dependent intrinsic apoptosis is p53-independent but stimulated by MLH1/ABL/p73/ GADD45 retrograde signaling [12]. p73, which is involved in MR was the only gene in this category that was
upregulated after $^{212}$Pb-TCMC-trastuzumab therapy with a calculated 8.3-fold increase compared to 1.1-fold decrease after $^{212}$Pb-TCMC-HulG therapy. In NER, DDB1, ERCC1, ERCC2, and XPC were seemingly upregulated albeit nonsignificantly, while in BER, no genes were found to be differentially expressed in any of the treatment groups. Among those genes related to DNA repair, $BTG2$ (7.0-fold increase) was marked upregulated while $Cry1$ (3.3-fold decrease) was downregulated by treatment with $^{212}$Pb-TCMC-trastuzumab. The downregulation of $Cry1$, however, did not reach statistical significance. In contrast, $^{212}$Pb-TCMC-HulG resulted in the upregulation of $BTG2$ (3.7-fold increase) and downregulation of $Cry1$ (1.0-fold decrease). Interestingly, among the genes identified in the profile, no genes related to DSB repair were differentially expressed.

$^{212}$Pb-TCMC-trastuzumab induces cell cycle arrest and apoptosis by the p73/GADD45 signaling pathway

Many genes involved in cell cycle arrest and apoptosis are regulated at the transcriptional level by p73 [13]. GADD45 is a well-defined downstream gene of p73 and p53, and is activated by p73. GADD45 has critical roles in negative cell growth control and apoptosis [14, 15]. In response to a DNA damage signal, activated ABL kinase induces phosphorylation of p73 and the p38 mitogen-activated protein (MAP) kinase pathway mediates this response [16]. $^{212}$Pb-TCMC-trastuzumab increased the expression of ABL, p73, and the GADD45 gene family compared to the nonspecific control or trastuzumab alone. To investigate the role of p73/GADD45-induced cell cycle arrest and apoptosis in LS-174T i.p. xenografts from mice treated with $^{212}$Pb-TCMC-trastuzumab, the expression of p73 and GADD45 at the protein level was first determined using immunoblots. Figure 1 shows that phosphorylation of p73 was enhanced by $^{212}$Pb-TCMC-trastuzumab treatment compared to the controls. Expression of the GADD45 genes, downstream of p73, was also enhanced in tumors treated with $^{212}$Pb-TCMC-trastuzumab, indicating that $^{212}$Pb-TCMC-trastuzumab increased the expression of genes involved in apoptosis and cell cycle arrest at both the transcriptional and protein level.

p38 activation is required for the ability of ABL to induce apoptosis, and p38 MAP kinase is required for the activation of p73 by ABL. Activation of MKK6/p38 is sufficient to increase p73 protein levels [16]. The GADD45 family, GADD45α and GADD45γ, have been found to utilize JNK/p38 pathways, leading to G2/M cell cycle arrest [17]. Based on gene profiles induced by $^{212}$Pb-TCMC-trastuzumab, MKK6, an activator of p38, which is required for ABL to induce apoptosis, was significantly upregulated. To examine whether activation of p73/GADD45 induced by $^{212}$Pb-TCMC-trastuzumab is mediated through JNK/p38 pathways, the expression of MKK6 and map kinase kinase 4 (MKK4) at the protein levels were determined using Western blot analysis. The results showed that $^{212}$Pb-TCMC-trastuzumab enhanced phosphorylation of MKK6, an activator of p38, but phosphorylation of MKK4, an activator of JNK was not observed (Fig. 1), suggesting that the stressful growth arrest conditions by $^{212}$Pb-TCMC-trastuzumab may be mediated by the p38 signaling pathway.

**Discussion**

Exposure to radiation leads to complex cellular responses that include changes in gene expression. This behavior appears to be different between low LET γ- or β−-irradiation and high LET α-particle irradiation. The rate of DNA repair in damaged cells after exposure to high LET α-irradiation has been shown to be much slower than the rate observed in cells exposed to low LET irradiation. This is attributed to the ability of α-particles to induce...
multiple ionizations within the DNA structure and in adjacent molecules, resulting in severe locally damaged sites that are less likely to be repaired [4, 18]. This renders $\alpha$-radiation highly efficient in cell killing. Incorporation of $\alpha$-emitters in radioimmunotherapeutics attempts to specifically target $\alpha$-radiation to pathologies in tissue to take advantage of this exquisite cell killing property. Toward this end, the therapeutic efficacy of $^{212}$Pb-TCMC-trastuzumab, an $\alpha$-emitting radioimmunotherapeutic, given as a single injection has been demonstrated [3]. To better understand the molecular basis of $^{212}$Pb-TCMC-trastuzumab-induced cell death in vivo, gene expression profiling was performed to screen for genes affected by $^{212}$Pb-TCMC-trastuzumab in LS-147T i.p. xenografts. The primary target of ionizing radiation in tissue is DNA and the biological responses to the DNA damage signal are complex. Thus, the experimental approach utilized in this study can only hope to identify major pathways likely to dominate the response.

Altogether, 84 genes involved in DNA damage signaling pathways were analyzed by a qRT-PCR array. The genes analyzed here are associated with the ATR/ATM (ataxia telangiectasia mutated) signaling network and transcriptional targets of the DNA damage response. The differentially expressed genes were functionally classified into biological categories (Table S3). Among them, a set of 22 genes were identified that seemed to be differentially expressed 24 h after administration of $^{212}$Pb-TCMC-trastuzumab (Tables S1, S2). The level of differential expression of half of these genes reached statistical significance. Many of the upregulated genes identified in this study are known to play key roles in the DNA damage response, apoptosis, and cell cycle arrest. These include ABL, ATM, $p73$, and the GADD45 family. The findings support the recent study from this laboratory that demonstrated that $^{212}$Pb-TCMC-trastuzumab induces G2/M arrest and apoptosis, in vivo, in the colon cancer LS-147T i.p. xenograft model [8]. The upregulated genes identified in these experiments could favor confinement of the DNA damage by delaying initiation of the cell cycle, leading to induction of apoptosis as a result of $^{212}$Pb-TCMC-trastuzumab therapy. As indicated in the results section, $p73$ appears to be involved in all three major biological responses to DNA damage, namely, DNA damage repair (MR), apoptosis, and cell cycle arrest.

Given the upregulated genes identified, it was hypothesized that $^{212}$Pb-TCMC-trastuzumab may induce G2/M arrest and apoptosis through the $p73$/GADD45 signaling pathway, which might be mediated by p38 kinase signaling. As indicated in Tables 1 and 2, both the ABL and ATM genes were found to be significantly upregulated. ATM is activated in response to DNA damage and signals the presence of DNA damage by phosphorylating targets involved in cell cycle arrest and DNA repair. In mammals, ATM functions as a critical regulator of the cellular DNA damage response. ABL is a nonreceptor tyrosine kinase that is potently activated in DNA damage and is one of several key molecules, including ATM, DNAPK, and p53, involved in coordinating the response to DNA insults [19–22]. In response to a DNA damage signal, activated ABL kinase induces phosphorylation of $p73$ and the p38 MAP kinase pathway mediates this response [14]. ABL has been known to stimulate JNK and p38 kinase and to play a central role in the apoptotic response to DNA damage [23, 24]. ABL/GADD45 control G2 arrest and apoptosis, whereas ABL/p73 is needed in apoptosis and survival. $p73$, a member of the tumor suppressor $p53$ family, is similar to $p53$ both functionally and structurally, and induces cell cycle arrest and cell death in response to DNA damage. $p73$ can activate the promoters of several $p53$ response genes including $p21$, BAX, MDM2, GADD45, and IGFBP3 [13, 23].

The GADD45 (growth arrest and DNA damage-inducible 45) gene family encodes three proteins, GADD45a, $\beta$, and $\gamma$. GADD45 proteins functions are similar, but not identical and their induction differs under diverse physiological conditions or in different cell types. The GADD45 family is $p73/p53$–regulated, a DNA damage-inducible biomarker and a negative growth-control gene that has also been found to be an important player in the DNA damage response, causing cell cycle arrest at the G2/M phase. GADD45 is also known to interact with key cell regulators such as $p21$, CDC2/CYCLIN B1, PCNA, $p38$, and MEEK4 [14, 15]. The GADD45 genes are rapidly regulated by $p53$ which may play a role in apoptosis by activating the JNK and/or p38 mitogen-activated protein kinase (MAPK) signaling pathways [24].

Figure 1 shows that $^{212}$Pb-TCMC-trastuzumab treatment induced phosphorylation of $p73$ and expression of the GADD45 family at the protein level, as evidenced by immunoblot analysis, suggesting that the $p73$/GADD45 signaling pathway is activated in response to $^{212}$Pb-TCMC-trastuzumab. Recently, $p73$ has been found to induce the JNK apoptotic signaling pathway in response to cisplatin in ovarian cancer cells [25]. The induction of apoptosis was shown to be dependent upon the interaction of GADD45 protein with MEEK4, an upstream activator of stress-induced p38/JNK kinases. GADD45a and GADD45$\gamma$ have also been reported to utilize both the p38 and JNK pathways to induce G2M arrest [17]. Figure 1 also shows that $^{212}$Pb-TCMC-trastuzumab therapy results in the phosphorylation of M KK6 (p38 kinase activator) and not M KK4 (JNK kinase activator), suggesting that activation of $p73$ induced by $^{212}$Pb-TCMC-trastuzumab may be mediated by p38 kinase signaling. In response to
\(\gamma\)-irradiation, p38 MAPK is an ATM-dependent event. MKK6 (MAP2K6, mitogen-activated protein kinase kinase 6) is involved in many cellular processes such as stress-induced cell cycle arrest, transcription activation, and apoptosis. The MKK6-p38 cascade forms an essential part of the signaling pathway leading to G2 arrest [26]. Although GADD45, IP6K3 (inositol hexakisphosphate kinase 3), and PCBP4 (Poly(rC)-binding protein 4) have been known to induce apoptosis mediated by p53, activation of p53 was not observed after 212Pb-TCMC-trastuzumab treatment. Apoptotic pathways triggered by high LET radiation do not require p53. The activation of p73 mediated by p38 may, therefore, play an important role in the initiation of apoptotic programs and cell cycle arrest in response to 212Pb-TCMC-trastuzumab in many tumors that lack a functional p53, but do express p73 (Fig. 2).

Cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome. Genes related to DNA repair that were identified to be differentially expressed significantly include ATM and BTG2. BTG2 (BTG family member 2) function may be related to cell cycle control and cellular response to DNA damage [27]. Interestingly, no modulation of genes involved in DSB repair was apparent while the expression of genes involved in DDB and single-strand DNA breaks were enhanced by 212Pb-TCMC-trastuzumab. These results suggest that 212Pb-TCMC-trastuzumab treatment would result in inefficient DSB repair at a time when a high degree of DSBs have occurred as a result of the targeted \(\alpha\)-irradiation. Severe DNA DSBs caused by \(\alpha\)-emitting radiation have been known to be inefficiently repaired, leading to cell death [4].

In conclusion, multiple genes were identified in the colon cancer LS-174T i.p. xenograft model used by this laboratory that responded to 212Pb-TCMC-trastuzumab therapy. Modulation of such genes points to their potential role in apoptosis, cell cycle, and DNA repair after exposure to acute high LET radiation. These genes could provide a potent research tool for examination of the therapeutic responses to and mechanisms of \(\alpha\)-particle RIT for the treatment and management of patients with disseminated peritoneal disease. Identifying genes pivotal in patients’ responses may also provide direction for optimizing combination therapies. Insight into these mechanisms could lead to improved strategies for RIT with \(\alpha\)-emitters and promote the clinical translation of such agents.

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**Conflict of Interest**

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. Genes upregulated in LS-174T i.p. xenografts by 212Pb-TCMC-trastuzumab.
Table S2. Genes downregulated in LS-174T i.p. xenografts by 212Pb-TCMC-trastuzumab.
Table S3. Functional gene grouping.
Table S4. Gene symbols and name.