Potentiation of High-LET Radiation by Gemcitabine: Targeting HER2 with Trastuzumab to Treat Disseminated Peritoneal Disease

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Abstract

Purpose: Recent studies from this laboratory with $^{212}$Pb-trastuzumab have shown the feasibility of targeted therapy for the treatment of disseminated peritoneal disease using $^{212}$Pb as an in vivo generator of $^{212}$Bi. The objective of the studies presented here was improvement of the efficacy of $\alpha$-particle radioimmunotherapy using a chemotherapeutic agent.

Experimental Design: In a series of experiments, a treatment regimen was systematically developed in which athymic mice bearing i.p. LS-174T xenografts were injected i.p. with gemcitabine at 50 mg/kg followed by $^{212}$Pb radioimmunotherapy.

Results: In a pilot study, tumor-bearing mice were treated with gemcitabine and, 24 to 30 h later, with 5 or 10 $\mu$Ci $^{212}$Pb-trastuzumab. Improvement in median survival was observed at 5 $\mu$Ci $^{212}$Pb-trastuzumab in the absence (31 days) or presence (51 days) of gemcitabine: 45 and 70 days with 10 $\mu$Ci versus 16 days for untreated mice ($P < 0.001$). Multiple doses of gemcitabine combined with a single $^{212}$Pb radioimmunotherapy ($10 \mu$Ci) administration was then evaluated. Mice received three doses of gemcitabine: one before $^{212}$Pb-trastuzumab and two afterwards. Median survival of mice was 63 versus 54 days for those receiving a single gemcitabine dose before radioimmunotherapy ($P < 0.001$), specifically attributable to $^{212}$Pb-trastuzumab ($P = 0.01$). Extending these findings, one versus two treatment cycles was compared. A cycle consisted of sequential treatment with gemcitabine, 10 $\mu$Ci $^{212}$Pb radioimmunotherapy, then one or two additional gemcitabine doses. In the first cycle, three doses of gemcitabine resulted in a median survival of 90 versus 21 days for the untreated mice. The greatest benefit was noted after cycle 2 in the mice receiving 10 $\mu$Ci $^{212}$Pb-trastuzumab and two doses of gemcitabine with a median survival of 196.5 days ($P = 0.005$). Pretreatment of tumor-bearing mice with two doses of gemcitabine before $^{212}$Pb radioimmunotherapy was also assessed with gemcitabine injected 72 and 24 h before $^{212}$Pb-trastuzumab. The median survival was 56 and 76 days with one and two doses of gemcitabine versus 49 days without gemcitabine. The effect may not be wholly specific to trastuzumab because $^{212}$Pb-HuIgG with two doses of gemcitabine resulted in a median survival of 66 days (34 days without gemcitabine).

Conclusions: Treatment regimens combining chemotherapeutics with high-LET targeted therapy may have tremendous potential in the management and care of cancer patients.

Despite recent advances in the treatment of ovarian and pancreatic cancer, new strategies remain a high priority. The majority of patients with either of these cancers routinely present with late-stage disease, and for patients that undergo surgery, removal of microscopic disease is difficult, if not impossible. Targeted radiation therapy with monoclonal antibodies (mAb), armed with a radionuclide that react with a “tumor”-associated antigen, may be efficacious in the coordinated strategy for the treatment and management of disease in these patients.

The exquisite cytotoxicity of targeted $\alpha$-particle radiation has been hypothesized as an appropriate therapeutic modality for treatment of smaller tumors/tumor burdens, disseminated disease, micrometastatic disease, and for eradication of malignant single cells. Because only three to six transversals of a cell nucleus result in an estimated dose of 70 to 100 cGy, $\alpha$-particle radiation is cytotoxic at a dose rate as low as 1 cGy/h (1, 2). The short path length associated with $\alpha$-particle radiation may also limit toxicity to normal tissues adjacent to tumor. Isotopes that are suitable for this application are limited by physical characteristics, such as half-life, or by commercial/economical ($^{211}$Bi) or production ($^{211}$At) availability. Within these boundaries, we chose to evaluate the feasibility of treating disseminated peritoneal disease with $\alpha$-particle radiation.
This laboratory recently showed the efficacy of two different α-emitting radionuclides in a peritoneal model for ovarian and pancreatic cancer using trastuzumab as the targeting moiety (3, 4). HER2 is overexpressed in several epithelial tumors, including 35% to 45% of all pancreatic adenocarcinomas, 25% to 30% ovarian cancers, and 4% to 83% colorectal adenocarcinomas (5–7). A specific dose response was observed when trastuzumab was radiolabeled with either 213Bi or 212Pb.

Studies that exploited 212Pb as an in vivo generator of 212Bi clearly showed the feasibility of this isotope for targeted therapy treating disseminated peritoneal disease (4). Specifically, whereas HER2 was targeted using trastuzumab, the results therein also showed that at the protein doses used, the mAb itself provided no therapeutic benefit (3). Thus, all responses originated from the site-specific delivery of the high-LET radiation. A specific dose response was observed, and a dose of 10 βCi was selected as the effective operating dose for future experiments. Its selection was based on the observation of minimal toxicity (weight loss) experienced by the mice that would also permit differences in responses to treatment regimens to be discerned (e.g., when 212Pb radioimmunotherapy was evaluated with other modalities such as chemotherapy; ref. 4). Median survival of mice bearing LS-174T i.p. tumor that received 10 βCi 212Bi-trastuzumab increased from 3 to 8 weeks. Radioimmunotherapy using 212Pb also showed an effective response in a human pancreatic carcinoma (Shaw xenograft previously described as unresponsive to radioimmunotherapy with 211Bi-trastuzumab (3)). Multiple dosing of 212Bi-trastuzumab was also evaluated in both animal models. Three doses of 212Pb-trastuzumab given at, approximately, monthly intervals increased median survival by 7.3-fold in the LS-174T i.p. xenograft model. However, no improvement in median survival was noted when applying a similar dose regimen in the Shaw xenograft model.

Gemcitabine (Gemzar, 2’,2’-difluoro-2’-deoxyuridine), a nucleoside analogue that inhibits DNA synthesis, has been found to have therapeutic efficacy as a single modality against a variety of tumors (8–10). Gaining Food and Drug Administration approval in 1998, Gemzar has rapidly become a standard of systemic therapy, and clinical trials are being conducted combining gemcitabine with radiotherapy (11–13, 16–24). Typical of systemic therapy, however, radiosensitization also affects normal tissues, resulting in dose-limiting toxicities that present daunting challenges. Although standard radiotherapy procedures do not easily or efficiently treat distant, undetected metastatic or disseminated disease, a series of preclinical studies have reported promising results with gemcitabine combined with targeted radiation therapy (13, 21, 22, 24). Specifically, gemcitabine has been administered with PAM4, an anti-MUC1 mAb. The gemcitabine was administered at the dose given to patients (1,000 mg/m²/wk), either as a single weekly dose or as three doses in a week. PAM4 also has been evaluated with two β-emitting radionuclides: 131I and 90Y (22, 24). Inclusion of gemcitabine was found to lower the maximum tolerated dose of 90Y-PAM4 by 2.6-fold; however, the combined modality was still effective in inhibiting s.c. tumor growth (22). Studies were also conducted at lower doses of 90Y-PAM4 (25 βCi), with the objective of lessening normal tissue toxicity. The lower dose of 90Y-PAM4 provided benefit and extended median survival (24).

Simply stated, the hypothesis of the studies reported herein was that gemcitabine would potentiate the therapeutic efficacy of 212Pb-trastuzumab in conjunction with targeted delivery of high-LET radiation. To the best of our knowledge, there have been a very limited number of reports of targeted delivery of high-LET radiation applied in a combination therapy scenario. Although others, as noted above, have pursued β-emitters, our focus has been on the discrete killing of metastatic and small disease with α-emitters. Secondary to addressing this hypothesis and showing that goal was to establish a multimodality regimen for the treatment of disseminated i.p. disease targeting the HER2 molecule.

**Materials and Methods**

**Cell lines.** The human colon carcinoma cell line (LS-174T) was used for in vivo studies. A human ovarian carcinoma cell line, SKOV-3 (American Type Culture Collection, Manassas, VA), that expresses high levels of HER2 was used for in vitro analyses (25). LS-174T was grown in supplemented DMEM as previously described (26). SKOV-3 cells were maintained in McCoy’s 5a supplemented with 10% fetal bovine serum and 1 mmol/L nonessential amino acids. All media and supplements were obtained from Quality Biologicals (Gaithersburg, MD).

**Chelate synthesis and mAb conjugation.** The synthesis, characterization, and purification of the bifunctional ligand TCMC has been previously described (27, 28). Trastuzumab was conjugated with TCMC by established methods using a 10-fold molar excess of ligand to mAb (29). The protein concentration was quantified by the method of Lowry (30). The number of TCMC molecules linked to the mAb was determined using a spectrophotometric-based assay (31).

**Radioimmunotherapy.** A 5- to 10-mCi 224Ra/212Pb generator was supplied by Alphamed, Inc. (Acton, MA). The preparation of the generator and radioimmunoabsorption procedures have been previously detailed (4) The radioimmunotherapy reaction included ascorbic acid (22 μg), 5 mol/L NH₄OAc to pH 4.5 to 5.0, and TCMC-trastuzumab (300 μg). Following a 1-h incubation at 37°C, the reaction was quenched with EDTA, and the radioimmunoglobulin was purified using a PD-10 desalting column (GE Healthcare, Piscataway, NJ). HuIgG (ICN, Irvine, CA) was similarly conjugated with TCMC and radiolabeled with 212Pb, as described above, as a negative control. A calibrated Ge(Li) detector (Model 60101185-P, EG&G/Ortec, Oak Ridge, TN) coupled to a multichannel analyzer Gamma Vision version 5.2 software (EG&G/Ortec) was used to determine the activity of the 212Pb by measurement of the 238.6 KeV γ-ray (43.6%).

**RIA.** Immunoreactivities of the radiolabeled preparations were assessed in a RIA as detailed previously using SKOV-3 (29). SKOV-3 cells express HER2 at ~5 × 10⁶ receptors per cell (32). LS-174T cells (75-90%) express HER2; however, with a mean fluorescence intensity of ~30, the expression is low (3). When used in a RIA, the percent bound (10-15%) by LS-174T cells is too low to discern differences in immunoreactivity.

**Therapy studies.** Radioimmunotherapy studies were done using 19 to 21 g female athymic mice (Charles River Laboratories, Wilmington, MA). The mice were injected i.p. with 1 × 10⁶ cells LS-174T as previously reported (33). Gemcitabine (Gemzar; Eli Lilly and Company, Indianapolis, IN) was prepared in PBS, and 1 mg (0.5 mL, 50 mg/ kg) was administered i.p. at the indicated times as described in Results for each experiment. 212Pb-TCMC-trastuzumab was administered to the mice 3 days after inoculation of tumor. Doses of 212Pb-TCMC-trastuzumab were prepared in PBS and administered (n = 7-10) via i.p. injection in 1 mL. The mice were weighed twice a week throughout...
their treatment regimens and for 3 to 4 weeks following the last injection of radioimmunotherapy or gemcitabine.

Mice were monitored and euthanized if found to be in distress, moribund, or cachectic. Mice were also euthanized when 10% to 20% weight loss occurred, or disease progression was evident (i.e., swollen abdomen, development of ascites, or obvious palpable nodules in the abdomen). All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee.

In experiment 1, athymic mice (n = 7-8) with 2-day i.p. LS-174T xenografts were given i.p. injections of gemcitabine followed 24 to 30 h later with $^{212}$Pb-trastuzumab or $^{212}$Pb-HuIgG. Additional groups of mice included those that received either radiolabeled antibody alone, gemcitabine only, or no treatment. Two doses of $^{212}$Pb (5 and 10 μCi) were compared.

Experiment 2 was designed to assess the effects of multiple doses of gemcitabine combined with a single treatment of $^{212}$Pb-trastuzumab. Following the same scheme as described above, mice (n = 8-9) bearing i.p. LS-174T xenografts were treated with gemcitabine followed by the $^{212}$Pb-labeled trastuzumab or HuIgG. In this instance, additional sets of mice were administered two more doses of gemcitabine at 1-week intervals and were compared with those that had received one treatment of gemcitabine.

In experiment 3, the treatment regimen was extended to two cycles in which a cycle consisted of gemcitabine administered 24 to 30 h before $^{212}$Pb radioimmunotherapy. Groups of mice (n = 12-20) then either received none, one, or two more doses of gemcitabine thereafter at weekly intervals. One half of all of these groups of mice then underwent a second cycle of treatment 3 weeks after the administration of the first $^{212}$Pb radioimmunotherapy.

A fourth experiment evaluated the potential of two doses of gemcitabine administered before $^{212}$Pb radioimmunotherapy. Mice (n = 10) with a 2-day tumor burden (LS-174T, i.p.) were injected with gemcitabine. A second dose was administered at 120 h followed 24 to 30 h later by $^{212}$Pb-trastuzumab or $^{212}$Pb-HuIgG. This was compared with mice that had received only one dose of gemcitabine as well as mice that were untreated, had received one, or had two doses of gemcitabine only, or $^{212}$Pb-trastuzumab, or $^{212}$Pb-HuIgG only.

**Statistical analyses.** A Cox proportional hazards model was used to test for the relationship between the treatment and survival (time to sacrifice or natural death). The dose level was treated as a linear covariate in the Cox model and tested whether the corresponding regression variable was zero using a likelihood ratio test.

For the animal weight data, the maximum percent reduction from baseline was estimated for each mouse. This was calculated as the ratio of the maximum reduction in weight from baseline during the 4-week monitoring period (when the animals were weighed between twice and thrice a week) divided by the baseline weight of the mouse. Boxplots were constructed for each dose level, which show the median, upper and lower quartiles, as well as identifying outliers. Differences between dose groups were tested using a Kruskal-Wallis test (nonparametric test for the relationship between the treatment and survival (time to sacrifice or natural death). The dose level was treated as a linear regression variable was zero using a likelihood ratio test.

Radiolabeling of trastuzumab with $^{212}$Pb resulted in a final product consistent with published studies (4). Immunoreactivity was evaluated using SKOV3 cells: values of 62.4% were obtained with trastuzumab, whereas no binding (0.1%) of $^{212}$Pb-HuIgG with was observed. Specific activities of 11.8 ± 5.7 and 11.7 ± 3.5 mCi/mg were obtained for $^{212}$Pb-trastuzumab and $^{212}$Pb-HuIgG, respectively.

Based on published data, 10 μCi $^{212}$Pb-trastuzumab was determined to be the administered i.p. dosage. However, in the event that gemcitabine proved to be an effective potentiator of α-particle radiation, a lower dose of $^{212}$Pb-trastuzumab (5 μCi) was also evaluated. Before initiating in vivo studies, the sensitivity of LS-174T, a colon adenocarcinoma cell line, and Shaw, a pancreatic carcinoma cell line, to gemcitabine were evaluated in a cytotoxicity assay. The IC$_{50}$ was determined to be 9 and 1.8 mmol/L, respectively (data not shown). These values were within range of literature values for other cell lines (34).

**Results**

Fig. 1. Effect of gemcitabine in combination with $^{212}$Pb radioimmunotherapy. Kaplan-Meier survival curves of mice (n = 7-8) bearing i.p. LS-174T xenografts that were treated with 1 mg gemcitabine (i.p.), and 24 h later, $^{212}$Pb-labeled trastuzumab or HuIgG was administered. The mice received gemcitabine alone (○), $^{212}$Pb-trastuzumab (●), gemcitabine followed by $^{212}$Pb-trastuzumab (□), $^{212}$Pb-HuIgG (▲), or gemcitabine followed by $^{212}$Pb-HuIgG (◇) at doses of 5 μCi (A) or 10 μCi (B) and compared with mice that did not receive any therapy (★).
10 μCi $^{212}$Pb-trastuzumab, the median survival improved to 70 days (Fig. 1B). The combination of gemcitabine with 5 and 10 μCi $^{212}$Pb-trastuzumab increased the median survival by 35 and 54 days, respectively. The improvement in survival was specific in that the median survival of the mice receiving 5 or 10 μCi $^{212}$Pb-HuIgG was only 20 and 24 days. When the mice were pretreated with gemcitabine and then administered $^{212}$Pb-HuIgG, the median survival was 35 and 55 days. At 140 days, of those mice that received 5 μCi $^{212}$Pb-trastuzumab, one of eight mice were still alive; two of eight of the gemcitabine/$^{212}$Pb-trastuzumab group survived; whereas none injected with gemcitabine and 5 μCi $^{212}$Pb-HuIgG remained alive. Meanwhile, mice that were untreated, treated with gemcitabine, or with 5 μCi $^{212}$Pb-HuIgG succumbed to disease by 20, 55, and 32 days, respectively. Gemcitabine in combination with 10 μCi $^{212}$Pb-trastuzumab or $^{212}$Pb-HuIgG resulted in 1 of 7 and 0 of 7 survival to 140 days. In the absence of gemcitabine, all of the mice receiving $^{212}$Pb-HuIgG were euthanized by 31 days, whereas one of seven animals injected with $^{212}$Pb-trastuzumab remained at the termination of the experiment at 158 days.

The standard regimen of gemcitabine therapy in patients involves three cycles of weekly gemcitabine followed by a week of rest. Within this context, a multi-dose therapy (study 2) was conducted in which mice were given gemcitabine (1 mg) followed by administration of $^{212}$Pb-trastuzumab (10 μCi) 24 to 30 h later. At 1 and 2 weeks after administration of the radioimmunotherapy, sets of mice were then given additional doses of gemcitabine. The study also included groups that received only a single dose of gemcitabine and $^{212}$Pb-labeled trastuzumab or HuIgG. Additional groups included mice injected with gemcitabine (one or three doses) alone, or $^{212}$Pb radioimmunotherapy alone (Fig. 2).

In the experiment just described, the mice receiving a single injection of gemcitabine and $^{212}$Pb-trastuzumab experienced a median survival of 39 days. This is an increase of 2 weeks compared with the untreated group, 9 days compared with mice receiving $^{212}$Pb-trastuzumab, and 17 days compared with mice receiving gemcitabine alone (Fig. 2A).

Some therapeutic benefit was derived from three doses of gemcitabine when combined with $^{212}$Pb-trastuzumab. The median survival of mice that received three weekly injections of gemcitabine was 35 days, an improvement of 13 days over that achieved with a single dose of gemcitabine (Fig. 2B). When $^{212}$Pb-trastuzumab was added into the treatment regimen, the median survival increased to 63 days. This improvement seems to be specific to $^{212}$Pb-trastuzumab. The median survival of mice receiving one dose of gemcitabine and $^{212}$Pb-HuIgG was 27 days, whereas three injections of gemcitabine resulted in a median survival of 38 days. Mice receiving $^{212}$Pb-trastuzumab showed a significantly longer survival than mice injected with $^{212}$Pb-HuIgG ($P = 0.01$). Three weekly doses of gemcitabine also seemed to improve the overall survival of tumor-bearing mice treated with $^{212}$Pb-trastuzumab. No mice remained of those receiving a single treatment of gemcitabine followed by $^{212}$Pb-trastuzumab 54 days after the radioimmunotherapy. In contrast, two of nine animals given three doses of gemcitabine and 10 μCi $^{212}$Pb-trastuzumab remained alive when the experiment was terminated at 133 days. Groups injected with 10 μCi $^{212}$Pb-HuIgG and either the one or three doses of gemcitabine were all euthanized by 27 and 46 days, respectively, due to progression of disease.

Differences among groups can also be discerned when changes in weights are compared as a measurement of toxicity. The maximum % relative weight reduction was calculated and plotted for each treatment group (Fig. 3). Gemcitabine alone does seem to result in weight loss that increased with each dose ($P = 0.09$, Kruskal-Wallis test). There is also increased toxicity by this measure when $^{212}$Pb-trastuzumab was incorporated as part of the regimen ($P = 0.07$). This higher toxicity was not observed in the corresponding groups that were given just $^{212}$Pb-HuIgG.

The efficacy of administering multiple cycles of $^{212}$Pb radioimmunotherapy with gemcitabine was examined in therapy study 3. As outlined in the scheme shown in Fig. 4, one versus two cycles of therapy was compared. A full therapy cycle was defined as gemcitabine given 24 to 30 h before $^{212}$Pb-trastuzumab followed thereafter by two additional injections of gemcitabine at 1-week intervals. Cycle 2 began immediately the week after completion of cycle 1 without rest. Control groups of mice included untreated and those that received single agents and combined modality with the non-targeting HuIgG.
Additionally, permutations on incomplete therapy cycles were included. The median survival of each of the groups is presented in Table 1. Consistent with the data just described, gemcitabine as a single agent, given once, had negligible effects on the LS-174T tumor. Following three doses, the median survival is 1.4-fold greater ($P = 0.033$). The median survival of the mice receiving one and two gemcitabine doses in cycle 2 differs little from the corresponding groups of cycle 1. The third dose, again, seems to have the greatest effect on median survival (62 days).

The data from cycle 1, when $^{212}$Pb-trastuzumab is administered as a single agent, show that the median survival increases to 66 days (versus 18.5 days for untreated mice; $P < 0.001$) and 33 days for $^{212}$Pb-HuIgG. Three weekly doses of gemcitabine in conjunction with $^{212}$Pb-trastuzumab resulted in the greatest therapeutic efficacy with a median survival of 90 days. This effect seems to be specific to trastuzumab in that the same treatment regimen with HuIgG resulted in a median survival of only 56 days.

Further improvement in median survival was observed for those groups that were carried into cycle 2 of the treatment regimen. Specifically, the greatest improvement was obtained in those mice that received the $^{212}$Pb-trastuzumab (total = 2) and two doses of gemcitabine (total = 4). The combination of one
dose of gemcitabine and $^{212}$Pb-trastuzumab resulted in a median survival of 90 days, a 4.86-fold increase of survival. Two doses of gemcitabine and $^{212}$Pb-trastuzumab resulted in a 10.6-fold increase in survival, with a median of 196.5 days. A third dose (total = 6) of gemcitabine does not seem to provide any benefit and may in fact be deleterious. The number of gemcitabine doses after a second injection of $^{212}$Pb-HuIgG failed to affect median survival. Throughout cycle 2, there is little difference observed between any of the $^{212}$Pb-HuIgG control groups. There is a significant difference in the median survival between the groups in cycle 1 and cycle 2 that received the two doses of gemcitabine ($P = 0.005$). The survival curves for the groups in cycle 2 that received a second administration of $^{212}$Pb radioimmunotherapy and two doses of gemcitabine are presented in Fig. 5. When the experiment was terminated after 260 days, 3 of 10 mice were still alive. In contrast, no animals remained in any of the control groups, with the exception of one mouse in the untreated set.

In vitro studies have shown that when cells are treated with gemcitabine for 2 h, there is an accumulation of the cell population into the S phase for up to 48 h. The cell cycle distribution then reverts to a pattern that resembles untreated cells by 72 h (11). It was hypothesized that multiple treatments of gemcitabine before the $^{212}$Pb radioimmunotherapy would result in greater arrest of cells in S phase, thereby increasing therapeutic efficacy. A study (therapy study 4) was conducted in which mice ($n = 7-10$) received gemcitabine 2 and 5 days after tumor implantation followed 24 h later by 10 $\mu$Ci $^{212}$Pb-labeled trastuzumab or $^{212}$Pb-HuIgG. Also included in the study were groups that were untreated or that had received only gemcitabine, $^{212}$Pb-trastuzumab, or $^{212}$Pb-HuIgG; two sets of mice were treated with a single dose of gemcitabine followed by either $^{212}$Pb-labeled trastuzumab or HuIgG.

Treatment with 10 $\mu$Ci $^{212}$Pb-trastuzumab (Table 2) resulted in a median survival of 49 versus 20 days for the untreated group. The median survival increased to 56 days with a single dose of gemcitabine given 24 h before the radioimmunotherapy.

Table 1. Median survival of athymic mice bearing i.p. LS-174T xenografts following combined modality treatment regimen of gemcitabine and $^{212}$Pb-radioimmunotherapy

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<th>Treatment</th>
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<th>Cycle 2</th>
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<tr>
<td></td>
<td>Untreated</td>
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<tr>
<td></td>
<td>Gemcitabine 2x</td>
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<tr>
<td>Gemcitabine 3x</td>
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<th>HuIgG</th>
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<td>HuIgG</td>
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<tr>
<td>Gemcitabine 1x</td>
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<td>33 (1.9)</td>
<td>56 (3.0)</td>
<td>38 (2.1)</td>
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<td>90 (4.9)</td>
<td>39 (2.1)</td>
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<tr>
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<td>55.5 (3.0)</td>
<td>92.5 (5.0)</td>
<td>40.5 (2.2)</td>
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</table>

NOTE: Athymic mice ($n = 6-10$) bearing i.p. LS-174T xenografts received one or two cycles of treatment consisting of gemcitabine injected 24 h before $^{212}$Pb-labeled antibody (10 $\mu$Ci) and then zero, one, or two doses of gemcitabine (1 mg) given at 1-wk intervals. Values in table expressed as median survival (therapeutic index). Therapeutic index is the median survival divided by the median survival of the untreated group.

Fig. 5. Potentiation of $^{212}$Pb-trastuzumab therapy by gemcitabine. Mice bearing 3-day LS-174 i.p. xenografts were pretreated with gemcitabine (i.p.) 24 h before $^{212}$Pb radioimmunotherapy. Mice were then given one or two additional doses of gemcitabine weekly. This treatment cycle was repeated in a second set of mice. Kaplan-Meier survival curves depicting the results of the mice that underwent two cycles of treatment as follows (refer to Fig. 4 for the groups): group 1 (●), group 6 (○), group 12 (■), group 14 (○), group 20 (▲), and group 22 (○).
The median survival of mice pretreated with two doses of gemcitabine before $^{212}$Pb-trastuzumab increased to 76 days ($P = 0.015$). In the groups of mice receiving the nonspecific $^{212}$Pb-HuIgG, median survival was 42 and 66 days for the single and two weekly doses of gemcitabine, respectively ($P = 0.31$). The median survival of mice injected with $^{212}$Pb-HuIgG only was 34 days. Treatment with two weekly gemcitabine doses alone resulted in some improvement in the median survival of the mice: 44 days for two gemcitabine doses versus 25 days after one dose ($P = 0.004$). Comparing the overall survival, 4 of 10 mice receiving two doses of gemcitabine before $^{212}$Pb-trastuzumab were alive at 150 days versus two mice that received only $^{212}$Pb-trastuzumab, whereas none of those receiving the gemcitabine alone and none of those that received a single dose of gemcitabine before $^{212}$Pb-trastuzumab remained alive. One mouse survived in each of the groups that were given $^{212}$Pb-HuIgG.

Using weight changes (Fig. 6) as an indicator of toxicity in this study, differences between the animals that received one or two doses of gemcitabine followed by $^{212}$Pb-trastuzumab were not significant ($P = 0.29$). In contrast, a statistical significance in weight changes was found between the groups given $^{212}$Pb-HuIgG after one or two doses of gemcitabine ($P = 0.006$).

### Discussion

HER2, expressed in a variety of epithelial cancers, is proving to be an ideal target for radioimmunotherapy (5, 6, 35, 36). An advantage of using trastuzumab as a vehicle for targeting $\alpha$-particle radiation over its use as monotherapeutic agent is that not every cell need express HER2. Indeed, the effectiveness of $\alpha$-particle radiation does not require high HER2 expression within the tumor, and with cytotoxicity occurring at a dose rate of 1 cGy/h, only a few $\alpha$-particles need be delivered to a tumor cell to inflict death (1 – 4, 37, 38). Particle decay radiates in all directions: neighboring cells may receive cytotoxic doses, and the bystander effect may compensate for not only tumor heterogeneity but may also overcome in part the challenge of tumor cell accessibility.

The high LET of $\alpha$-particle radiation and short path length, although not ideal for large burden disease, has been proposed as ideal for treatment of smaller tumor burdens, micrometastatic disease, and disseminated disease (3, 4, 39, 40). These same physical characteristics may also lessen normal tissue toxicity. Those $\alpha$-emitters that have been used in radioimmunotherapy applications include $^{212}$Bi ($t_{1/2} = 60.6$ min), $^{213}$Bi ($t_{1/2} = 45.6$ min), $^{211}$At ($t_{1/2} = 7.2$ h), and $^{225}$Ac ($t_{1/2} = 10$ days), and the advantages/disadvantages of each of these radionuclides has been discussed elsewhere (1, 3, 4, 40 – 46). Studies from this laboratory have shown the effectiveness of both $^{213}$Bi- and $^{212}$Pb-trastuzumab in the treatment of peritoneal disease (3, 4). Although not an $\alpha$-emitter itself, $^{212}$Pb, which decays to the $\alpha$-emitter $^{212}$Bi, has been exploited as an in vivo generator (47). The decay of $^{212}$Pb to $^{212}$Bi results in a $\beta$-emission of only an $E_{\beta_{max}}$ of 0.3 MeV. There is then an $\alpha$- and $\beta$-emission as $^{212}$Bi decays to $^{208}$Tl ($6.1$ MeV) and $^{212}$Po ($2.3$ MeV), respectively. Another $\alpha$-emission (8.8 MeV) occurs when $^{212}$Po decays to $^{208}$Pb, and a $\beta$-emission (1.8 MeV) occurs with the decay of $^{208}$Tl also to $^{208}$Pb. Although the 2.3 MeV $\beta$-emission may contribute

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**Table 2.** Median survival of athymic mice bearing i.p. LS-174T xenografts following combined modality treatment regimen of gemcitabine and $^{212}$Pb-radioimmunotherapy

<table>
<thead>
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<th>Treatment</th>
<th>Median survival (therapeutic index)</th>
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<tr>
<td>Untreated</td>
<td>20 (1.0)</td>
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<td>25 (1.3)</td>
</tr>
<tr>
<td>Gemcitabine 2×</td>
<td>44 (2.2)</td>
</tr>
<tr>
<td><strong>Trastuzumab</strong></td>
<td></td>
</tr>
<tr>
<td>mAb</td>
<td>49 (2.5) 34 (1.7)</td>
</tr>
<tr>
<td>Gemcitabine 1× + mAb</td>
<td>56 (2.8) 42 (2.1)</td>
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<tr>
<td>Gemcitabine 2× + mAb</td>
<td>76 (3.8) 66 (3.3)</td>
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NOTE: Athymic mice ($n = 10$) bearing i.p. LS-174T xenografts were treated with one or two doses of gemcitabine (1 mg, i.p.) before radioimmunotherapy with 10 μCi of $^{212}$Pb-labeled antibody.

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**Fig. 6.** Effect of multiple gemcitabine doses before $^{212}$Pb-trastuzumab radioimmunotherapy on animal weight as an indicator of toxicity. Data presentation is as described for Fig. 3. Tumor-bearing mice received one or two doses of gemcitabine before $^{212}$Pb radioimmunotherapy. Groups included mice that were given either $^{225}$Pb-Herceptin or $^{212}$Pb-HuIgG as well as no therapy at all.
to the therapeutic efficacy of the $^{212}$Pb radioimmuno-therapy, the two α-emissions with energies of 6.1 and 8.8 MeV are the dominating therapeutic contributors. In vitro studies have shown superior cytotoxicity of $^{212}$Pb over $^{212}$Bi. If any benefit were being derived from the β-emission, then one might expect the data of $^{212}$Bi-Herceptin to more closely resemble that obtained with the $^{212}$Pb-trastuzumab. The possible contribution of the β-emission may be more evident in the initial studies conducted with $^{212}$Pb-trastuzumab (4). Even when a larger tumor burden was present at the time of therapy with $^{212}$Pb-trastuzumab, therapeutic efficacy was observed. This is consistent with the hypothesis that β-radiation is more appropriate for the treatment of larger tumor lesions/burdens. Additionally, effective β-emission doses are generally an order of magnitude greater than those employed here. Lastly, dosimetry studies by Hamacher and Sgouros regarding the use of α-emission/β-emission – targeted radiation largely discount the accompanying β-emission as contributing meaningful benefit (48). A far more likely scenario to combine an α-emission with a β-emission would be to fold in a full dose of a β-emitter such as $^{177}$Lu. One goal of the studies presented herein was to explore the potential of combining the chemotherapeutic agent gemcitabine to enhance the efficacy of $^{212}$Pb-trastuzumab radioimmunotherapy. The LS-174T tumor xenograft has been proven an effective model to show the efficacy of $^{212}$Pb radioimmuno-therapy and has been used by other investigators as a model for ovarian cancer (33, 49). A second tumor model using a human pancreatic carcinoma xenograft (Shaw), a far less aggressive model, has also been employed to validate results (3, 4).

Overall survival for pancreatic patients (all stages) is <5% (50). This dire statistic reflects the fact that the majority of patients are diagnosed with advanced disease. Systemic therapy rather than surgical resection or radiation therapy then becomes the treatment option for the patient. This limited choice highlights the need for developing new approaches and refining strategies for the management of pancreatic cancer patients (51). Considering that 35% to 40% of patients have pancreatic cancer that express HER2, targeted therapy. Gemcitabine has been found to interfere with DNA synthesis by (a) depleting deoxynucleotide pools through the inhibition of ribonucleotide reductase and (b) by interfering with DNA synthesis either directly or by interfering with elongation of the DNA strand by blocking DNA polymerases (14, 55–57). Inhibition of chromosome repair following irradiation has also been shown by increasing the frequency of chromosome breaks (58, 59). This cytotoxic activity of gemcitabine has been found to be effective against a variety of tumor types, including pancreatic, head and neck, lung, breast, bladder, and ovarian (54).

Milas et al. published a set of studies wherein they sought to optimize gemcitabine in combination with external beam therapy. Gemcitabine was found to enhance the radioresponsiveness of sarcomas (SA-NH tumors) in a time-dependent fashion (21). The greatest inhibition of tumor growth was obtained when a single dose of gemcitabine (50 mg/kg) was administered 24 h before irradiation. Data from other investigators, although done at a higher dose of gemcitabine (60 mg/kg), not only confirmed these findings but also showed that these higher doses did not provide any additional benefit (23).

Based on these reports, gemcitabine (50 mg/kg) given 24 h before radioimmunotherapy was selected as the operating dose of the studies described herein. The first experiment explored the potential of gemcitabine with two different doses of $^{212}$Pb-trastuzumab (5 and 10 μCi); the median survival was 31 and 45 days using 5 or 10 μCi $^{212}$Pb-trastuzumab, respectively, consistent with previous data as a monotherapy (4). When gemcitabine was administered before radioimmunotherapy, a clear improvement was observed with a 1.6-fold increase in median survival observed at both doses. Concurrently, the LS-174T tumor was minimally responsive to gemcitabine monotherapy. When compared with the untreated group, median survival increased 3.2- and 4.3-fold from the combination of gemcitabine with 5 and 10 μCi $^{212}$Pb-trastuzumab. This enhancement in therapeutic efficacy is superior to that previously reported in the literature (21, 24). Comparisons with other published radioimmunotherapy studies remain difficult, however, because tumor models, radionuclide, route of administration, the targeted molecule, and antibody differ. The fact that gemcitabine also increased median survival of mice that were injected with $^{212}$Pb-HuIgG, the nonspecific control antibody, attests to the radiosensitizing action of gemcitabine, which is then further exploited by the specific targeting of $^{212}$Pb-trastuzumab.

Gemcitabine is administered to pancreatic patients weekly for up to 7 weeks, with a week of rest followed by three more weekly treatments. As a component of optimizing a multimodality treatment regimen, a $^{212}$Pb-trastuzumab therapy was studied to evaluate the benefit of weekly doses versus one gemcitabine dose given before radioimmunotherapy. Gemcitabine was administered 24 h before $^{212}$Pb-trastuzumab followed by two additional weekly doses of gemcitabine to mimic the regimen used in clinical trials evaluating the effectiveness of gemcitabine in patients undergoing radiotherapy (19). Gemcitabine given thrice with $^{212}$Pb-trastuzumab not only increased median survival but also improved overall survival: 22% of the animals receiving the three doses of gemcitabine were still alive when the experiment was terminated at 133 days (Fig. 2).

To further optimize a chemo-radioimmunotherapy regimen, an experiment was executed that extended the previous study to compare one versus two cycles of three weekly doses of gemcitabine with $^{212}$Pb radioimmunotherapy. Gemcitabine given thrice with $^{212}$Pb-trastuzumab and then divided into groups that received either no, one, or two additional doses of gemcitabine at 1-week intervals. At the end of that cycle, one half of each of those
groups of mice underwent a second treatment cycle. Consistent with the earlier study, $^{212}$Pb-trastuzumab radioimmunotherapy with three doses of gemcitabine showed the highest therapeutic index of 4.84 (treatment group median survival divided by the median survival of the untreated group), and the response was specific to trastuzumab. A higher therapeutic index (10.60) was obtained during the second course of treatment. Interestingly, that value was obtained after two doses of gemcitabine with the radioimmunotherapy. In other words, mice that received two 10-μCi doses of $^{212}$Pb-trastuzumab and a total of four 1-mg doses of gemcitabine showed the greatest response. In this group of mice, there was a rest period of 2 weeks before the second cycle of treatment began. It should also be noted that the greatest difference between $^{212}$Pb-trastuzumab and $^{212}$Pb-HuLgG groups was observed in this treatment group, with median survivals of 196.5 and 52 days, respectively.

Following treatment with gemcitabine, in vitro studies have shown an accumulation of cells in S phase for up to 48 h (9, 11, 17, 60). In vivo, the effect may be longer because the therapeutic enhancement of radiotherapy was observed when gemcitabine was given anywhere from 24 to 96 h before irradiation (13). An additional dosing regimen of gemcitabine was explored in an attempt to maximize the properties of gemcitabine as a radiosensitizer with this source of targeted radiation. Tumor-bearing mice were treated with gemcitabine 2 and 5 days after tumor implantation followed 24 h later with $^{212}$Pb-trastuzumab. Some therapeutic benefit was observed when two gemcitabine doses were administered to the mice before the radioimmunotherapy. However, the advantage of trastuzumab specificity may also have been diminished. The median survival of mice that received $^{212}$Pb-trastuzumab was 76 days, whereas it was 66 days for $^{212}$Pb-HuLgG. Pursuit of this particular treatment regimen will require careful further refinement of gemcitabine dose, timing, and frequency.

The maximum tolerated dose of $^{212}$Pb-trastuzumab had been determined to be between 20 and 40 μCi (4). The effective operating dose (10 μCi) for the studies described herein was chosen (a) due to the minimal toxicity (weight loss) experienced by the mice and (b) to permit differences in responses to treatment regimens to be discerned (e.g., when $^{212}$Pb radioimmunotherapy was evaluated with other modalities such as chemotherapy). In fact, studies with treating a s.c. pancreatic tumor (CaPan 1) with $^{90}$Y-PAM4 had determined the maximum tolerated dose to be 260 μCi (61). When gemcitabine is combined with $^{90}$Y-PAM4, the maximum tolerated dose is actually lowered to 100 μCi (21). Gemcitabine (2 mg) was given every 3 days, for a total of five injections. Those investigators found that the combination of radioimmunotherapy and gemcitabine extended the period of tumor growth inhibition, with a median survival of ~ 10 to 12 weeks. When two cycles of gemcitabine were administered with concomitant $^{90}$Y-PAM4, tumor regression was observed, and the median survival was ~ 21 weeks (22). A lower dose (25 μCi) of $^{90}$Y-PAM4 was also evaluated with the same dose of gemcitabine (6 mg) administered per week albeit as a single dose (versus three injections per week in the study previously mentioned; ref. 21). In this case, median survival was extended from 16 up to 24 weeks for the $^{90}$Y-PAM4 alone and with gemcitabine, respectively. The latter treatment regimen may have been better tolerated by the mice as evidenced by lesser changes in body weight during the treatments. It is not surprising that the lower dose of $^{90}$Y-PAM4 may have been more effective, certainly from the view point that lower normal tissue toxicity was probably incurred. The use of gemcitabine permitted the evaluation of whether radiosensitization of high-LET radiation could be significant. Although the mechanism of interaction between gemcitabine and high-LET radiation versus low-LET radiation may in fact be different, or not, the determination of this remains an independent study. This would be very interesting to execute in that differential modulation of gene expression between high- and low-LET radiation has been noted (62). As such, studies are planned to be incorporated in parallel to future therapy studies.

These investigations suggest that regimens combining chemotherapeutics and high-LET radioimmunotherapy may have tremendous potential in the management and treatment of cancer patients. Therapeutic regimens employing paclitaxel and carboplatin in concert with targeted α-particle radiation are currently under evaluation.

References

Gemcitabine Potentiation of High-LET Radioimmunotherapy


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Potentiation of High-LET Radiation by Gemcitabine: Targeting HER2 with Trastuzumab to Treat Disseminated Peritoneal Disease

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