Combination Therapy with Pretarget CC49 Radioimmunotherapy and Gemcitabine Prolongs Tumor Doubling Time in a Murine Xenograft Model of Colon Cancer More Effectively Than Either Monotherapy

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ABSTRACT
Pretarget radioimmunotherapy (RIT) is a multistep strategy for cancer therapy designed to reduce nontarget organ exposure by uncoupling the tumor targeting moiety from the radioactive ligand. Using this approach, we and others have demonstrated objective responses to therapy among patients with non-Hodgkin’s lymphoma, with less hematological toxicity than is typically seen at equivalent doses of conventional RIT in the same patient population. In the present study, we show that combination therapy with gemcitabine (200 mg/kg on days 1 and 1) and Pretarget RIT (400 μCi 90Y-labeled DOTA-biotin on day +1) is superior to Pretarget monotherapy (400 or 800 μCi 90Y) as well as to gemcitabine monotherapy in nude mice bearing established human LS174T colon cancer xenografts. For the targeting moiety, we used a murine anti-TAG-72 (CC49) single-chain Fv-streptavidin (scFvSA) fusion protein that has been shown to be safe and well-tolerated in humans. The median number of days to tumor volume doubling in the gemcitabine-only studies (200 mg/kg) was 10.4 ± 5.5 days; in the Pretarget 400 μCi dose-only studies, tumor doubling time was 6.7 ± 4.9 days; and in combination therapy studies, it was 23.9 ± 7.2 days (P < 0.0001 versus Pretarget or gemcitabine monotherapy). There were no consistently significant differences among the two monotherapy regimens and the combination therapy regimen with respect to peripheral blood cell counts, nor were there significant differences in bone marrow colony-forming activity among the three treatment groups. These data indicate that gemcitabine can be combined with Pretarget RIT to increase antitumor response, without increasing hematological toxicity, in a murine xenograft model.

INTRODUCTION
Mab-based RIT is a promising treatment modality for hematological and, to a lesser extent, solid malignancies (reviewed in Refs. 1 and 2). The efficacy of RIT is limited by the following: slow elimination of the targeting agent from the blood, resulting in dose-limiting radiation exposure of hematopoietic tissue (3); intrinsic variability in radiosensitivity of the tumor (4); immunogenicity of the targeting agent, limiting the number of doses of RIT that can be administered (5); and low and/or heterogeneous uptake of the labeled antibody by the tumor target (6).

Pretarget RIT is a multistep method that attempts to address the first limitation by uncoupling the targeting agent from the radionuclide. The radionuclide is administered in a separate step, after facilitated clearance of non-tumor-bound targeting agent from circulation (7–9). Because the radionuclide is delivered on a small molecule (M, <1,000) that is rapidly eliminated from the body, nontarget organ exposure to circulating radiation is effectively reduced by this approach. Objective responses to Pretarget RIT have been demonstrated among patients with non-Hodgkin’s lymphoma, with less hematological toxicity than is typically seen at equivalent doses of conventional RIT in the same patient population (10, 11). These studies used the CD20 Mab C2B8 (Rituximab) conjugated to SA as the targeting agent (10) or a CD20 fusion protein composed of recombinant scFv derived from Mab B9E9 and SA (11, 12).

CC49 is a murine IgG1 Mab, shown to react with the disaccharide sialyl Tn epitope, expressed on the high-molecular-weight mucin TAG-72 (13). This epitope is expressed on ~85% of human adenocarcinomas including most colon, breast, pancreatic, ovarian, prostate, non-small cell lung, and gastric cancers. It is not appreciably expressed on normal adult tissues, with the exception of secretory endometrium (14). CC49 has been used in RIT in a variety of clinical studies and, in the majority of cases, has demonstrated successful tumor localization (15–20). However, it has not often produced objective antitumor responses, presumably because of the inability to deliver sufficiently high doses of radioactivity to the tumor
before reaching dose-limiting toxicity. Hence, we decided to evaluate a CC49 scFv/SA fusion protein in the Pretarget format, combined with gemcitabine (2', 2'difluoro-2', deoxycytidine). We reasoned that combining the marrow-sparing effect of Pretarget RIT with the radiation-potentiating effect of gemcitabine (21, 22) might enable us to augment the antitumor activity of RIT while minimizing non-target organ toxicity. Furthermore, gemcitabine is a Food and Drug Administration-approved agent for the treatment of tumors that generally express the CC49 antigen, such as non-small cell lung and pancreatic cancer (23).

MATERIALS AND METHODS

Genetic Constructs. The targeting agent used in this study was expressed as a genetic fusion of the single-chain variable regions (scFv) of murine CC49 with the full-length, genomic SA of Streptomyces avidini. The heavy-chain (VH) and light-chain (VL) DNA fragments of CC49 were synthesized by PCR sewing reactions using a set of oligonucleotides designed from their DNA sequence data (GenBank accession nos. L14549 and L14553, respectively), which had been further optimized based on Escherichia coli codon usage. The PCR fragments were cloned, and DNA sequences were confirmed. The VH and VL fragments were cloned into an E. coli periplasmic expression vector as described previously (12, 24).

Fusion Protein Expression and Purification. The soluble, mature CC49 scFv/SA fusion protein was produced in the periplasmic space of E. coli XL1-Blue (Stratagene, La Jolla, CA), as described previously (12, 24). Briefly, transformants containing the scFv/SA plasmid were grown at 30°C in a 14-liter fermentor using a galactose fed-batch protocol. The culture was induced with isopropyl-β-D-thiogalactopyranoside (0.2 mM) at 6 h postinoculation and harvested 46 h later. The periplasmic scFv/SA expression levels were quantified using a rhodamine-derivatized biotin high-performance liquid chromatography assay. Fusion protein was purified to >95% homogeneity from crude cell lysates by immunoblot affinity chromatography.

Fusion Protein Characterization. CC49 fusion protein was analyzed according to previously described methods (12) and shown to have the expected molecular weight (M, 176,000), to be immunoreactive with bovine submaxillary mucin, and to bind biotin at an average of 3.7 of 4 possible sites. The biotin dissociation rate was essentially identical to that of recombinant SA. In vivo, the fusion protein targeted LS174T human colon carcinoma xenografts in nude mice, exhibiting a terminal elimination half-life of ~10 h.

Pretarget Biodistribution and Therapy Studies. All of the animal studies were conducted under the supervision of the NeoRx Animal Care and Use Committee. For all studies using the Pretarget RIT regimen, animals were maintained on a bunitin-deficient diet (Purina Biotin Deficient Diet 5836; Purina Mills, Richmond, IN) from 5 days before study initiation until 5 days after administration of the radioligand (7).

Biodistribution studies were conducted using female BALB/c nu/nu mice (B & K Universal, Kent, WA) bearing 100–200 mm3 LS174T human colon adenocarcinoma (CL 188: American Type Culture Collection, Manassas, VA) xenografts. To obtain tumor-bearing animals, tissue culture-maintained LS174T cells (5 × 106) were injected s.c. in the flanks 5–6 days before the initiation of the study. CC49 fusion protein was radioiodinated using N-succinimidyl-p-[125I]iodobenzyl (NEN Research Products, Boston, MA) as described previously (25). Groups of five mice were given injections (t = 0 h) of 0.6 µg of [125I]-fusion protein. Animals were given a single 100-µg i.v. injection (t = 20 h) of the synthetic clearing agent (sCA), biotin/LC-NM-(GalNAc)16 (26). In a final step, mice were given injections i.v. (t = 24 h) of 1.0 µg (10 µCi) of 111In-labeled DOTA-Biotin (7). Blood samples were collected immediately before sacrifice and dissection of mice at t = 26, 48, 72, and 144 h. Incorporation radioactivity was measured using a gamma counter (Packard Cobra II Gamma Counter, Packard Instruments, Meriden, CT). For therapy studies, the same mass dose of reagents and schedule of administration were used, except that the fusion protein was unlabelled and the DOTA-biotin ligand was labeled with either 400 or 800 µCi of 90Y instead of 111In.

Gemcitabine Therapy Studies. Gemcitabine (Gemzar; Lilly Corporation, Indianapolis, IN) was solubilized in sterile saline and administered i.p. in doses ranging from 50 to 200 mg/kg.

Combination Therapy Studies. Combination therapy studies were performed according to the scheme in Fig. 1. Gemcitabine was administered i.p. on days −1 and +1. Pretarget RIT was administered in three i.v. steps beginning on day 0, with injection of the fusion protein, followed 20 h later by the clearing agent, and followed 4 h later by 90Y-labeled DOTA-biotin. Occasionally, gemcitabine was given as a single dose on day +1. The dose of gemcitabine was either 100 or 200 mg/kg, and the dose of 90Y was either 400 or 800 µCi, depending on the experiment. The mass of fusion protein and clearing agent was never varied.

Tumor Measurements. Tumor growth was assessed by taking two-dimensional measurements of tumor diameter using a micrometer. Tumor volume was calculated using the formula: V = π/6(L + W)2H, where L and W are orthogonal measurements of tumor diameter.

BrdUrd Incorporation in Tumor Xenografts. The effect of gemcitabine on cell cycle progression was assessed by measuring BrdUrd incorporation in tumors from treated ani-

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4 Unpublished data.
mals. The low dose of BrdUrd used was significantly less than doses conventionally used to inhibit DNA synthesis and has been shown not to affect cell cycle progression at this level (27). Tumor-bearing mice were treated with gemcitabine (50 or 200 mg/kg, i.v.) at various times before the injection of BrdUrd (5 mg/mouse, i.p.). Two h later, the tumors were removed and frozen in OCT medium (Sakura, Ltd., Tokyo, Japan) in liquid nitrogen. Cryostat sections were made using a Frigocut 2800 cryostat (Reichert-Jung, Heidelberg, Germany) set at −20°C. Sections (7-μm) were cut and placed on chrome-alum gel-subbed slides (Esco, Portsmouth, NH). Slides were dried and fixed in ice-cold acetone for 90 s. After fixation, slides were dried again and stored at −70°C until stained. BrdUrd uptake was visualized with a BrdUrd staining kit (Zymed, South San Francisco, CA; Cat. no. 93-3943) that stained positive cells brown. Slides were counterstained with hematoxylin, dehydrated in a series of alcohols, cleared in Histoclear (National Diagnostics, Atlanta, GA), and coverslipped with HISTOMOUNT (National Diagnostics) for light microscopic analysis. Photomicrographs of tumor sections were taken through a light microscope (BX40) with a Nikon Coolpix 995 digital camera.

**Toxicity Studies.** Hematopoietic toxicity of Pretarget RIT monotherapy, gemcitabine monotherapy, and combination therapy was evaluated as follows. Non-tumor-bearing BALB/c mice were given i.v. injections of PBS, gemcitabine (2 doses of 200 mg/kg, 48 h apart), or Pretarget RIT (400 or 800 μCi) according to the schedule described above. Alternatively, mice were injected with 200 μCi 90Y-labeled DOTA-biotin/CC49 fusion protein (referred to as complex) to mimic a radiolabeled whole antibody. The complex was prepared by reacting CC49 fusion protein (4.12 mg/ml or 24 μM) with 32 μM 90Y-labeled DOTA-biotin for 3 min, followed by the addition of ascorbic acid (0.5 mg/ml) and DTPA (5 mM, final concentration) to minimize radiolysis and chelate any free radiometal, respectively. The radiochemical purity of the complex, as assessed by high-performance liquid chromatography with a γ-ray detector, was greater than 99%. Blood was sampled from the retroorbital plexus at baseline (before study initiation) and on days 7 and 14 posttreatment. Blood was collected in tubes containing EDTA, and complete blood count analysis was performed by Phoenix Central Laboratory (Everett, WA).

Bone marrow colony-formation assays were performed using marrow harvested from the long bones of mice treated as described above. Femurs were removed from mice on days 7 or 14 posttreatment, and the bone marrow was flushed using a 22-g needle and 2 ml of Iscove’s Modified Dulbecco’s Medium-2% fetal bovine serum (BioWhittaker,Walkersville, MD). Bone marrow cells were counted using a hemocytometer and 2 × 10⁴ cells were cultured in 1.1 ml of Methocult medium (Stemcell Technologies, Vancouver, BC). Fourteen days after initiation of the culture, the number of CFU-GM (cluster of more than 20 cells) in each dish was counted using an inverted microscope (Nikon) at ×4 power.

**Data Analyses and Statistics.** Response to therapy was assessed based on the number of days required for tumor volume to double after the initiation of therapy on day 0 (28). A reduction of ≥50%, sustained for at least 7 days, was considered a partial response (PR). A complete response (CR) was one in which the tumor disappeared for a minimum of 7 days. A cure (C) was defined as disappearance of the tumor and failure to regrow during the period of the study (typically >34 days).

A Mann-Whitney U test was used to determine the level of significance between groups using mean days-to-tumor-volume-doubling as the variable. Alternatively, t tests were used to compare the effect of gemcitabine and PBS on hematopoietic toxicity. A regression model was fit, incorporating gemcitabine (mg/kg), dose of 90Y (μCi), a variable indicating whether Pretarget RIT was used, and a variable indicating whether gemcitabine dosing was done over a single day or multiple days. Thus, the model was designed to estimate the effects of gemcitabine, 90Y, and their interaction with and without Pretarget RIT as well as to determine the effect of multiple dosing. Changes in animal body weight because of therapy were not taken into account in these analyses. The regression analyses were performed using SAS statistical software V8.2 (SAS Institute, Cary, NC).

**RESULTS**

**Biodistribution of Pretarget CC49 Fusion Protein.** We assessed the ability of CC49 fusion protein to localize radioactivity to tumor in nude mice bearing established, human LS174T colon carcinoma xenografts. This was accomplished using 125I-fusion protein, followed by clearing agent, followed by 111In-labeled DOTA-biotin.

As can be seen in Fig. 2A, the fusion protein localized to both tumor and liver. Liver uptake and retention is attributable to complexation of the SA-containing fusion protein with biotin-containing clearing agent. The resultant complex binds to Asialo-GM1 receptors on hepatocytes via the N-acetylglactosamine moiety on the clearing agent, leading to internalization and catabolism over time. A slight, transient increase in kidney concentration of the iodine radiolabel can also be seen in Fig. 2A. This is believed to be caused by hepatic metabolism of 125I-fusion protein and subsequent renal excretion of the radiolabel. Because the targeting agent is not normally labeled in Pretarget RIT, the presence of radioactivity in nontarget organs in this study is inconsequential from a therapeutic perspective. A high concentration of 125I-fusion protein targeted the tumor, relative to the blood, in which it was retained with peak levels of >5%ID/g (≈230 pm/g).

Colocalization and retention of 111In-labeled DOTA-biotin at the tumor were also observed after administration of CC49 fusion protein and clearing agent (Fig. 2B). The highest concentration, both stoichiometrically and relative to blood pool concentration, occurred at the tumor at all time points. Peak concentrations of 111In-labeled DOTA-biotin at the tumor were 22–28%ID/g and occurred by 2 h postadministration of the DOTA-biotin. Tumor:blood ratios increased from 4:0.2 h postadministration, to >1600 by 24 h. The area under the curve (AUC) for blood was 28, whereas the AUC for tumor was 1394, resulting in a specificity index of almost 50. 111In-labeled DOTA-biotin concentration in blood and most well-perfused soft tissues was very low, generally <2%ID/g. Significant liver uptake of 111In-labeled DOTA-biotin was not observed, indicating that nontumor bound fusion protein was efficiently cleared via the clearing agent, rendering it unavailable to bind subsequently administered radiolabeled DOTA-biotin. These data demonstrate that CC49 fusion protein is an effective tar-
the injection of CC49 scFvSA to assess biodistribution of pretargeted
Additional groups of mice were sacrificed at 26, 48, 72, or 144 h after
injection of CC49 scFvSA (100 mg/kg). Animals were given i.v. injections of 125I-labeled
cC49 scFvSA (600 µg) at t = 0 h, clearing agent at t = 20 h, and
111In-labeled DOTA-Biotin (1.0 µg) at t = 24 h. Mice were sacrificed
at 2, 24, 48, and 120 h after the injection of CC49 scFvSA (A). Additional groups of mice were sacrificed at 26, 48, 72, or 144 h after
the injection of CC49 scFvSA to assess biodistribution of pretargeted
111In-labeled DOTA-biotin (B). Tissues sampled were Bl, blood; Ta,
tail; Lu, lung; Ll, liver; Sp, spleen; St, stomach; Ki, kidney; In, intestine;
Tu, tumor. Data are presented as the mean percentage injected dose (%) of injected dose per gram of tissue of each whole organ ± SD.

Fig. 2 Biodistribution of 111In-labeled DOTA-Biotin (111In-DOTA-biotin) in a Pretarget RIT study using LS174T xenograft-bearing nude mice (n = 5/group). Animals were given i.v. injections of 125I-labeled
CC49 scFvSA (600 µg) at t = 0 h, clearing agent at t = 20 h, and
111In-labeled DOTA-Biotin (1.0 µg) at t = 24 h. Mice were sacrificed
at 2, 24, 48, and 120 h after the injection of CC49 scFvSA (A). Additional groups of mice were sacrificed at 26, 48, 72, or 144 h after
the injection of CC49 scFvSA to assess biodistribution of pretargeted
111In-labeled DOTA-biotin (B). Tissues sampled were Bl, blood; Ta,
tail; Lu, lung; Ll, liver; Sp, spleen; St, stomach; Ki, kidney; In, intestine;
Tu, tumor. Data are presented as the mean percentage injected dose (%) of injected dose per gram of tissue of each whole organ ± SD.

Effect of Gemcitabine on LS174T Cell Cycle Progression. To determine a suitable dose and schedule of administration for gemcitabine in combination therapy studies, we evaluated the effect of the drug on cell cycle progression in vivo in tumor-bearing nude mice. This was accomplished by measuring BrdUrd incorporation into tumor DNA.

The results indicate that gemcitabine (200 mg/kg) blocked DNA synthesis in the tumor by about 90% as early as 2 h postinjection (Fig. 3). This effect was sustained for at least 24 h, with more than 50% reduction still evident 40 h later. By contrast, a 50-mg/kg dose of gemcitabine effectively inhibited DNA synthesis for only about 24 h (data not shown).

On the basis of these data, a dose of 200 mg/kg of gemcitabine on days −1 and +1 appeared to be the most suitable for subsequent combination therapy studies, because the period of cell cycle arrest would coincide with the period of Pretarget RIT treatment. Nonetheless, in several studies, we evaluated a lower dose of 100 mg/kg and at one, rather than two, doses, as discussed below.

In Vivo Antitumor Activity of Monotherapy and Combination Therapy. We assessed the efficacy of Pretarget RIT monotherapy, gemcitabine monotherapy, and combination therapy in the LS174T xenograft model, according to the schedule depicted in Fig. 1, using tumor-volume-doubling time as the study end point. The goals of the study were to demonstrate an improvement in antitumor effect with the combination therapy regimen and to assess its hematological toxicity.

Untreated, LS174T is an aggressively growing xenograft with a mean tumor-volume-doubling time of 5.25 ± 0.7 days (n = 6 studies, see Table 1). As expected, the CC49 fusion protein alone had no effect on doubling time whereas a slight (1.4-fold) decrease in tumor doubling time was seen with 90Y-labeled DOTA biotin.

Using the full Pretarget regimen at 90Y doses of 400 and 800 µCi did not significantly delay mean tumor doubling time relative to untreated controls (6.7 ± 4.9 days and 14.9 ± 8.3 days, respectively), although the 800-µCi dose did result in a single cure. Gemcitabine monotherapy (200 mg/kg, on day −1 and +1) also failed to significantly delay mean tumor doubling time (10.4 ± 5.5 days) relative to untreated controls. However, the addition of two doses of gemcitabine (200 mg/kg, on day −1 and +1) to Pretarget RIT, at either the 400- or the 800-µCi dose, significantly delayed mean tumor doubling time (23.9 ± 7.2 and 36.5 ± 12, respectively; P < 0.0001) relative to either monotherapy (Table 1). Nine partial responses and one cure were seen among 29 animals by combining gemcitabine with Pretarget RIT at 400 µCi, whereas one partial response was seen in a group of eight mice treated with the combination at the 800-µCi dose.

Next, we evaluated the effect of a lower dose of gemcitabine on the antitumor activity of the combination therapy. Gemcitabine was administered at 100 mg/kg on days −1 and +1, with Pretarget RIT at 400 µCi. Again, a significant delay in mean tumor doubling time was seen with the combination therapy relative to the corresponding monotherapies (see Table 1; P < 0.005). However, when the lower dose of gemcitabine was combined with the higher dose of Pretarget (800 µCi), a significant delay in mean tumor doubling time was seen only relative to gemcitabine treatment alone (Table 1; P < 0.0006). Overall, these data suggest that combination therapy with gemcitabine and Pretarget RIT leads to significant enhancement of antitumor activity relative to monotherapy with either agent and that this effect is dependent on the dose of gemcitabine used.

In Fig. 4, we compare the effects of each monotherapy and combination therapy on tumor growth, expressed as percentage of initial tumor volume over time in three different studies. Gemcitabine or Pretarget RIT as monotherapy is only moderately effective in delaying tumor growth of the LS174T xenograft. Again, the combination therapy at the 400- and 800-µCi dose of Pretarget was superior to either monotherapy (Fig. 4, A and B). However, fractionating the dose of gemcitabine over a 4-day period (50 mg/kg on days −1 to +2) failed to
enhance the therapeutic effect of Pretarget RIT at the 400-μCi dose (Fig. 4C).

The results of the above studies are reflected in animal survival, as shown in Kaplan-Meyer plots (Fig. 5). The combination of gemcitabine at the two-dose schedule of 200 mg/kg and Pretarget RIT resulted in an extended survival, relative to either monotherapy, regardless of the dose of Pretarget RIT used.

Table 1 Effect of schedule and dose of Pretarget RIT, gemcitabine, or combination therapy on growth of LS174T xenografts in mice

<table>
<thead>
<tr>
<th>Schedule and dose</th>
<th>No. of studies/Total no. of mice</th>
<th>Level of significance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Estimated median (±SD) time to 200% of initial volume (days)</th>
<th>Response</th>
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<td></td>
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<td>36.5 ± 12</td>
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<sup>a</sup> Level of significance comparing combined therapy with Pretarget alone/combined therapy with gemcitabine alone at equivalent doses.

<sup>b</sup> PR, partial response. Tumor volume decreases by 50% for a minimum of 7 days.

<sup>c</sup> CR, complete response. Tumor disappears for a minimum of 7 days.

<sup>d</sup> C, cure. Tumor disappears and fails to regrow for more than 34 days plus absence of tumor on necropsy.
Lack of Toxicity with Combination Therapy. Improvements in antitumor activity with combination chemotherapy are often achieved at the expense of increased toxicity. To assess this, we compared the body weights of animals treated with gemcitabine or with Pretarget RIT monotherapy and combination therapy. As shown in Fig. 6, there was a transient weight loss of 20% in animals receiving either gemcitabine alone or gemcitabine plus Pretarget. After about 4–5 days, however, weight gain resumed and, by the end of the study, there was no longer a significant difference in body weight among the treatment groups. With respect to Pretarget RIT monotherapy, there was no weight loss at the 400-µCi dose, but there was a transient drop with the 800-µCi dose. This drop was less pronounced than with the gemcitabine-treated animals but was of approximately the same duration, after which weight gain resumed.

These results suggest that combination therapy with gemcitabine and Pretarget RIT is well tolerated in nude mice. However, the dose-limiting toxicity in humans for gemcitabine is myelosuppression. Hence, we evaluated peripheral blood cell counts in vivo and bone marrow colony-forming activity ex vivo in non-tumor-bearing BALB/c mice exposed to PBS, gemcitabine (200 mg/kg for two doses), and 99Y-labeled DOTA-biotin (400 µCi), or gemcitabine plus 99Y-labeled DOTA-biotin, administered according to the schedule outlined in Fig. 1.

As shown in Table 2, there was no significant decrease in WBC count among the experimental groups and the corresponding controls, with the exception of the gemcitabine-Pretarget RIT group on day 7. However, this difference was seen only when compared with the gemcitabine monotherapy group. There was, however, a statistically significant decrease in the number of peripheral blood platelets in the gemcitabine-Pretarget RIT group on day 14 (P < 0.05). Of unknown significance, the gemcitabine-only group showed a statistically significant increase in the number of WBCs and platelets relative to the control on day 7 that was not observed at baseline or on day 14.

Table 3 compares the RBC count and CFU-GM count among the experimental groups and the corresponding controls.
Gemcitabine alone resulted in a significant but reversible decrease in RBCs on day 7, relative to the PBS control and relative to \( ^{90} \)Y-labeled DOTA-biotin alone. There were no consistent differences in the number of CFU-GM among any of the experimental groups, relative to the corresponding controls, indicating that the combination therapy is no more myelosuppressive than either of the monotherapies.

Lack of Hematological Toxicity Is Attributable to Rapid Clearance of \(^{90}\)Y-labeled DOTA-biotin. Pretarget RIT is distinguished from conventional RIT in that the radioisotope \(^{90}\)Y, in this case, is delivered as a low-molecular-weight chelate that clears rapidly from the circulation, predominantly via renal excretion (7). When used in conjunction with a facilitated clearance step to remove non-tumor-bound targeting agent from the circulation, the net effect is a reduction in the exposure of nontarget, radiosensitive organs, such as the bone marrow, to radiation. The hematological toxicity studies above indicated that 400 \( \mu \)Ci of \(^{90}\)Y, delivered on DOTA-biotin, can be combined safely with gemcitabine, as predicted from our knowledge of DOTA-biotin pharmacokinetics. However, we wondered whether the same amount of radioactivity delivered as conventional RIT would be similarly safe.

To address this question, we complexed \(^{90}\)Y-labeled DOTA-biotin with CC49 fusion protein before administration to simulate the longer half-life species that would be used in conventional RIT. We then evaluated this complex in BALB/c mice, alone and in combination with gemcitabine. At 400 \( \mu \)Ci of \(^{90}\)Y, the complex alone (without gemcitabine) resulted uniformly in death. This observation is consistent with earlier studies in which \(^{90}\)Y was directly conjugated to the Mab and the MTD of a directly conjugated Mab was established at 200 \( \mu \)Ci (7). Hence, in the studies presented in Tables 2 and 3, the complex was formed using a sublethal dose of 200 \( \mu \)Ci of radioactivity.

As shown in Table 2, the combination of \(^{90}\)Y-complex with gemcitabine exhibited significantly greater hematological toxicity than the combination of uncomplexed radioligand and gemcitabine. WBC count was significantly decreased on day 7 and was still significantly decreased on day 14, although the count appeared to be rising again. The platelet count was also significantly decreased on day 7 and continued to decrease through day 14. Likewise, the number of CFU-GM in bone marrow on days 7 and 14 were severely reduced (Table 3). However, there was not as pronounced an effect on RBC count. These results suggest that the lack of hematological toxicity seen when Pretarget RIT is combined with gemcitabine is attributable to the relatively short-life of the low-molecular-weight, radiolabeled ligand used in Pretarget, as compared with a longer-lived radiolabeled antibody or antibody fragment. Hence, Pretarget RIT may be uniquely suited to combination therapy with a radiation-potentiating agent such as gemcitabine, because it provides enhanced antitumor activity without a concomitant increase in toxicity.

DISCUSSION

Pretarget RIT has proven to be a versatile method of delivering comparatively high doses of radioactivity to tumors while sparing the marrow. Early studies using a NRLu-10/SA conjugate to pretarget \(^{90}\)Y to Ep-CAM antigen-positive adenocarcinomas in humans suggested that the MTD of radioisotope was in the range of 100 mCi/m\(^2\), considerably more than with conventional RIT. Unfortunately, the Ep-CAM antigen proved to be a poor target for therapy, because it is expressed in relatively high levels not only on tumor but on normal gut as well (29). It is noteworthy, however, that even at these high doses of radioactivity, few objective responses were attained.

More recent human experience with CD20 targeting agents in Pretarget RIT suggest that objective responses can be attained in non-Hodgkin’s lymphoma at doses as low as 15 mCi/m\(^2\) in heavily pretreated patients (30). Even patients with mantle cell lymphoma have been observed to respond. These studies suggest that the MTD of radioactivity is at least 30 mCi/m\(^2\) and may be 50 mCi/m\(^2\) or more (10).

Despite the success of RIT (both conventional and Pretarget) in lymphoma, it is unlikely that this success will be repeated in the vast majority of solid tumors. Solid tumors are generally more resistant to radiation than hematological tumors are and, depending on the stage at which treatment is applied, may also be bulkier and less well vascularized, making it difficult for antibodies or antibody fragments to penetrate the tumor effectively. Hence, it seems reasonable to assume that effective
control of solid tumors may require the combination of RIT (either conventional or Pretarget) with chemotherapy. In this regard, drugs such as gemcitabine, 5-fluorouracil, and paclitaxel are attractive candidates for combination therapy because they are effective as single agents and they also function as radiation-potentiating agents.

Over the past decade, several preclinical studies of combination RIT and radiation-potentiating chemotherapy have been undertaken using such drugs as 5-Iododeoxyuridine, paclitaxel, and 5-fluorouracil (28, 31, 32). In the majority of the studies, antitumor effects were enhanced in the combination-therapy group(s) relative to the control (drug only, RIT only, and no therapy) groups. However, toxicity, particularly myelosuppression, was not systematically investigated in these studies, although most of these agents are myelosuppressive to one degree or another.

Gemcitabine has been characterized as an efficient radiosensitizing drug for a variety of cell lines including the colon carcinoma HT29 for which a 2-fold increase in radiation sensitivity has been demonstrated (33). Cytotoxic activity and radiation-potentiating activity are believed to be mediated by two different phosphorylated metabolites (34). Difluorodeoxycytidine diphosphate inhibits ribonucleotide reductase, leading to perturbation of deoxynucleoside triphosphate (dNTP) pools, which is believed to be critical in radiation potentiation as well as in cell cycle redistribution, whereas a triphosphate metabolite appears to be critical for the cytotoxic activity of the drug (22).

For in vivo therapy, a twice-weekly therapy of gemcitabine was shown to be an efficient radiation-potentiating agent against murine squamous cell flank tumors when used in combination with external sources of radiation (35). A recent study combining gemcitabine and an 131I-labeled Mab, PAM4, demonstrated antitumor effects in a CaPan I pancreatic carcinoma xenograft model (36). In this model, five doses of gemcitabine (100

Table 2  Hematopoietic toxicity (WBC and platelets) of gemcitabine and radiation (data compiled from three studies)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC (×10^3/μl)</th>
<th>Platelet (×10^3/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>PBS control</td>
<td>8.6 ± 3.0 (28)</td>
<td>4.1 ± 1.6 (14)</td>
</tr>
<tr>
<td>Gemcitabine control</td>
<td>8.7 ± 2.5 (28)</td>
<td><strong>11.0 ± 11.4 (14)</strong></td>
</tr>
<tr>
<td>PBS + Pretarget</td>
<td>9.1 ± 1.9 (22)</td>
<td>3.2 ± 1.1 (11)</td>
</tr>
<tr>
<td>Gemcitabine + Pretarget</td>
<td>8.0 ± 2.8 (22)</td>
<td>2.9 ± 3.7 (11)</td>
</tr>
<tr>
<td>PBS/90Y-complex</td>
<td>9.3 ± 2.2 (21)</td>
<td>0.6 ± 0.6 (11)</td>
</tr>
<tr>
<td>Gemcitabine/90Y-complex</td>
<td>7.9 ± 2.4 (22)</td>
<td>0.4 ± 0.4 (11)</td>
</tr>
</tbody>
</table>

* Mean ± SD (number of mice).
  | Mice received injections of Pretarget RIT (400 μCi).
  | Mice received injections of 90Y-labeled DOTA-biotin/CC49 scFvSA complex to mimic radiolabeled whole antibody (200 μCi).
  | Significant difference from PBS control group (P < 0.05).
  | Significant difference from gemcitabine control group (P < 0.05).

Table 3  Hematopoietic toxicity [RBC and bone marrow (BM) colony formation] of gemcitabine and radiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RBC* (×10^3/μl)</th>
<th>No. of BM colonies/plate**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>PBS control</td>
<td>10.2 ± 0.6 (24)</td>
<td>10.0 ± 0.7 (14)</td>
</tr>
<tr>
<td>Gemcitabine control</td>
<td>10.2 ± 0.6 (27)</td>
<td><strong>7.7 ± 0.9 (14)</strong></td>
</tr>
<tr>
<td>PBS/PRIT</td>
<td>10.2 ± 0.6 (22)</td>
<td>9.9 ± 0.7 (10)</td>
</tr>
<tr>
<td>Gemcitabine/PRIT</td>
<td>10.2 ± 0.4 (20)</td>
<td><strong>8.4 ± 1.0 (11)</strong></td>
</tr>
<tr>
<td>PBS/90Y complex</td>
<td>10.4 ± 0.2 (20)</td>
<td><strong>8.8 ± 1.4 (11)</strong></td>
</tr>
<tr>
<td>Gemcitabine/90Y complex</td>
<td>10.3 ± 0.3 (20)</td>
<td>8.0 ± 0.9 (7)</td>
</tr>
</tbody>
</table>

* Mean ± SD (number of mice). Data were compiled from three studies.
  | Mean ± SD (number of plates counted). Data were compiled from two studies.
  | Mice received injections of PRIT (400 μCi).
  | Mice received injections of 90Y-tritium-labeled DOTA-biotin/fusion construct complex to mimic radiolabeled whole antibody (200 μCi).
  | Significant difference from PBS control group (P < 0.05).
  | Significant difference from gemcitabine control group (P < 0.05).
mg/kg) were administered with either 100 or 200 μCi of radio-labeled tumor-specific or nonspecific Mab. Treatment with either gemcitabine or 131I-labeled PAM4 alone was not effective in delaying tumor growth. However, combination therapy resulted in tumoristasis, whether a tumor-specific or a nonspecific Mab was used. In these studies, a single 200-μCi dose of radiolabeled Mab with gemcitabine appeared to be the MTD, underscoring the limitations in using RIT with or without a radiopotentiating agent.

In the present study, we found the combination of Pretarget RIT and gemcitabine to be significantly better than either mono-therapy in antitumor activity using an LS174T colon carcinoma xenograft model. The combination therapy resulted in a statistically significant delay in tumor-volume-doubling time relative to either of the monotherapies and appeared to be dependent on the dose of gemcitabine. Objective tumor remissions were seen for the combination therapy at both the 400-μCi and the 800-μCi doses of Pretarget; fewer were seen at the 800-μCi dose relative to the 400-μCi dose. This is likely because there was only a single study at the 800-μCi dose versus four studies at the 400-μCi dose. Animal survival correlated with tumor response.

Most importantly, the combination-therapy regimen was no more toxic than either monotherapy with respect to peripheral blood cell counts, in vitro colony-forming activity, and maintenance of body weight. This was in contrast to simulated conventional RIT, in which a complex of targeting agent and radiolabeled ligand was lethal at the same dose of 90Y when combined with gemcitabine. Furthermore, the optimal dose of gemcitabine found in this study for the mouse xenograft model (200 mg/kg for two doses) is comparable with the recommended human dose (1000 mg/m²; Refs. 36 and 37).

Pretarget RIT using the CC49 targeting agent used in the present study has been evaluated recently in humans. Nine patients with advanced colorectal carcinoma received the Pretarget regimen with 10 mCi/m² of 90Y. The targeting agent generally localized well to tumor, and the therapy as a whole was safe and well tolerated (38). The next step in the evaluation of this therapeutic modality is dose escalation of the radioligand, perhaps in conjunction with gemcitabine in a CC49 antigen-positive and gemcitabine-sensitive tumor, such as pancreatic adenocarcinoma.

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Combination Therapy with Pretarget CC49 Radioimmunotherapy and Gemcitabine Prolongs Tumor Doubling Time in a Murine Xenograft Model of Colon Cancer More Effectively Than Either Monotherapy

Scott S. Graves, Erica Dearstyne, Yukang Lin, et al.


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