Intraperitoneal Pretarget Radioimmunotherapy with CC49 Fusion Protein

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Abstract

Purpose: This study examined a pretarget radioimmunotherapy strategy for treatment of an i.p. tumor model (LS174T).

Experimental Design: The strategy used regional administration (i.p.) of a novel targeting molecule composed of four CC49 anti–tumor-associated glycoprotein 72 (TAG-72) single-chain antibodies linked to streptavidin as a fusion protein (CC49 fusion protein); 24 hours later, a synthetic clearing agent was administered i.v. to produce hepatic clearance of unbound CC49 fusion protein/synthetic clearing agent complexes. Four hours later, a low molecular weight radiolabeled reagent composed of biotin conjugated to the chelating agent 7,10-tetra-azacyclodecane-1,11-diacetic acid (DOTA) complexed with 111In-, 90Y-, or 177Lu-DOTA-biotin was injected.

Results: Radiolocalization to tumor sites was superior with i.p. administration of radiolabeled DOTA-biotin as compared with i.v. administration. Imaging and biodistribution studies showed excellent tumor localization of radioactivity with 111In- or 177Lu-DOTA-biotin. Tumor localization of 111In-DOTA-biotin was 43% ID/g and 44% ID/g at 4 and 24 hours with the highest normal tissue localization in the kidney with 6% ID/g at 48 and 72 hours. Therapy studies with 90Y-DOTA-biotin at doses of 400 to 600 μCi or 177Lu-DOTA-biotin at doses of 600 to 800 μCi produced significant prolongation of survival compared with controls (P = 0.03 and P < 0.01).

Conclusions: Pretarget radioimmunotherapy using regional administration of CC49 fusion protein and i.p. 90Y- or 177Lu-DOTA-biotin represents a successful therapeutic strategy in the LS174T i.p. tumor model and this strategy may be applicable to human trials in patients with i.p. ovarian cancer.

A major limitation of radioimmunotherapy of solid tumors with radiolabeled monoclonal antibodies (mAb) is normal tissue toxicity, especially to the bone marrow. In an attempt to maximize radionuclide deposition into tumor sites while minimizing exposure to the bone marrow, investigators have designed pretarget radioimmunotherapy strategies. This strategy is based on an initial targeting molecule designed to localize at tumor sites with a subsequent low molecular weight radioisotope ligand administered which can bind to the targeting molecule. One pretarget strategy used a bifunctional mAb with one binding site for a tumor antigen and a second binding site for the radioactive ligand (1–6). The unlabeled bifunctional antibody was administered and allowed to circulate for several days to achieve optimal tumor deposition. Next, the radioligand was administered and showed a rapid tissue distribution and short plasma half-life. This strategy allowed tumor localization of the radioligand to occur rapidly, in a matter of a few hours, with limited radiation doses to the bone marrow. This approach improved radionuclide delivery to tumors in animal models and enhanced scintigraphic imaging in man. A drawback of this design is that the single antigen combining site for tumor antigen resulted in a compromised binding affinity.

A second pretarget strategy took advantage of the high-affinity streptavidin-biotin system by conjugating one of the pair to a mAb for targeting and the other member of the pair in a radioligand preparation. In this way, the mAb retained its affinity for tumor antigen while the short-lived radioligand had high binding affinity to the mAb conjugate (1, 3, 7–9). This design seemed to be attractive in animal models and with imaging studies in man. A major drawback was the high-affinity binding of radioligand with any residual mAb in the plasma or extravascular space. The use of an additional step (9–12) to clear circulating unbound antibody conjugates improved the biodistribution of the radioligand. More recently, this three-step pretarget regimen was modified to use a novel targeting molecule generated by recombinant DNA technology (13–15). A genetic construct composed of a single-chain antibody linked to a single chain of streptavidin when transfected in E. coli for protein production forms stable...
tetramers composed of four single-chain antibodies fused to a streptavidin molecule (four-chain molecule). This reagent has four antigen combining sites and 4 biotin binding sites with a molecular weight of 176,000 Da. It has been used quite effectively in animal model systems of lymphoma and colon cancer using anti-CD20 antibody (B9E9) and anti–tumor-associated glycoprotein 72 (TAG-72) antibody (CC49) constructs (13, 14). The anti-CD20 fusion protein did well in a pilot clinical trial of pretarget radioimmunotherapy in non-Hodgkin’s lymphoma (16) whereas the CC49 fusion protein was studied in a pilot trial of pretarget radioimmunotherapy in metastatic colon cancer (17).

Our ongoing experience with i.p. radioimmunotherapy for recurrent or relapsed ovarian cancer (18) led us to consider a pretarget radioimmunotherapy strategy for regional therapy. This strategy has previously been tested as a regional (i.p.) treatment (19) and motivated us to examine the CC49 fusion protein as an i.p. component of the three-step pretarget regimen in an i.p. TAG-72-positive LS174T tumor model (20, 21).

Materials and Methods

Production of CC49 fusion protein and biotin clearing agent. Schism and his group at the National Cancer Institute developed murine CC49 as a second-generation, high-affinity anti-TAG-72 molecule (22) which has been used in phase I trials in ovarian cancer (23). This genetically engineered molecule retained high binding affinity to TAG-72 with excellent tumor localization and therapeutic efficacy (22, 24). We have produced the CC49 fusion protein composed of four CC49 single-chain antibodies linked to streptavidin. A genetic fusion of the single-chain variable regions (scFv) of murine anti-TAG-72 antibody (CC49) to a single chain of genomic streptavidin of Streptomyces avidinii was produced. The scFv gene consists of the V\_H and V\_L regions, which are separated by a 25-mer Gly4Ser linker. The fusion gene encodes a mature protein of 423 amino acids with a calculated monomeric molecular weight of 43,971. The tetrameric structure is due to the streptavidin component, which spontaneously forms a soluble, stable homotetramer on secretion into the cytoplasm periplasm. The fusion protein has a molecular weight of 176,000 Da with four antigen-binding sites and four biotin-binding sites. Methods for CC49 fusion expression and purification are previously described (13, 14).

Functional characterization was assessed by immunoreactivity and biotin binding/dissociation assays. A competitive immunoreactivity ELISA assay was used to measure the binding of horseradish peroxidase–labeled murine CC49 whole antibody to bovine submaxillary mucin ( Sigma Chemical). The bovine submaxillary mucin was immobilized onto solid support (6.2-mm polystyrene beads; Precision Glass, Chicago, IL) at a concentration of 10 \mu g/bead. A single bead was incubated for 1 hour in duplicate with 1 \mu g of radiolabeled antibody diluted in 1% bovine serum albumin at a concentration of 10 ng/ml in the absence and presence of increasing concentrations of unlabeled fusion protein. The beads were then washed with 4 mL of PBS. The radioactivity remaining on each bead was counted on a gamma counter and percent binding was calculated. The inverse percent binding was plotted versus inverse concentration of unlabeled fusion protein. The inverse of the y intercept was determined as percent immunoreactive fraction. The 125I-CC49 fusion protein showed 86.2% binding to mucin-coated beads in this assay. 7,10-Tetraazacyclododecane-N,N',N''N'''-tetraacetic acid (DOTA)-biotin was labeled with 111In (New England Nuclear), 90Y (New England Nuclear), or 177Lu (University of Missouri Research Reactor, St. Louis, MO) as described elsewhere (9) and showed >90% binding to avidin-coated beads by passing a known amount of radiolabeled fusion protein through an avidin-coated bead column and calculating percent binding.

Cell line. The LS174T human colon cancer cell line was obtained from the American Type Culture Collection (Herndon, VA). The cells were maintained in Eagle's MEM + 10% fetal bovine serum, L-glutamine, and nonessential amino acids at 37°C and 5% CO\(_2\).

Biodistribution and imaging studies. All animal studies were conducted with the approval of the University of Alabama at Birmingham Animal Care and Use Committee. Six- to eight-week-old female athymic nude mice (BALB/c, National Cancer Institute Frederick Cancer Research facility, Frederick, MD) were placed on a biotin-deficient diet ( Purina Biotin Deficient Diet 5836, Purina Mills, Richmond, IN) 5 days before administration of CC49 fusion protein in each study for a period of 10 days. Imaging studies were done with an Anger 420/550 Mobile Radiosotope Gamma camera (Technicare, Solon, OH) equipped with a pinhole collimator at 4 and 22 hours after i.p. or i.v. injection of the 111In- or 177Lu-DOTA-biotin.

For biodistribution experiments, animals were sacrificed and tissues counted on a well-type gamma scintillation counter with results expressed as % of injected dose (ID)/g of tissue. Based on prior animal studies of pretarget radioimmunotherapy and preliminary studies, the pretarget format was i.p. administration of 800 \mu g of CC49 fusion protein followed 20 hours later by 100 \mu g of synthetic clearing agent (i.v.) and radiolabeled DOTA-biotin (5 mg) 4 hours post synthetic clearing agent. Subsequent time points were expressed as duration from DOTA-biotin administration.

Intraperitoneal tumor model. Athymic nude mice were injected i.p. with 5 × 10\(^5\) LS174T cells. At 9 days after tumor injection, the pretarget regimen for biodistribution, imaging, or therapy was initiated. For therapy studies, survival analysis was conducted for each experiment. Kaplan-Meier survival curves were constructed per treatment group, then plotted and compared, and median survival times were computed. A log-rank test (26) was conducted to examine differences in survival between groups. If the overall log-rank test was significant ( P < 0.05) over all groups, pairwise comparisons were also conducted. In the event that an animal’s survival time was greater than the time of study termination, the day of study termination was substituted for that animal’s survival time and treated as censored in the analysis. The great majority of the animals survival times were not censored (i.e., most died before study termination, resulting in very few censored values).
Results

Imaging and biodistribution of $^{111}$In-DOTA-biotin following CC49 fusion protein. To examine the effects of DOTA-biotin route of administration, mice received 800 µg of CC49 fusion protein i.p., 100 µg of synthetic clearing agent at 20 hours, and 50 µCi of $^{111}$In-DOTA-biotin (5 mg) administered i.v. or i.p. 4 hours later. As seen in Fig. 1, the i.p. route had greater tumor localization than the i.v. route, probably reflecting the rapid excretion of intravascular DOTA-biotin.

To examine the biodistribution of the CC49 fusion protein (targeting agent) as compared with the radiolabeled DOTA-biotin, CC49 fusion protein was labeled with $^{125}$I and DOTA-biotin was radiolabeled with $^{111}$In using the format described above with both agents administered by i.p. route. As seen in Fig. 2A, the $^{125}$I-CC49 fusion protein had its primary sites of localization in the liver (4.7% ID/g) at 24 and 48 hours, reflecting the hepatic clearance of the synthetic clearing agent-CC49 fusion complex, and in the tumor (12.1% ID/g) at 24 hours, reflecting the binding of CC49 fusion protein to tumor TAG-72 antigen. In contrast (Fig. 2B), the biodistribution of $^{111}$In-DOTA-biotin had tumor localization of 43% ID/g and 44% ID/g at 4 and 24 hours, reflecting the biotin binding to tumor-bound CC49 fusion protein (streptavidin). The highest normal tissue exposure was the kidney with 6% ID/g at 48 and 72 hours, reflecting the urinary excretion of DOTA-biotin. The low liver uptake of DOTA-biotin (~2% ID/g) reflects the intracellular location of the synthetic clearing agent-CC49 fusion complex (16). This experiment illustrates well the amplification of isotope delivery to tumor and low normal tissue exposure of the low molecular weight isotope carrier molecule (800 Da).

Imaging and biodistribution of $^{177}$Lu-DOTA-biotin following CC49 fusion protein. To examine a second radioisotope, 100 µCi of $^{177}$Lu-DOTA-biotin were used in the same pretarget schema. Figure 3 illustrates the excellent localization in tumor nodules (37.7 ± 8.3% ID/g and 22.6 ± 8.3% ID/g at 5 hours postinjection; 17.7 ± 13.8% ID/g and 25.2 ± 11.5% ID/g at 24 hours postinjection) with minimal normal tissue exposures. Figure 4A and B illustrates the excellent imaging of multiple i.p. tumor nodules.

Animal tumor therapy studies with $^{90}$Y-DOTA-biotin. Mice bearing i.p. LS174T tumors underwent pretarget radioimmunotherapy at 9 days after tumor cell injection via administration of 800 µg CC49 fusion protein i.v., synthetic clearing agent after 20 hours, and 400 or 800 µCi of $^{90}$Y-DOTA-biotin after 4 hours. Controls included mice who received 800 µg of CC49 fusion protein or mice who received 800 µCi of $^{90}$Y-DOTA-biotin without prior CC49 fusion protein. As seen in Fig. 5, mice receiving pretarget radioimmunotherapy with 800 µCi $^{90}$Y-DOTA-biotin had 60% early deaths and 40% long-term survivors. Mice receiving pretarget radioimmunotherapy with 400 µCi $^{90}$Y-DOTA-biotin had a 48-day median survival, which...
was significantly longer (log-rank test; $P = 0.03$) than that for mice receiving CC49 fusion protein only (35-day median survival) or 800 μCi $^{90}$Y-DOTA-biotin only (23-day median survival). From other independent studies with same amount of cells injected in the same kind of mice, the median survival for 26 untreated control mice was 29 days (95% confidence interval, 28-33 days). A repeat study using pretarget radioimmunotherapy with 400 and 600 μCi $^{90}$Y-DOTA-biotin had median survivals of 53 and 54 days, which were significantly longer than controls ($P = 0.03$), and a third experiment using 400 μCi $^{90}$Y-DOTA-biotin had a median survival of 57 days, which was significantly longer than controls ($P < 0.01$). Thus, the most effective dose with least radionuclide toxicity was in the 400 to 600 μCi range.

**Animal tumor therapy studies with $^{177}$Lu-DOTA-biotin.** Mice bearing i.p. LS174T tumors underwent pretarget radioimmunotherapy at 9 days after tumor cell injection via administration of 800 μg of CC49 fusion protein i.p., 100 μg of synthetic

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**Fig. 3.** Biodistribution of $^{177}$Lu-DOTA-biotin at 5 (■) and 24 (▲) hours after i.p. injection in athymic nude mice bearing LS174T peritoneal tumors following pretargeting with 800 μg unlabeled CC49 fusion protein i.p. and 100 μg synthetic clearing agent i.v. 4 hours later. HT, heart; LI, liver; ST, stomach; SI, small intestine; CE, cecum; SP, spleen; LU, lung; KI, left kidney; RKI, right kidney; MU, muscle; BL, blood; RO, reproductive organs; TU MN, main tumor bound to small intestine; TU OT, other i.p. tumors.

**Fig. 4.** Imaging of 100 μCi $^{177}$Lu-DOTA-biotin at 4 hours (A) and 24 hours (B) after i.p. injection in athymic nude mice bearing peritoneal LS174T tumor xenografts following pretargeting with 800 μg unlabeled CC49 fusion protein i.p. and 100 μg synthetic clearing agent i.v. 4 hours later.
the difference is significant ($P$ (95% confidence interval, 38-54 days). Based on a log-rank test, i.v. and treated with 400 or 800 $A$ significantly longer than seen with controls (37 days; $P$ survivals of 57 and 60 days, respectively, which were $A$ receiving 600 and 800 $A$ clearing agent i.v. 20 hours later, and 600 or 800 $A$ Survivals of nude mice bearing peritoneal LS174T tumors injected i.p. with $A$ Ci 177Lu-DOTA-biotin (Fig. 4) was 48 days (95% confidence interval, 38-54 days). Based on a log-rank test, the difference is significant ($P$ = 0.026).

## Discussion

Prior studies by our group have shown good tumor localization by radiolabeled CC49 to a variety of tumor types (27–29) without evidence of localization to normal gastrointestinal tissues (23). This supported the hypothesis that a pretargeting strategy based on CC49 would provide less cross-reactivity with normal tissues (gastrointestinal mucosa) than was previously seen with pretargeting based on NR-LU-10 antibody (30). Toward this end, we have produced a novel tetrameric fusion protein composed of four chains of CC49 scFv and streptavidin. This study has examined this fusion protein and radiolabeled DOTA-biotin given by the i.p. (regional) route.

Prior clinical trials have administered radiolabeled mAb i.p. as a single agent for the treatment of ovarian cancer (23, 31–33) and in combination with Taxol as a radiosensitizer (18, 34). These trials had evidence of antitumor efficacy thought to be due to a favorable therapeutic ratio achieved following regional administration. Despite the regional route of delivery, bone marrow suppression was the dose-limiting toxicity. In the pretargeting approach described in this report, fusion protein was administered i.p. and allowed to localize in tumor. The fusion protein was then eliminated from the circulation with a clearing agent and the radiolabeled small molecule (DOTA-biotin) was administered. The latter was bound to the pretargeted fusion protein in the tumor and the remaining radioactivity was rapidly eliminated from normal tissues by renal excretion.

The pretarget protocol described here resulted in high tumor uptake of both preadministered CC49 fusion protein and radiolabeled DOTA-biotin following regional i.p. administration. The i.p. route of radiolabeled DOTA-biotin was superior to an i.v. route of administration. Furthermore, the high tumor/normal tissue uptake of radiolabeled (111In or 177Lu) DOTA-biotin was sufficient to provide high-contrast images of multiple tumor nodules throughout the peritoneum, which was validated at necropsy. The level of uptake of radiolabeled DOTA-biotin was substantially less in normal tissues than in tumors as determined by gamma counting.

177Lu is currently being studied as a radioimmunotherapy isotope because of its favorable characteristics, which include a 64-hour half-life and a relatively high $β$ energy (2.3 MeV maximum). The mean range (the distance in which 60% of decay energy is deposited) for 177Lu is 2.76 mm. Because 177Lu is unsuitable for quantitative imaging, many groups are using 111In biodistribution data to predict dose for 177Lu administrations. DOTA is a bifunctional chelating agent that produces stable conjugates with $111In$ or $90Y$. The “matched pair” approach using $111In/90Y$-DOTA-biotin (the former for imaging and the latter for therapy) is a very attractive option. These can both be bound by DOTA-biotin and generally produce similar biodistributions (35). The preliminary pretargeting therapy results obtained with 90Y-DOTA-biotin against the peritoneal LS174T tumor model are promising. There was extended animal survival obtained with 400 or 600 $μCi$ 90Y-DOTA-biotin whereas higher doses produced toxicity. This is a similar dose range for marrow suppression previously reported for pretargeting with the CC49 fusion protein and 90Y-DOTA-biotin (14).

A second option is 177Lu, which also has a potential for therapy in this pretargeting protocol (36). 177Lu is a rare earth element (lanthanide) with a physical half-life of 6.7 days and with intermediate energy and range $β$ emissions (average energy of 133 keV, maximum energy of 497 keV) that penetrate 0.2 to 0.3 mm in soft tissue. 177Lu also emits two
relatively low-abundance, low-energy γ rays (113 and 208 keV) that allow imaging with a gamma camera. It is possible that \(^{177}\)Lu-DOTA-biotin will produce less normal tissue toxicity than \(^{90}\)Y-DOTA-biotin in radiosensitive tissues such as bone marrow, gastrointestinal tract, and kidney. In the initial study, 600 or 800 μCi \(^{177}\)Lu-DOTA-biotin produced prolonged survival as compared with control animals, similar to that seen with \(^{90}\)Y-DOTA-biotin. \(^{177}\)Lu-DOTA-biotin seemed less toxic than \(^{90}\)Y-DOTA-biotin. At the 800 μCi dose, \(^{90}\)Y-DOTA-biotin caused 60% early (toxic) deaths whereas this dose produced no evidence of toxicity with \(^{177}\)Lu-DOTA-biotin.

In conclusion, these preclinical results suggest that the three-step pretarget radioimmunotherapy strategy is applicable to regional therapy of i.p. tumor sites. Bone marrow suppression will need to be monitored along with toxicity to other key organs including the gastrointestinal tract, kidney, and liver. These studies support the development of a regional pretarget radioimmunotherapy strategy for recurrent or relapsed ovarian carcinoma.

References

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