Targeting of HER2 Antigen for the Treatment of Disseminated Peritoneal Disease

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ABSTRACT
The studies reported herein demonstrate the efficacy of α-particle–targeted radiation therapy of peritoneal disease with Herceptin as the targeting vehicle. Using the CHX-A-DTPA linker, Herceptin was radiolabeled with indium-111 and bismuth-213 with high efficiency without compromising immunoreactivity. A pilot radioimmunotherapy study treating mice bearing 5-day LS-174T (i.p.) xenografts, a low but uniform HER2 expressing, human colon carcinoma, with a single dose of 213Bi-CHX-A”-Herceptin, proved disappointing. This defined the effect of tumor burden/size on tumor response to radioimmunotherapy with α-radiation. A more successful experiment with a lower tumor burden (3 days) in mice followed. A specific dose-response (P = 0.009) was observed, and although a maximum-tolerated dose was not determined, a dose of 500 to 750 µCi was selected as the operating dose for future experiments based on changes in animal weight. Median survival was increased from 20.5 days for the mock-treated mice to 43 and 59 days with 500 and 750 µCi, respectively. The therapeutic effectiveness of 213Bi-CHX-A”-Herceptin was also evaluated in a second animal model for peritoneal disease with a human pancreatic carcinoma (Shaw). The results of this study were not as dramatic as with the former model, and higher doses were required to obtain an increase in survival of the mice (P = 0.001).

INTRODUCTION
Overexpression of HER2 (HER2/neu, c-erb-B2) in a range of epithelial cancers makes an ideal target for radioimmunotherapy (1). Targeted radiation therapy, i.e., the delivery of therapeutic radionuclides via monoclonal antibodies (mAbs), has reemerged as a viable option for the treatment and management of cancer patients. The field has been revitalized by the success of and Food and Drug Administration-approval of Zevalin and Bexxar for the treatment of lymphohematopoietic malignancies (2, 3).

Radioimmunotherapy for treatment of solid tumors has not been as successful and as such requires both definition and additional refinement of optimal conditions in its application. When designing a radioimmunoconjugate, the disease being treated and the therapeutic regimen dictate radionuclide selection. The β-emitting radionuclides, e.g., yttrium-90 (t1/2 = 6.7 days, Emax = 2.3 MeV) and iodine-131 (t1/2 = 8.04 days, Emax = 0.81 MeV), have dominated in radioimmunotherapy applications primarily because of availability and facile-labeling procedures (4).

The path length characteristic of β-emitters can be disadvantageous, and the decay energy of 90Y is only deposited in tumors with diameters of ≥1 cm (5). In contrast, the discrete energy emissions of α-particle decays (4 to 9 MeV) are directly deposited over a short distance in tissue (40 to 100 μm), resulting in high linear energy transfer (6). α-Particle radiation is cytotoxic with only three to six transversals of a cell’s nucleus, resulting in an estimated dose of 70 to 100 cGy (dose rate as low as 1 cGy/hour; refs. 6, 7). The shorter path may be advantageous, limiting toxicity to normal tissues adjacent to tumor. The α-emitting radionuclides may be ideal for specific treatment of smaller tumors/tumor burden, disseminated disease, and micrometastatic disease. α-Emitters currently under investigation for radioimmunotherapy applications include bismuth-212 (t1/2 = 60.6 minutes), 211Bi (t1/2 = 46.5 minutes), actinium-225 (t1/2 = 7.2 hours), and actinium-225 hr (t1/2 = 10 days; refs. 6, 8–14). Clinical trials have been conducted validating the potential of targeted radiation therapy with 211Bi, which has an added benefit of possessing low energy, low abundance γ-emissions that allow performance of real-time external imaging suitable for dosimetry (15, 16). The isotope used in this study, 211Bi, is a decay product of 225Ac and is obtained from a 225Ac/211Bi generator (17).

HER2 (HER2/neu, c-erb-B2) is a M disparate 185,000 membrane-bound receptor with tyrosine kinase activity (epidermal growth factor receptor 2), which interacts with other members of the epidermal growth factor receptor family and other ligands. These receptors stimulate intracellular signal transduction pathways involved in the control of cell growth. HER2 is overexpressed in a variety of epithelial tumors: ~25 to 30% of breast and ovarian cancers, 35 to 45% of all pancreatic adenocarcinomas, and up to 90% of colorectal carcinomas (1, 18). Overexpression of this oncogene is associated with poor prognosis and aggressive tumor attributes. Herceptin (trastuzumab) is a humanized mAb that targets HER2 and has been shown to have antitumor activity in a dose-dependent manner (19).

As a monotherapy, Herceptin has resulted in a response...
rate of 12 to 20% in metastatic breast cancer patients (20, 21). Therapeutic efficacy has been improved when combined with chemotherapy (22). Although approved by the Food and Drug Administration in 1998 for the treatment of advanced-stage HER2/neu+ patients, a large percentage of eligible patients fail to respond to treatment and/or relapse. As the therapeutic efficacy of Herceptin is cytostatic, radioimmunotherapy offers an opportunity to complement this intrinsic activity by incorporating radiation into the treatment regimen. In vivo studies have shown the feasibility of targeting HER2/neu antigen and the parental murine mAb 4D5, radiolabeled with $^{131}$I or $^{67}$Ga, inhibited the growth of HER2-expressing xenografts in mice (23, 24).

The hypothesis for this study was that Herceptin radiolabeled with $^{213}$Bi as a therapeutic agent was 2-fold. First, treatment of disseminated peritoneal disease would demonstrate efficacy with $^{213}$Bi. Second, Herceptin therapy could be extended to the treatment of malignancies with low HER2 expression. This report details the in vitro and in vivo analysis of radiolabeled Herceptin. Dose ($\mu$Ci) studies were then conducted to define maximum-tolerated dose and to establish the therapeutic dose for future experiments in athymic mice bearing LS-174T xenografts. The efficacy of $^{213}$Bi-CHX-A'-Herceptin was also evaluated in a human pancreatic carcinoma xenograft animal model.

**MATERIALS AND METHODS**

**Cell Lines.** The human colon carcinoma cell line, LS-174T was grown in supplemented DMEM as described previously (25). Shaw, a human pancreatic carcinoma cell line, and N87, a human gastric carcinoma cell line that expresses high levels of HER2, were grown in supplemented RPMI 1640 (7, 26). All media and supplements were obtained from Quality Biologicals (Gaithersburg, MD).

**Flow Cytometric Analysis.** HER2 expression of the LS-174T and Shaw cell lines was assessed with flow cytometric techniques by methods described previously (27). The cells (10,000 events collected) were analyzed with a FACS Calibur with CellQuest software (BD Biosciences, San Jose, CA).

**Chelate Synthesis and mAb Conjugation.** The synthesis, characterization, and purification of the bifunctional ligand, CHX-A-DTPA, has been described previously (28). Herceptin was conjugated with the CHX-A'-DTPA by established methods with a 10-fold molar excess of ligand to mAb (27). Protein was quantified by the method of Lowry and the number of CHX-A' molecules linked to the mAb was determined with the yttrium-Arsenazo(III)-based assay (29, 30).

**Radiolabeling of Herceptin.** Radiolabeling of CHX-A'-Herceptin with indium-111 [200 $\mu$g in 0.15 mol/L ammonium acetate buffer (pH 7.0)] was performed by adding 1 mCi in 1 to 2 $\mu$L of $^{111}$In chloride (Perkin-Elmer, Boston, MA). The reaction was quenched after 30 minutes with 0.1 mol/L EDTA (3 $\mu$L) to scavenge-free radionuclid, and the radiolabeled product was purified with a PD-10 desalting column (Amersham Biosciences, Piscataway, NJ).

The labeling of CHX-A'-Herceptin with $^{213}$Bi was accomplished as follows. $^{213}$Bi (7.8 to 8.4 mCi) was eluted from a $^{225}$Ac ($\sim$15 mCi) generator (Oak Ridge National Laboratories, Oak Ridge, TN) as described previously (17). To prevent radioysis, 100 $\mu$L (220 mg/mL) of ascorbic acid solution were added to the $^{213}$Bi, the pH adjusted to 4.5 to 5 with 5 mol/L ammonium acetate, and then the CHX-A'-Herceptin (300 $\mu$g) was added. After a 12-minute incubation at room temperature, EDTA was added to scavenge uncomplexed $^{213}$Bi. The radiolabeled mAb was purified by size exclusion high-performance liquid chromatography with a TSK-3000 column (TosoHaas, Montgomeryville, PA) equilibrated in PBS (pH 7.4) at a flow rate of 1 mL/minute or on PD-10 desalting columns. HulG (ICN, Irvine, CA), conjugated with the CHX-A' DTPA ligand and radiolabeled with $^{213}$Bi, served as a negative control in the radioimmunotherapy studies.

**Radioimmunoassay.** Immunoreactivities of the radiolabeled preparations were assessed in a live-cell radioimmunoassay as detailed previously (27). Serial dilutions of radiolabeled Herceptin (~200,000 to 12,500 cpm in 50 $\mu$L of 1% BSA in PBS) were added to test tubes containing N87 cells (1 x $10^6$/200 $\mu$L) resuspended in PBS/BSA (pH 7.2). After a 2-hour incubation at 4°C, the cells were washed, pelleted, and counted in a $\gamma$-scintillation counter (Wizard One, Perkin-Elmer, Shelton, CT). The percentage binding was calculated for each dilution and averaged. The specificity of the radiolabeled Herceptin was confirmed by incubating cells with radiolabeled Herceptin and 5 $\mu$g of unlabeled Herceptin.

**In vivo Studies.** All in vivo studies were performed with 4 to 6-week-old female athymic (nu/nu) mice (Charles River Laboratories, Wilmington, MA).

**Tumor Targeting.** Mice received s.c. injections in the flank with either 1 x $10^6$ LS-174T cells in 0.2 mL of medium or with 4 x $10^6$ Shaw cells in 200 $\mu$L of a 1:1 mix of Matrigel (BD Biosciences). Mice were used in studies when the tumor xenografts maximal diameter measured 0.4 to 0.6 cm. Mice ($n = 5$) received i.v. injections of $^{111}$In-CHX-A'-Herceptin (~7.5 $\mu$Ci) and sacrificed by exsanguination at time points 24 to 168 hours. The blood, tumor, and major organs were collected, wet-weighed, and counted in a $\gamma$-scintillation counter. The percent injected dose per gram (%ID/g) and SD were calculated.

**Therapy Studies.** Radioimmunotherapy studies were performed with 4 to 6-week-old female athymic (nu/nu) mice (Charles River Laboratories). The mice received i.p. injections of $1 \times 10^8$ cells LS-174T in 1 mL of medium or PBS as reported previously (31). $^{213}$Bi-CHX-A'-Herceptin was administered to the mice 3 or 5 days after tumor implantation. Radioimmunotherapy studies with mice bearing Shaw xenografts were performed 6 to 7 days after i.p. implantation of $1.5 \times 10^7$ cells. Doses of $^{213}$Bi-CHX-A'-Herceptin were administered i.p. to the mice ($n = 5$–10) in 1 mL of PBS.

The mice were weighed twice a week for 4 weeks after injection of $^{213}$Bi-Herceptin. Disease progression presented as bloating, severe weight loss, or as was noticeable, palpable nodules in the abdomen. 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 if bloating or tumor nodules were apparent. All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee.
Statistical Analyses. A Cox proportional hazards model was used to test for a dose-response relationship between the dose of $^{213}$Bi-CHX-A”-Herceptin 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 parameter was 0 with 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 initial 4 week period divided by the baseline weight of the mouse. Box plots were constructed for each dose group and the normal organ distribution of the radioimmunoconjugate was established to ensure each animal developed tumor.

RESULTS

Studies performed were designed to evaluate in vitro and in vivo properties of radiolabeled Herceptin. Conjugation of CHX-A” DTPA to Herceptin routinely resulted in a chelate to protein ratio of 2 to 3:1, similar to previously reported results (27). Radiolabeling of CHX-A”-Herceptin with $^{111}$In was efficient (83.3%) with a specific activity of 23.3 mCi/mg. The immunoreactivity of $^{111}$In-Herceptin was 56.6% bound to the HER2-positive N87 cells, which could be inhibited with an excess of Herceptin, reducing the binding to 7.9%. The in vitro analysis of $^{111}$In-Herceptin indicated that 98.4% of the radioactivity was associated with the IgG peak as determined by size exclusion high-performance liquid chromatography.

Flow cytometric analyses of the two cell lines indicated that HER2 is expressed on 76.7% and 90.4% of LS-174T and Shaw cells, respectively. However, overall expression was low with a mean fluorescence intensity of 30.3 for the former cell line and 40.4 for the latter. The gastric carcinoma cell line N87, included in this analysis as a reference, has $1 \times 10^6$ receptors per cell, had a mean fluorescence intensity of 899.5 with 99.8% of the cells positive for HER2 (32).

Athymic mice bearing s.c. LS-174T xenografts ($n = 5$) received i.v. injections of $^{111}$In-Herceptin to establish and define tumor targeting and normal organ distribution of the radioimmunoconjugate. The $^{111}$In-CHX-A”-Herceptin demonstrated excellent tumor targeting (Table 1) with a %ID/g of 29.0 ± 14.9 at 24 hours and 35.02 ± 6.51 at 48 hours, remaining high, through to the end of the study with a %ID/g of 26.94 ± 4.27 at 168 hours. The normal tissue with the highest %ID/g was the liver with a peak value of 6.94 ± 1.74 at 72 hours. The remainder of the organs presented with less than 5 %ID/g; the femur peaked at 48 hours at 1.86 ± 1.06 %ID/g.

The maximum tumor targeting was associated with the IgG peak as determined by size exclusion high-performance liquid chromatography.

The gastric carcinoma cell line N87, which could be inhibited with an excess of Herceptin, reducing the binding to 7.9%.

To this end, a tumor growth study was initiated in which mice with the highest cell inoculum was $10^9$ LS-174T or Shaw cells, respectively. However, overall expression was low with a mean fluorescence intensity of 30.3 for the former cell line and 40.4 for the latter. The gastric carcinoma cell line N87, included in this analysis as a reference, has $1 \times 10^6$ receptors per cell, had a mean fluorescence intensity of 899.5 with 99.8% of the cells positive for HER2 (32).
mock treatment had succumbed to disease. Unfortunately, in this experiment, there is also a lack of a relationship between survival of the mice and the 213Bi-CHX-A”-Herceptin doses given (P = 0.57). In addition, when the 500-μCi dose of 213Bi-CHX-A”-Herceptin is compared with the same dose of 213Bi-CHX-A”-HuIgG, there is no difference in survival between the two groups (P = 0.88), suggesting a lack of specificity. Alternately, the lack of a specific dose-response, and the rapidity with which the mock-treated mice succumbed to disease suggests that the tumor burden was simply far too established at the time radioimmunotherapy was given.

To test this hypothesis, a second experiment of the same design was executed with mice (n = 10) with a 3-day tumor burden. Under these conditions, increased survival of tumor-bearing mice was observed in those mice receiving i.p. injections of 213Bi-CHX-A”-Herceptin, 50% of the mock-treated mice were dead 20.5 days post-implantation of the tumor cells (Fig. 2A). Mice treated with 213Bi-CHX-A”-HuIgG (500 μCi) showed a marginal increase of survival with a median survival of 25 days. The median survival for the groups that received injections of 250, 500, and 750 μCi of 213Bi-CHX-A”-Herceptin was 25, 43, and 59 days, respectively. The dose-response relationship between survival and 213Bi-CHX-A”-Herceptin was found to be highly significant (P = 0.009). Furthermore, the response was specific, and the animals receiving 213Bi-CHX-A”-Herceptin showed a significant improvement in survival over those that had received 213Bi-CHX-A”-HuIgG (P = 0.009).

In a separate experiment, weights of the mice treated with either 213Bi-CHX-A”-Herceptin or -HuIgG (n = 7 to 12) were monitored and recorded twice weekly for 4 weeks after radioimmunotherapy as an indicator of toxicity. The data (Fig. 2, B and C) were estimated as the maximum percent reduction in weight for each mouse over the 4-week period and calculated as the ratio of maximum weight reduction divided by the initial weight of the mouse. The data are expressed with box plots for each of the dose levels (Fig. 2B): mock-treated, and 250, 500, 750, and 1000 μCi. There is a strong dose-response relationship of toxicity with increasing dosage (P = 0.0001, Kruskal-Wallis ANOVA). Furthermore, comparison of 500 μCi of 213Bi-CHX-A”-Herceptin to the same dose of 213Bi-CHX-A”-HuIgG (Fig. 2C) indicates no difference in toxicity between the two vehicles (P = 0.93, Wilcoxon rank-sum test).

Herceptin is currently used for treatment/management of breast cancer patients based on its ability to block or inhibit tumor cell growth and proliferation. To assess the possibility that Herceptin itself was impacting tumor growth, tumor-bearing mice (n = 10) received i.p. injections of 300 μg of unlabeled Herceptin. As depicted in Fig. 3, a single injection of unlabeled Herceptin failed to prolong survival in these animals. In fact, all of the Herceptin-treated animals succumbed to the tumor by day 16, virtually congruent with the mock-treated group (P = 0.58).

Evaluation of the antitumor efficacy of 213Bi-CHX-A”-Herceptin in a second tumor model was also of interest. As noted earlier, flow cytometric analysis of the Shaw cell line

![Kaplan-Meier survival curves. Increasing μCi doses of 213Bi-CHX-A”-Herceptin were given i.p. to mice bearing 5-day LS-174T i.p. xenografts. The injected doses were (■) mock-treated, (◇) 250 μCi, (■) 500 μCi, ( ○) 750 μCi, (▲) 1000 μCi 213Bi-Herceptin, and ( ▼) 500 μCi of 213Bi-HuIgG used as a nonspecific control.](image-url)

![Kaplan-Meier survival curves. Increasing μCi doses of 213Bi-CHX-A”-Herceptin were given i.p. to mice bearing 3-day LS-174T i.p. xenografts. The injected doses were (■) mock-treated, (◇) 250 μCi, (■) 500 μCi, ( ○) 750 μCi, (▲) 1000 μCi 213Bi-Herceptin, and ( ▼) 500 μCi of 213Bi-HuIgG used as a nonspecific control (A). Affects of 213Bi-CHX-A”-Herceptin radioimmunotherapy on animal weights. The maximum relative weight reduction was calculated for each treatment group and presented as box plots (B). Specificity of the affect of the radioimmunotherapy is illustrated with a comparison between the mice that received either 500 μCi of 213Bi-CHX-A”-Herceptin or 213Bi-HuIgG (C). The light line is the median, the upper region of the box represents the third quartile, the lower portion is the first quartile, the brackets delineate 1.5 times the interquartile range, and the lines outside of the brackets represent outlying observations.](image-url)
indicated similar levels of HER2/neu expression as the LS-174T cell. A tumor-targeting study with mice (n = 5) bearing s.c. Shaw xenografts was performed. Similar to the results reported for the LS-174T tumor xenografs, excellent tumor targeting was obtained with the highest tumor %ID/g (34.00 ± 10.15) occurring at 72 hours (Table 1). The %ID/g of the blood begins at 14.96 ± 2.30 (24 hours) and exhibited a steady decline throughout the study to end with 3.64 ± 1.64 (168 hours). In this experiment, the normal tissue that presented with the highest %ID/g was spleen, which had an initial value of 9.06 ± 2.72 at 24 hours and decreased to 4.35 ± 2.63 by 168 hours.

Tumor growth studies indicated that the Shaw cell line would grow as a disseminated peritoneal tumor. Mice (n = 5) received injections of 12.5, 25, 50, and 75 × 10^6 cells; all mice that received 12.5 × 10^6 cells developed tumor throughout the peritoneum. In contrast to the LS-174T xenografts, which developed discrete nodules, Shaw tumor growth presented as a thin coating throughout the peritoneum that resulted in thickening of the abdominal wall and diaphragm. On the basis of the radioimmunotherapy studies with the LS-174T tumor, a small study was conducted to assess efficacy of 213Bi-CHX-A'-Herceptin in mice (n = 10) bearing 5-day Shaw (i.p.) xenografts. A single 500-μCi dose of 213Bi-CHX-A'-Herceptin was compared with a group of mice that received 500 μCi of 213Bi-CHX-A'-HuIgG, as well as to a group of mock-treated mice (Fig. 4A). Surprisingly, no difference in animal survival was discerned between the specific and nonspecific antibody. In the mock-treated group, 50% of the animals were still alive 48 days post-treatment. Both 213Bi-labeled antibodies increased the survival of animals; 50% of the animals that received injections of Herceptin and HuIgG were alive at 98 days and 107 days, respectively. Additionally, unlabeled Herceptin was found to have no impact on the growth of the Shaw tumor (Fig. 3); no difference in survival was found between the Herceptin and mock-treated groups (n = 10, P = 0.55).

A second radioimmunotherapy study was then conducted with the Shaw peritoneal model. Escalating doses of 213Bi-CHX-A'-Herceptin (0, 0.5, 1, and 2 mCi) were given to mice (n = 10) i.p. 6 days after tumor implantation; one group of mice received 1 mCi of 213Bi-HuIgG (Fig. 4B). Half of the mock-treated animals were dead 15 days after tumor implantation, whereas the median survival for the animals receiving 0.5, 1, and 2 mCi of 213Bi-CHX-A'-Herceptin was 26, 28, and 26 days, respectively. The relationship between survival and these doses was found to be significant (P = 0.001) versus the control group. The median survival for animals that received 1 mCi of 213Bi-CHX-A'-HuIgG was 22 days. Survival was increased by 6 days for the animals receiving 1 mCi of 213Bi-CHX-A'-Herceptin; however the difference in survival was not statistically significant (P = 0.11) versus the specific mAb.

DISCUSSION

Studies reported herein demonstrate efficacy of α-particle-targeted radiation therapy of peritoneal disease with Herceptin as the targeting vehicle. The feasibility and potential of immunoconjugates radiolabeled with α-particle emitters have been demonstrated in preclinical and clinical investigations (11–14, 23, 33–36). Despite that treatment of disseminated peritoneal...
disease has routinely been proposed as an appropriate utilization of this class of radionuclides, few studies have addressed this hypothesis (11, 13, 36, 37). Investigators have concentrated on clinical use of α-particle radioimmunotherapy for treatment of lymphohematopoietic diseases or for locoregional administration for the treatment of glioma (16, 38).

Overexpression of HER2 in a variety of epithelial cancers makes this an ideal target for radioimmunotherapy (1, 18, 23, 24). Objective responses in breast cancer patients receiving Herceptin therapy ranges from 12 to 20% and in ovarian cancer patients a disappointing 7.5% (20, 21, 39). Patient selection for Herceptin therapy is based on immunohistochemical staining of biopsy samples for HER2 expression; scores of 2+ or 3+ are eligible. This screening criteria excludes patients with low HER2-expressing disease. For example, of 837 patients screened for the clinical trial just noted, only 95 patients (11.4%) met the 2+/3+ scoring criteria, whereas 158 (18.9%) patients with a score of 1+ were ineligible (40). Radioimmunotherapy with Herceptin potentially could greatly expand the population eligible for treatment.

The majority of ovarian and pancreatic cancer patients routinely present with late-stage disease. For patients undergoing surgery, removal of microscopic disease is difficult, if not impossible. Platinum-based chemotherapies have improved response rates of patients with ovarian carcinoma; however, 50% of patients with confirmed complete responses relapse (41). Only a small percentage of pancreatic cancer patients are considered resectable and then the 5-year survival rate remains only 5 to 10% (42). These patient populations are ideal for targeted radiation therapy with an α-emitting radionuclide. Furthermore, with HER2 overexpressed in 25 to 30% of ovarian cancer and 35 to 45% of the pancreatic cancers, Herceptin, because of its availability, seems a logical targeting vector for radioimmunotherapy in these patient populations (1, 18).

The cytotoxic effect of α-particle radiation may be as low as a dose rate of 1 cGy/hour; therefore, cell death may occur from only a few 211Bi molecules localized per tumor cell (6, 7). Additionally, not all cells need be targeted by a radioimmunoconjugate. Because of omni-directional particle decay, sufficient cytotoxic doses may be delivered to neighboring cells while sparing normal tissue. To some degree, this bystander effect may overcome the heterogeneous nature of tumors, as well as the lack accessibility to cells by a radioimmunoconjugate within the tumor environment (43). Bystander effects from another perspective are the cellular responses, direct or indirect, to an agent, in this context, to α-particles. These responses for example include gene mutations, increased complexity of DNA lesions, induction of apoptosis, and cell cycle arrest (43).

Conjugation of Herceptin with the acyclic CHX-A'-DTPA ligand resulted in a chelate-to-protein ratio comparable with that obtained previously with this and other mAbs (12, 13, 33, 44, 45). Regardless of radiolabeling with 111In or 211Bi, labeling efficiency and specific activity of the final products were reasonable and consistent (12, 13, 33, 44). Furthermore, radiolabeling of CHX-A'-Herceptin immunonoconjugate with 111In yielded a product with better specific activity and with greater efficiency what has been reported previously (45). More importantly, radiolabeling of Herceptin with an α-emitter was not detrimental to the antibody. The integrity and the immunoreactivity of the mAb were maintained, comparable with the 111In-labeled Herceptin.

The LS-174T cell line, in fact, has been used as a model for peritoneal disease in radioimmunotherapy studies with other mAbs as delivery vectors (7, 31, 36). Biodistribution data obtained with the LS-174T and Shaw xenograft models resulted in greater tumor uptake in what has been reported for studies with 111In- or 131I-labeled mAb 4D5 targeting HER2-transfected MCF-7 or NIH3T3 tumors (23, 24). In the case of 131I-4D5, the tumor reached a maximum of 25%ID/g at 25 hours and rapidly declined to ~5 by 120 hours. This latter study illustrates an inappropriate selection of radionuclide wherein rapid disappearance of the radionuclide results from dehalogenation post-internalization (46).

The analysis of the LS-174T and Shaw cell lines by flow cytometry was not predictive of the in vivo data targeting the HER2 molecule. Determination of the number of HER2 molecules per cell for both cell lines with conventional radioimmunoassays has been attempted. The quantitation to date has been thwarted by the low receptor number on both of these cell lines as evidenced by flow cytometric analysis.

When mice bearing a 3-day tumor burden (i.p.) were treated with 211Bi-CHX-A'-Herceptin, a specific dose-dependent response of increased survival was observed. Not surprisingly, because of the aggressive nature of the model, a lack of tumor response was obtained when mice with a 5-day tumor burden were treated. Similar observations have been noted in which larger tumor burdens were unresponsive to radioimmunotherapy with a radioimmunoconjugate labeled with an α-emitting radionuclide. This observation is consistent with the hypothesized merits of α-versus β-emitting radionuclides (10, 11, 36). The α-emitters are postulated to be ideal for the treatment of smaller tumors/tumor burdens, disseminated disease, and micrometastatic disease (8–12, 14, 47). In contrast, 90Y, a β-emitting radionuclide, is more appropriate for tumor lesions that are ≥1 cm, a function of a long path length and energy decay (5). A direct comparison has been published in which the effectiveness of 90Y and 211Bi was conducted in two animal models for lung cancer (44). Reduction of larger tumor burdens was more effective with the 90Y-labeled mAb, although acute lung damage occurred. Radioimmunotherapy targeting of HER2 with 90Y- and 131I-labeled 4D5 has resulted in a delayed tumor growth (23, 24). The lack of cure is likely attributable to the small tumor burden at the time of radioimmunotherapy, which reflects inappropriate matching of radionuclide with disease burden (48).

An increase in survival was also observed in the Shaw xenograft model at higher doses. However, the response appears to be a general response to radiation. The difference in survival between specific and nonspecific radioimmunotherapy was only 6 days and was not found to be significant. The results of this study illustrate the differences in animal models, the inherent radiosensitivities of tumors, and the importance of assessing radioimmunoconjugates in more than one model.

Determination of a maximum-tolerated dose was elusive, which was consistent with a previous study examining the effectiveness of a 211Bi-labeled immunoconjugate with a domain-deleted mAb (17). None of the animals succumbed to radiation death at the doses given; isotope generation and tol-
erable injection volumes were the limiting factors. In the present study, using animal weights as a harbinger of toxicity, mice receiving 1 mCi of $^{213}$Bi-CHX-A$^+$-Herceptin experienced the greatest weight loss. On the basis of these results, 500 to 750 μCi were established for use in future experiments as a maximum effective dose. This decision was also based on the desire to combine radioimmunotherapy with other modalities, such as chemotherapeutics, that would alter tumor sensitivity to radiation.

The injection volume of the radioimmunoconjugate may be a contributing factor to the inability to determine a real maximum-tolerated dose. The peritoneum is a large cavity throughout which the tumor xenographs are dispersed. A large delivery volume (1 mL) was chosen for these studies to ensure that the peritoneal cavity and organs would be accessed by the radioimmunoconjugate. In fact, it has been shown that higher injection volumes not only enhanced delivery of an agent in the peritoneum, but there is also a lowered maximum concentration of the agent achieved in the blood along with extended retention (49). The pharmacokinetic outcome is a modest reduction in the area under the concentration-time curve. Higher injection volumes therefore would result in lower normal organ toxicity.

A single dose of unlabeled 4D5 (3 μg) was also found to have little effect on the retardation of tumor growth in previous studies (23, 24). In the studies presented herein with 100-fold more Herceptin, survival of animals bearing either the Shaw or LS-174T i.p. xenograft was unaffected. This latter tumor model, however, showed the greatest increase in survival after radioimmunotherapy with $^{213}$Bi-CHX-A$^+$-Herceptin, a persuasive argument for the treatment of patients with radioimmunotherapy that are unresponsive to treatment by the unarmed mAb.

Direct comparison of studies dealing with treatment of i.p. disease with α-emitters is a challenge at this time. The short list of published studies that are available (<10) differ in radioisotopes, tumor models, chelation chemistries, dosing regimens, and whether therapy studies were actually executed (11, 13, 14, 36, 37). For example, Borchardt et al. (37) evaluated Herceptin targeting of HER2. However, the radiolabel was $^{225}$Ac, and the tumor model studied was SKOV-3-NMP2. $^{225}$Ac has a 10-day half-life and was actually being used as an in vivo generator of $^{213}$Bi. This tumor xenograft also expresses high levels of HER2. In another study, the potential of $^{213}$Bi was explored for i.p. radioimmunotherapy of gastric cancer; however, although targeted was reported, no actual therapy studies were presented (13). Mice bearing LS-174T i.p. xenografts were reported to experience a prolonged survival after treatment with $^{212}$Bi-B72.3 (36). The caveat to this by the author’s own admission was that ~20% of the dose leaked to the kidneys within hours of administration, a result of inadequate chemistry for the $^{213}$Bi.

Studies reported herein have demonstrated the feasibility of locoregional administration of an intact mAb to target a short-lived radionuclide for the treatment of disseminated peritoneal disease. The effectiveness of Herceptin radiolabeled with an α-emitting radionuclide is attributed to both the nature of the disease and accessibility of the tumor. Radioimmunotherapy via systemic administration, i.e., i.v., or for the treatment of less accessible tumor burdens may not be an appropriate application of an intact mAb such as Herceptin labeled with $^{213}$Bi. Intact mAbs have slower plasma pharmacokinetics and demonstrate limited penetrance of solid tumors (50, 51). In conjunction with tumor heterogeneity, their use as delivery vehicles for short-lived radionuclides may be limited (50). Molecules such as Fab’ fragments, scFvs, or peptides might be more appropriate with short-lived radionuclides (50, 51). These molecules unfortunately present a new set of problems pertaining to renal uptake and clearance (50).

Circumvention of many of the difficulties has been achieved with domain-deleted mAbs (50). A $^{213}$Bi-labeled humanized domain-deleted mAb has recently been shown to be effective in the treatment of s.c. tumor xenografts (17). The biological half-life of this mAb form is a reasonable match to the physical half-life of $^{213}$Bi despite having a $M^*_f$ (~132,000 (52). This mAb variant is also not subject to the high renal accretion that occurs with the smaller mAb forms, e.g., fragments and peptides.

Radioimmunotherapy targeting the HER2 molecule is appealing in that it may prove beneficial even for those patients with a lower expression of the receptor that would not normally be eligible for immunotherapy. The literature abounds with reports describing the enhancement of anti-HER2 antibody antitumor activity when combined with chemotherapeutics (53). α-Particle radioimmunotherapy offers the opportunity of complementing the intrinsic cytostatic therapeutic efficacy of Herceptin with high linear energy transfer radiation. Studies are currently underway examining the potential of combining modalities such as targeted radiation therapy with chemotherapeutics and radiosensitizers and ultimately to combine these modalities with radiotherapy.

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Targeting of HER2 Antigen for the Treatment of Disseminated Peritoneal Disease

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