An α-Particle Emitting Antibody ([213Bi]J591) for Radioimmunotherapy of Prostate Cancer

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ABSTRACT

A novel α-particle emitting monoclonal antibody construct targeting the external domain of prostate-specific membrane antigen (PSMA) was prepared and evaluated in vitro and in vivo. The chelating agent, N-[2-amino-3-(p-isothiocyanatophenyl)propyl]-trans-cyclohexane-1,2-diamine-N,N,N',N''-pentaaacetic acid, was appended to J591 monoclonal antibody to stably bind the 213Bi radiometal ion. Bismuth-213 is a short-lived (t1/2 = 46 min) radionuclide that emits high energy α-particles with an effective range of 0.07–0.10 mm that are ideally suited to treating single-celled neoplasms and micrometastatic carcinomas. The LC50 prostate cancer cell line had an estimated 180,000 molecules of PSMA per cell; J591 bound to PSMA with a 3-nM affinity. After binding, the radiolabeled construct-antigen complex was rapidly internalized into the cell, carrying the radiometal inside. [213Bi]J591 was specifically cytotoxic to LC50. The LD50 value of [213Bi]J591 was 220 nCi/ml at a specific activity of 6.4 Ci/g. The potency and specificity of [213Bi]J591 directed against LC50 spheroids, in an in vitro model for micrometastatic carcinoma, also was investigated. [213Bi]J591 effectively stopped growth of LC50 spheroids relative to an equivalent dose of the irrelevant control [213Bi]HuM195 or unlabeled J591. Cytotoxicity experiments in vitro were carried out in an athymic nude mouse model with an i.m. xenograft of LC50 cells. [213Bi]J591 was able to significantly improve (P < 0.0031) median tumor-free survival (54 days) in these experiments relative to treatment with irrelevant control [213Bi]HuM195 (33 days), or no treatment (31 days). Prostate-specific antigen (PSA) was also specifically reduced in treated animals. At day 51, mean PSA values were 104 ng/ml +/- 54 ng/ml (n = 4, untreated animals), 66 ng/ml +/- 16 ng/ml (n = 6, animals treated with [213Bi]HuM195), and 28 ng/ml +/- 22 ng/ml (n = 6, animals treated with [213Bi]J591). The reduction of PSA levels in mice treated with [213Bi]J591 relative to mice treated with [213Bi]HuM195 and untreated control animals was significant with P < 0.007 and P < 0.0136, respectively. In conclusion, a novel [213Bi]-radiolabeled J591 has been constructed that selectively delivers α-particles to prostate cancer cells for potent and specific killing in vitro and in vivo.

INTRODUCTION

Metastatic prostate cancer that is resistant to hormone therapy has been treated with nonspecific, systemic bone-seeking agents such as 32P, 89Sr, and 153Sm (1–3), having the palliation of pain as the objective end point. Specifically targeted radiotherapy using prostate tumor-specific CC49 antibody labeled with 211At has been evaluated in two clinical trials with patients having metastatic prostate carcinoma. In one trial, antibody localization was seen in 11 of 14 patients along with significant myelosuppression, however, it was estimated that there were low radiation doses to tumor (~1000 cGy); there was no repeat dosing due to human antimurine antibody responses, and no patients met the radiographic or PSA2 criteria for objective response (4). In the second trial, a high degree of tumor localization was observed in 13 of 15 patients, but human antimurine antibody response was seen in all patients; some degree of antitumor effect, as measured by pain relief, was seen in 6 of 10 symptomatic patients. However, once again, no patients met the radiographic or PSA criteria for objective response (5).

Several mAbs that target prostate cancer were modified with the N-[2-amino-3-(p-isothiocyanatophenyl)propyl]-trans-cyclohexane-1,2-diamine-N,N,N',N''-pentaaacetic acid (SCN-CHXA’’-DTPA) moiety, labeled with 213Bi, and their in vitro potency toward cancer cell lines was evaluated (6). This comparative study of potential therapeutic antiprostate mAbs resulted in the selection of J591 as the IgG to study further. J591 targets the external domain of PSA (7–9). PSA is a highly restricted prostate epithelial cell integral membrane glycoprotein, providing an immunogenic extracellular domain. PSA is expressed by a high proportion of prostate carcinomas, and its expression is further increased in higher-grade cancers, in metastatic disease and in hormone-refractory prostate cancers. There are several mAbs targeting the external domain of PSA that have been developed and described in the literature (7–9).

The construction of an α-particle emitting IgG radiotherapeutic moiety represents a “new class” of potent and specific radiotherapeutics used only recently in humans (10, 11). The radionuclide 213Bi is a short-lived (t1/2 = 46 min) α-particle emitting metal-ion generated from the decay of Ac-225. Bismuth-labeled HuM195, an anti-CD33 antibody, demonstrated specific and potent cell killing ability when directed against a leukemia cell line (12). A robust, reliable 225Ac/213Bi generator system was developed at Memorial Sloan-Kettering Cancer Center (13, 14) and used to treat 18 patients with 80 doses of [213Bi]HuM195 in a Phase I clinical trial (11). Clinical conditions for reproducible radiolabeling of antibodies and quality assurance testing were developed (15). Feasibility for human use and therapeutic activity were demonstrated. The low abundance, low energy γ-emissions associated with the decay of 213Bi (440 KeV, frequency of 16% per disintegration) were used in evaluating the biodistribution patterns of [213Bi]HuM195 in humans and in performing dosimetry calculations (16–18).

α-Particle therapy has been proposed for use in single-cell disorders, such as leukemias. The purpose of the current study was to evaluate the biochemistry, biology, and cytotoxicity of a potent [213Bi]-labeled J591 construct in a series of in vitro and in vivo models of prostate cancer to determine whether RIT with α-particles also would be suitable for clinical use against prostate cancer. Such an approach would be particularly appropriate after debulking radiation or surgery to reduce the size of metastatic deposits of cancer.

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The abbreviations used are: PSA, prostate-specific antigen; mAb, monoclonal antibody; PSA, prostate-specific membrane antigen; RIT, radioimmunotherapy; DTPA, diethylentriaminepentaacetic acid.

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**MATERIALS AND METHODS**

**J591.** The murine mAb J591, reactive with the external domain of PSMA, was obtained from the laboratory of Dr. Neil Bander (Department of Urology, New York Presbyterian Hospital-Weill Medical College of Cornell University; Refs. 7–9).

**Conjugation of Bisfunctional Chelate to J591.** J591 was conjugated to N-[2-amino-3-(p-isothiocyanatophenyl)propyl]-trans-cyclohexane-1,2-diamine-N,N',N,N'-pentaaacetic acid (SCN-CHX-A-DTPA), a backbone substituted derivative of DTPA (19), using an Amicon, Inc. (Beverly, MA) continuous buffer exchange/dialysis apparatus (20). The average number of chelates per antibody ranged from one to three as determined by the yttrium arsenazo spectrophotometric method (21). Immunoactivity of the construct was determined as described (12).

**Radioisotope Production, Radio labeling, and Purification of [212Bi]J591.** Bismuth-212 was produced (22–24) from an 222Ac-source generously supplied by the Institute for Transuranium Elements (Karlsruhe, Germany) or purchased from Oak Ridge National Laboratory (Oak Ridge, TN). The 222Ac was fashioned into a radionucleide generator that produced 212Bi using published procedures (13). Radiolabeling and purification of the mAb construct with 212Bi was carried out using published methods (12). The labeling efficiency of the reaction and purity of the final product was determined routinely using instant TLC with silica gel impregnated paper (Gelman Science Inc., Ann Arbor, MI; Ref. 12).

**Bismuth-213 Activity Determination.** Bismuth-213 activity was measured with a Squibb CRC-17 Radioisotope Calibrator (or equivalent model; E. R. Squibb and Sons, Inc., Princeton, NJ) set at 775 and multiplying the displayed activity value by 10. The activity value reported by the CRC calibrator was verified by counting a sample aliquot point source at a fixed geometric on a pulse height multichannel analyzer (Canberra Industries, Meriden, CT). The counting efficiency of the 213Bi 440 KeV γ-emission was determined using a plot of counting efficiency verses the γ-energy of standard radionuclide sources (13).

**Binding Affinity and Determination of the Number of Antigen-binding Sites.** A nonlinear regression curve fit of the data obtained for the specific bound [111In]J591 was performed to determine the J591 equilibrium binding affinity with LNCaP cells and the number of J591-binding sites per cell. Briefly, the [111In]J591 construct was labeled, purified, and characterized in a similar fashion to the CHX-A-DTPA-HuM195 construct (12). Freshly prepared LNCaP cells at 4°C (5E4 cells/tube) were reacted with a 50-fold excess of unlabeled J591 and serial increments of [111In]J591 (in triplicate) or just the serial increments of [111In]J591 (in triplicate) to generate the equilibrium J591-binding curves. A nonlinear regression method was used to fit a curve to the plot of cpm per cell pellet versus added radiolabeled antibody. B max was corrected for the immunoactivity of the preparation and used to determine the number of antigen-binding sites per cell. An apparent Kd was read from the fit curve at 0.5 times B max and versus the γ-energy of standard radionuclide sources (13).

**RESULTS**

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**Equilibrium Binding Affinity and the Number of Antigen-binding Sites.** A nonlinear regression curve fit analysis (R2 = 0.995) of the [111In]J591 equilibrium binding data yielded an immunoactivity corrected B max value of 13,744 cpm per 50,000 LNCaP cells (Fig. 1). Using this value of B max, it was estimated that there were 180,000 molecules of J591 bound per cell. The equilibrium binding affinity, Kd, was taken from the fit curve at 0.5 times B max and estimated to be 3 nM.

**Modulation of Cell Surface Antibody-Antigen Complexes.** The [111In]J591-PSMA (cell surface antibody-antigen) complex was rap-
idle internalized immediately after binding to the cell. Approximately 66% of the complex was internalized after 2 h, and 79% was internalized after 4 h (Fig. 2). The total cell-associated counts and, therefore, the cell surface counts continued to increase as a function of time through 24 h.

Cell Kill against Single Cells in Vitro. LNCaP cell kill by [213Bi]J591 proved to be both specific activity dependent and activity concentration dependent (Fig. 3). The LD50 value of [213Bi]J591 was 220 nCi/ml at a specific activity of 6.4 Ci/g, 315 nCi/ml at a specific activity of 3.8 Ci/g, and 4400 nCi/ml at a specific activity approaching 0.06 Ci/g. At the low specific activities represented by the last group, there is essentially no specific binding of the α-particle emitting J591 to the targets. Thus, this level of cell kill represents the nonspecific cytotoxicity in the system used here. Additional experiments evaluated [213Bi]J591 against a PSMA-negative tumor cell line, SKOV3. The LD50 value derived was 13,000 nCi/ml at a specific activity level of 1.6 Ci/g (data not shown). Therefore, cytotoxicity was specific activity related, dose related, and antigen specificity dependent.

Cell Kill against Multicellular Spheroids in Vitro. We have also investigated the potency and specificity of [213Bi]J591 directed against LNCaP spheroids by assessing the change in volume of the spheroid as a function of time following various treatments. The spheroids initially comprised ~1000 LNCaP cells. The volumes of all of the spheroids treated with a single dose of [213Bi]J591 decreased markedly over a 2-month period (Fig. 4). Their size was more than 100-fold smaller than the untreated spheroid control group in this time period. The [213Bi]HuM195-treated spheroids demonstrate a 1-week delay in growth, followed by an increase in volume. Two other control groups, untreated spheroids and spheroids exposed to unlabeled J591 (0.027 mg/ml), showed similar increases in volume to each other as a function of time with an almost 100-fold increase in spheroid volume after 1 month.

LNCaP Tumor Model in Mice. LNCaP tumor cells were mixed with Matrigel (Becton Dickinson Labware) and xenografted into athymic nude mice, 8 weeks of age. Mice received an i.m. injection of 6–7E6 LNCaP tumor cells mixed with Matrigel in the right hind leg at a volume of 0.25 ml. Tumor growth in vivo was assessed histologically at days 2, 3, 5, 7, and 10 (Table 1). At the time RIT was administered (day 2), the tumors were characterized histologically as disorganized cell clusters and nodules each comprised of several thousands of cells. The nodules were not vascularized and not encapsulated. On day 3, the tumors were more organized and were becoming vascularized, but still not encapsulated. By the 5th day, vascularization was more pronounced, and on day 7 the tumors were encapsulated. The purpose of the RIT studies (described below) was to examine the ability of the agent to treat the tumors before encapsulation and neovascularization.

Cell Kill in Vivo. A single course of the [213Bi]J591 drug, administered in four daily doses, improved (P < 0.0031) median tumor-free survival (Fig. 5) of LNCaP xenografted mice relative to mice treated with [213Bi]HuM195 or untreated controls. The median tumor-free survival times were 31 days (n = 4, untreated animals), 33 days (n = 6, [213Bi]HuM195-treated animals), and 54 days (n = 6, [213Bi]J591-treated animals).

PSA is an important surrogate marker for prostate cancer burden in humans (28). It can also be used in mice with prostate cancer cell
Fig. 4. Cytotoxicity of $^{213}$BiJ591 on LNCaP spheroids. LNCaP spheroid volume versus time following treatment is shown: ● $^{[213]}$BiJ591; ○ $^{[213]}$BiHuM195; ■ J591; ▲ no treatment growth control. Experiments were conducted as described in “Materials and Methods.”

Table 1 Histopathological characterization of LNCaP tumor xenografts implanted i.m. in athymic nude mice, 8 weeks of age

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Morphology</th>
<th>Nodule size (mm)</th>
<th>Vascularized</th>
<th>Encapsulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Nodules and scattered cells</td>
<td>0.1 × 0.2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Small nodules</td>
<td>0.2 × 1.0</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>Nodules</td>
<td>0.5 × 1.3</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>Nodules</td>
<td>1.0 × 2.0</td>
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<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>Nodules</td>
<td>1.0 × 2.0</td>
<td>++</td>
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</table>

DISCUSSION

$\alpha$-Particle emitting radiolabeled antibody constructs have been proposed as potent, selective agents to kill single cells (30, 31). We have shown this in vitro and in humans using an $\alpha$-particle emitting anti-CD33 mAb for treatment of leukemia (10–12, 15, 16). Tens of billions to hundreds of billions of individual leukemia cells in the blood and marrow were killed safely. The kinetics and geometry of single-cell killing, however, might not be predictive of killing micrometastatic clusters of tumor cells as would be expected with the early spread of carcinomas. Therefore, we now ask whether an $\alpha$-particle emitting agent would be useful in a model for solid tumors, such as prostate cancer, that form micrometastatic disease in the bone marrow. In this study, we describe for the first time the construction of a prostate-specific, $\alpha$-particle emitting agent capable of: (a) binding and internalizing into target cells; (b) selectively killing both individual cells and 1000-cell spheroid clusters; and (c) prolonging tumor-free survival and reducing PSA in mice bearing prostate cancer xenografts.

Table 2 $P$ for the observed PSA values between $^{[213]}$BiJ591-treated mice and the $^{[213]}$BiHuM195-treated mice and controls

| Day 30 | $^{[213]}$BiJ591 (n = 12) | $P < 0.0443$ (n = 10) |
| Day 51 | $^{[213]}$BiJ591 (n = 6)  | $P < 0.007$ (n = 6)   |
| Controls | $^{[213]}$BiHuM195 | $P < 0.0192$ (n = 10) |

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LNCaP cell kill in vitro using $^{213}$BiJ591 proved to be both specific activity dependent and activity concentration dependent. There was a 20-fold difference in the LD$_{50}$ values in vitro, spanning a 100-fold range in specific activities, indicating the specificity of the drug for cells expressing PSMA. Additional data demonstrated that $^{213}$BiJ591 did not specifically kill a PSMA-negative tumor cell line, SKOV3, supporting the fact that the drug does not target or destroy tissue that does not express PSMA. Therefore, $^{213}$BiJ591 was an effective and specific radiolabeled agent for ablation of individual prostate cancer cells in vitro. This is an advantage of using $\alpha$-particle emitting radionuclides because the particles transfer their energy in a region that is in very close proximity to the target.

We have also investigated the potency and specificity of $^{213}$BiJ591 directed against LNCaP spheroids initially comprising $\sim$1000 cells in vitro. Spheroids of this size approximate the micrometastatic disease condition and can serve as a therapeutic test for investigating the potency and specificity of $\alpha$-particle emitting IgG directed against multicellular targets. The $^{213}$BiJ591 must be efficacious against the spheroids if it is expected to be clinically effective against metastatic prostate cancer. A single dose of the $^{213}$BiJ591 drug was effective in arresting the growth of LNCaP spheroids relative to an equivalent dose of $^{213}$BiHuM195, an irrelevant control mAb that has a minimal effect against the LNCaP spheroids, a similar dose of unlabeled J591, and untreated spheroids. Control experiments exhibited similar spheroid growth kinetics, with the average spheroid volume increasing almost 100-fold over a period of 1 month. The $^{213}$BiHuM195-treated spheroids exhibited a delay in growth by approximately 1 week, presumably due to nonspecific irradiation effects. It was estimated that random hits from $\alpha$ particles emitted from nonspecific IgG dispersed in the media could result in delivery of about 1% of the radiation dose as compared with a $^{213}$Bi-labeled specific mAb (27). Other experiments$^4$ have shown that anti-PSMA IgG penetrates one to two cell layers into spheroids during the time period (45–90 min) that $^{213}$Bi would be expected to be most active. The $^{213}$BiJ591-treated spheroids decreased by volume in about 10% over a 3-week period with no further growth during the 2-month duration of the experiment. These data were repeated in a second experiment with similar results. Because the spheroid model may resemble the micrometastatic disease situation, it was apparent that $^{213}$BiJ591, despite incomplete penetration into the spheroid mass, was able to specifically target large cell clusters, destroy targeted cells, and interrupt further growth of the unirradiated spheroid core. Additional studies ongoing in this area are focusing on the use of a multiple dosing schedule that would effectively peel away the remaining cell layers that might still be viable. A multiple dosing schedule may eradicate the disease point source in its entirety while targeting any other remaining or new disease sites; however, the schedule and dose details need to be better understood.

The two therapeutic experiments carried out in vivo demonstrated the ability of $^{213}$BiJ591 to improve the duration of tumor-free survival and to suppress PSA relative to controls in an animal model. Following the trends observed in the spheroid cytotoxicity experiments, unlabeled J591 was ineffective in treatment and was comparable with no treatment. In addition, the $^{213}$BiHuM195 demonstrated a minimal effect in delaying tumor growth (2–3 days) and in decreasing PSA values relative to controls. Table 2 compares the Ps for the observed PSA values between the $^{213}$BiJ591-treated mice and the $^{213}$BiHuM195-treated mice and controls. The tumor model used in this study was not optimal for examining the efficacy of an $\alpha$-particle emitting radioimmunotherapeutic agent because an $\alpha$-particle has a pathlength of two to four cell diameters. However, in the absence of reliable metastatic models for prostate cancer in vivo, we used an i.m. tumor model and treated at a relatively early time point following tumor inoculation; this allowed us to observe an effect due to the $^{213}$BiJ591 agent that resulted in tumor growth delay and a lower PSA value relative to controls. The specific $^{213}$BiJ591 was directed against clusters of cells that at the time of RIT were approximately the size of the spheroids that were studied in vitro. As with the spheroids, the tumors in vivo comprised nodules containing 1000–2000 LNCaP cells that were not vascularized and not encapsulated.

We have been successful in a Phase I clinical trial using $^{213}$BiHuM195 (10, 11) to treat 18 patients with acute myelogenous leukemia. Ten of 12 evaluable patients had reductions in peripheral blood leukemia cells, and 12 of 18 patients had decreases in bone marrow blasts. Doses up to 1 mCi/kg were used safely, and there was no acute toxicity seen. In the treatment of leukemia there may be ~1 kg of tumor present (1E12 cells) with ~20,000 HuM195 molecules bound by individual HL60 leukemia cells (32).

The clinical situation in prostate cancer may favor the use of a $^{213}$Bi-labeled antibody based on: (a) low tumor burden; (b) anti-PSMA-IgG-PSMA internalization; and (c) the relatively large number of PSA-binding sites per cell. In comparison with leukemia, there should be at least 10–100 times less prostate cancer tumor burden at the time of treatment, even with widespread micrometastatic lesions. Furthermore, J591 results in the internalization of the J591-PSMA complex into LNCaP cells (8), which favors the therapeutic use of $^{213}$Bi-labeled IgG constructs because internalized radionuclides (a) will tend to remain with the cell and not be released and carried away from the target and (b) decays within the cell will necessarily deposit energy within the cell. We undertook the measurement of the radionuclide-labeled construct-antigen complex internalization to determine the amount of drug internalized initially and as a function of time. Cellular internalization of $\alpha$-particle emitting radionuclides ensures that the $^{213}$Bi particulate decay traverses the cell, enhancing the cytotoxicity relative to surface-bound species. We anticipate the ability to internalize much of the targeted, cell bound $^{213}$BiJ591 activity within a 4-h period allowing the $^{213}$Bi to decay and deliver its dose efficiently within the target cell. Additionally, it has been observed that the LNCaP cells continue to bind J591 and internalize it following exposure to the construct, presumably by expression of new (recycled) antigen-binding sites (Ref. 8 and this study). From a therapeutic standpoint, this modulation phenomenon works to therapeutic advantage because $^{213}$BiJ591 in circulation can continue to be bound even after initial saturation of the cell binding sites. The LNCaP cells bind...
\[ 180,000 \text{ molecules of J591, which was about a 10-fold greater number of binding sites than the number of CD33 sites per leukemia cell. Assuming that prostate cancer cells express similar levels of PSMA in vivo, typical therapeutic specific activities of [\text{123}^{\text{Bi}}]J591 (10 \text{ Ci/g}) would result in delivery of \approx 210 \text{ Bi-atoms per cell. It has been demonstrated that several 210 Bi-atoms targeted per cell may result in a lethal dose of } \alpha \text{ particles to half of these cells.} \]

In summary, the radioimmunopharmaceutical described in this study, [\text{123}^{\text{Bi}}]J591, has been demonstrated to potently and specifically eradicate individual prostate cancer cells and 1000 cell spheroid clusters in vitro and inhibit tumor growth and suppress PSA in vivo in animal models. These features support the investigation of such an agent further in the human clinical setting to treat metastatic prostate cancer.

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