Anti-CD45 pretargeted radioimmunotherapy using bismuth-213: high rates of complete remission and long-term survival in a mouse myeloid leukemia xenograft model

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ABSTRACT

Pretargeted radioimmunotherapy (PRIT) using an anti-CD45 antibody (Ab)-streptavidin (SA) conjugate and DOTA-biotin labeled with beta-emitting radionuclides has been explored as a strategy to decrease relapse and toxicity. Alpha-emitting radionuclides exhibit high cytotoxicity coupled with a short path-length, potentially increasing the therapeutic index and making them an attractive alternative to beta-emitting radionuclides for patients with Acute Myeloid Leukemia (AML). Accordingly, we have used $^{213}\text{Bi}$ in mice with human leukemia xenografts. Results demonstrated excellent localization of $^{213}\text{Bi}$-DOTA-biotin to tumors with minimal uptake into normal organs. After 10 minutes, $4.5 \pm 1.1\%$ of the injected dose of $^{213}\text{Bi}$ was delivered per gram of tumor. Alpha imaging demonstrated uniform radionuclide distribution within tumor tissue 45 minutes after $^{213}\text{Bi}$-DOTA-biotin injection. Radiation absorbed doses were similar to those observed using a beta-emitting radionuclide ($^{90}\text{Y}$) in the same model. We conducted therapy experiments in a xenograft model using a single-dose of $^{213}\text{Bi}$-DOTA-biotin given 24 hours after anti-CD45 Ab-SA conjugate. Among mice treated with anti-CD45 Ab-SA conjugate followed by 800 $\mu$Ci of $^{213}\text{Bi}$- or $^{90}\text{Y}$-DOTA-biotin, 80% and 20%, respectively, survived leukemia-free for >100 days with minimal toxicity. These data suggest that anti-CD45 PRIT using an alpha-emitting radionuclide may be highly effective and minimally toxic for treatment of AML.
INTRODUCTION

For over a decade, antibodies (Abs) conjugated to a radionuclide emitting particulate radiation have been used in the management of leukemia in an effort to deliver targeted doses of radiation to bone marrow, spleen, and other sites of disease, while sparing normal organs. This radioimmunotherapy (RIT) approach has been employed to achieve significant remissions in patients with acute myeloid leukemia (AML), particularly when used at high-doses of radioactivity in conjunction with myeloablation.\textsuperscript{1-10} One of the major limitations of this approach, however, has been the pharmacokinetic properties of the Ab protein. Abs accrete slowly in solid tumors and are eliminated slowly from the circulation. Use of radiolabeled Abs, therefore, result in prolonged exposure in radiosensitive tissues, particularly marrow, due to the extended time within the circulation. Additionally, the extended time required for tumor localization of the Ab may result in loss of tumoricidal potency of the radionuclide due to ongoing isotopic decay. To address this shortcoming, the pretargeted (P)RIT system has been developed. This system differs from conventional RIT in that it uncouples the targeting agent from the radioisotope, which is administered in a separate step after facilitated clearance of non-tumor bound targeting agent.\textsuperscript{11} Because the radioisotope can be delivered on a small molecule (<1 kDa) that is rapidly excreted through the kidneys, normal organ exposure to circulating radiation is effectively reduced by this approach. It has been demonstrated that PRIT technology can further amplify the amount of radiation delivered to CD45\textsuperscript{+} tissues and at the same time diminish the radiation dose to non-targeted cells.\textsuperscript{12-15}

A variety of radionuclides have been investigated for RIT of leukemias, where the types of emissions employed have primarily focused on the use of beta-particles (\textsuperscript{131}I, \textsuperscript{90}Y, \textsuperscript{188}Re). Over the past several years interest has developed in targeting alpha-emitters to leukemia cells for RIT.\textsuperscript{8,16} As opposed to the relative non-specific cytotoxicity
of beta-emitting constructs due to the crossfire effect, alpha particle decay of radionuclides such as $^{213}$Bi, $^{211}$At, and $^{225}$Ac results in high energy (6-8 MeV) delivery over a very short distance (50-80 μm). The short path-length may provide a therapeutic advantage for targeting leukemic cells in the marrow, and thus prevent the exposure of many normal hematopoietic stem cells to non-specific irradiation. Therefore, the novel approach of PRIT combined with very short half-life of alpha emitters may have the potential to further optimize the administration of radionuclide therapy and improve outcomes for leukemia patients.

To assess the merits of alpha- versus beta-emitting CD45 PRIT for leukemia, we report here comparative biodistribution and therapy experiments using human leukemia xenografts implanted in athymic mice. We have demonstrated excellent localization to HEL leukemia tumor sites using both alpha- and beta-emitting radionuclides with minimal uptake into normal organs due to elimination of non-specific radiation exposure from blood-borne radiolabeled Ab after anti-CD45 Ab-SA pretargeting. The target-to-non-target therapeutic ratios (based on radiation dose) obtained using PRIT with $^{213}$Bi were similar to those observed using $^{90}$Y. Using a novel alpha-camera, we have also shown that $^{213}$Bi-DOTA-biotin uniformly distributes within tumor tissue 45 minutes after injection. Lastly, data from comparative PRIT experiments suggest that anti-CD45 PRIT using an alpha-emitting radionuclide may allow for intensification of the targeted radiotherapy, with diminished toxicity, to sites of leukemic involvement in order to decrease the risk of relapse.
METHODS

Cell Lines, Antibodies, and Production of Ab-SA Conjugates

All cell lines were obtained and maintained as described previously. The hybridoma cell lines expressing the murine anti-human IgG₁ CD45 Ab BC8 and the isotype-matched human anti-bovine herpesvirus-1 (BHV-1) Ab, employed as non-specific negative control, and all Ab-SA conjugates were produced as previously described.

Radiolabeling

DOTA-biotin was synthesized and labeled with either $^{90}$Y (PerkinElmer, Inc. Waltham, MA) or $^{213}$Bi (isolated from $^{225}$Ac; Department of Energy, Oak Ridge, TN) as previously described. Radiochemical purity was typically greater than 99% as determined by HPLC for each construct, and labeling efficiencies were >90%.

Biotinylated Clearing Agent

A synthetic biotinylated CA containing 16 N-acetyl-galactosamine residues per dendrimeric molecule (Aletheon Corporation, Seattle, WA) was used to eliminate excess Ab-SA molecules from the circulation before the administration of radiolabeled biotin. The N-acetyl-galactosamine residues have a high affinity for hepatic asialoglycoprotein receptors and thus facilitate the rapid hepatic clearance of residual Ab-SA conjugates from the bloodstream and their endocytosis into liver cells.

Mice

Female BALB/c mice, 6 to 12 weeks old, were purchased from Harlan Sprague Dawley (Livermore, CA). The animals were housed under protocols approved by the Fred Hutchinson Cancer Research Center (Seattle, WA) Institutional Animal Care and
Use Committee. Results of all mouse studies are representative of at least three experiments.

**Biodistribution Studies**

For murine biodistribution experiments, all mice were placed on a biotin-deficient diet (Animal Specialties, Hubbard, OR) at least 5 days prior to injection of Ab-SA conjugates and radiobiotin. Mice were subsequently injected with $1 \times 10^6$ HEL cells subcutaneously (sc) in the flank. Five days later mice were given 1.4 nmol unlabeled BC8 (300 µg) or BHV-1 (isotype-matched negative control) Ab-SA conjugate intravenously (iv) via tail vein. Mice were then administered 5.8 nmol (50 µg) of CA iv 22 hours after each Ab-SA followed by delivery of 1.2 nmol (1 µg) of $^{90}$Y-DOTA-biotin or $^{213}$Bi-DOTA-biotin (50 µCi) 2 hours after CA. At 10, 45, and 90 minutes after injection of $^{213}$Bi-DOTA-biotin and 1, 24, and 72 hours after $^{90}$Y-DOTA-biotin injection, tumors and normal organs were excised, weighed, and the percent administered activities per gram of tumor or organ (% ID/g) were determined as previously described. In studies to reduce the non-specific radionuclide uptake in the kidney, groups of 5 mice received 2,3-dimercapto-1-propanesulfonic acid (DMPS; Sigma-Aldrich, St. Louis, MO) in the drinking water (1.2 mg/ml) 24 hours prior to $^{213}$Bi-DOTA-biotin.

**Radiation Dosimetry**

Radiation absorbed doses were calculated for circulating blood and for each excised organ, tissue, and tumor based on wet tissue weights and time-activity plots that were constructed from the biodistribution data. To determine the total number of radioactive transformations (decay) in each tissue, we fit an exponential function to the time-activity data by least-squares regression analysis, and then integrated the area under each curve from time of injection to infinity. The total number of decays were multiplied by the energy released from $^{90}$Y and $^{213}$Bi per decay. We accounted for the energy absorbed
fractions in that tissue and for all other neighboring tissues.\textsuperscript{20} This method is sensitive to the size, shape, and anatomical placement of organs and tissues in the mouse that can result in cross-organ doses from incorporated $^{90}$Y. For alpha particles from $^{213}$Bi, a self-organ absorbed fraction of 1.0 was assumed and a cross-organ absorbed fraction of 0 due the very short range of alpha particles.

**Alpha Camera Imaging**

The alpha camera is a digital autoradiography technology utilizing an optical registration of photons emitted from a scintillator dedicated to quantitative imaging of alpha particles in tissues ex vivo.\textsuperscript{21} To perform alpha camera experiments, tumors were placed in a cryomold containing a cryoprotective gel immediately after dissection. The molds were placed on a pre-cooled aluminum block that was sunken in liquid nitrogen. The frozen tissues were placed in a HM 520 cryostat-microtome (Microm International GmbH, Walldorf Germany) set to a temperature of -19°C. Two consecutive cryosections, used for histological comparison were cut and transferred to a glass slide for haematoxylin and eosin (H&E) staining and for immunohistochemistry (IHC). Sections (10-16 $\mu$m) were cut and transferred to the top surface of a piece of scintillation sheet for the imaging procedure. Blue photons that impinged upon the scintillator were optically imaged with a charge-coupled device detector. The pixel intensities in acquired images were linear to activity of the alpha-emitting radionuclide in the imaged tissue cryosection, allowing quantitative analysis of activity distribution at a spatial resolution approaching the microscopic level.\textsuperscript{21} The acquired and background corrected alpha images were used quantitatively by analyzing and comparing the intratumoral activity distribution of the alpha-emitter $^{213}$Bi at different micrometer scales. Circular regions of interest (ROI) of different sizes were defined within the imaged tumors and the mean (±
standard deviation; SD) pixel intensities were compared for ROI of different size and spatial location.

**Pretargeted Radioimmunotherapy Studies using Human Leukemia Xenografts**

To compare PRIT using either $^{90}$Y- or $^{213}$Bi-DOTA-biotin, groups of 10 mice were placed on biotin-deficient diet at least 5 days prior to study onset. Selected groups of mice were also treated with DMPS as described above. Mice were then injected sc with $1 \times 10^{10}$ HEL cells and two days after tumor injection, mice received 1.4 nmol unlabeled BC8 (300 µg) or BHV-1 Ab-SA conjugate iv. Mice were subsequently given 5.8 nmol (50 µg) of CA iv 22 hours later. Two hours following CA administration, groups of mice received 1.2 nmol (1 µg) of DOTA-biotin labeled with increasing doses of either $^{90}$Y (112, 800 and 1200 µCi) or $^{213}$Bi (400 and 800 µCi). Mice were monitored as previously described and were euthanized if xenografts exceeded 10% of total body weight, caused obvious discomfort, or impaired ambulation. Tumor volumes (mm$^3$) were calculated as previously described. Time-to-tumor growth and time-to-death were treated as time-to-event end points, and Cox regression was used to test for a dose-response effect. Mice were assigned levels of 1 to 5 and a factor treating these levels as a continuous linear variable was then included in a Cox regression model to test for a dose-response effect (*i.e.*, to ask if the risk of failure was associated with a decrease in dose).

In a series of toxicity experiments, mice surviving more than 6 months after therapy were euthanized. Blood was obtained for complete blood counts. Serum was used to assay for hepatic function by determining levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate transaminase (AST) and renal function by obtaining levels for blood urea nitrogen (BUN) and creatinine (Cr). Averages and
standard errors for hematology and chemistry data are reported for each group of mice studied.

RESULTS

Biodistributions of Radioactivity after PRIT using Anti-CD45 Ab-SA Conjugate followed by $^{90}$Y-DOTA-biotin or $^{213}$Bi-DOTA-biotin

The biodistributions of $^{90}$Y- and $^{213}$Bi-DOTA-biotin were evaluated following administration of an Ab-SA conjugate directed against human CD45$^+$ leukemia xenografts given sequentially with a dendrimeric N-acetylglucosamine-containing CA. These pretargeted biodistribution studies were performed using HEL leukemia-bearing mice injected with 1.4 nmol of anti-CD45 BC8 Ab-SA, followed 22 hours later by 5.8 nmol of CA and 3 hours later mice were administered either 1.2 nmol $^{90}$Y-DOTA-biotin or $^{213}$Bi-DOTA-biotin. Groups of 5 mice each were sacrificed after 1, 24, and 72 hours after delivery of $^{90}$Y-DOTA-biotin and 10, 45, and 90 minutes after injection of $^{213}$Bi-DOTA-biotin. The uptake in tumors for mice treated with $^{90}$Y-DOTA-biotin was $7.8 \pm 4.3 \%\text{ID/g}$ 1 hour after injection, peaking at $12.2 \pm 4.7 \%\text{ID/g}$ 24 hours after injection (Fig. 1A). The targeted concentrations of $^{90}$Y radioactivity remained at relatively high levels in tumors over time, measured at $4.7 \pm 1.9 \%\text{ID/g}$ 72 hours after injection. The amount of radioactivity remained relatively high in the blood and normal organs 1 hour after delivery of $^{90}$Y-DOTA-biotin with $3.1 \pm 0.2\% \text{ID/g}$ remaining in the blood and $2.2 \pm 0.2\% \text{ID/g}$ in the kidneys. The tumor-to-normal organ ratios of $^{90}$Y radioactivity using pretargeted BC8 Ab-SA ranged from 3:1 (blood) to approximately 16:1 (muscle) after 24 hours.

In groups of mice where $^{213}$Bi was the radionuclide employed, high uptake was demonstrated in tumors 10 minutes after injection at $5.9 \pm 2.5 \%\text{ID/g}$ (Fig 1B). The
tumor localization of $^{213}$Bi-DOTA-biotin remained steady with a measured uptake of 5.3 ± 0.8 %ID/g after 45 minutes and 4.1 ± 3.4 %ID/g and 90 minutes after injection. The use of pretargeted $^{213}$Bi-DOTA-biotin, however, also showed high uptake in the kidneys, measured at 5.4 ± 2.0 %ID/g 10 minutes after injection, which remained relatively constant to the 90 minute time-point (5.2 ± 0.3% ID/g). At all time points, control animals injected with non-specific $^{213}$Bi-labeled non-binding isotype-matched Ab-SA conjugate exhibited negligible tumor uptake of the radiolabel demonstrating the specificity of targeting in these experiments (Fig 1D).

**Renal Uptake and Dosimetry**

In an effort to reduce non-specific uptake of radioactivity in the kidney, a metal chelator (DMPS) was administered in the drinking water for animals that subsequently received $^{213}$Bi-DOTA-biotin. In particular, DMPS has been shown to be effective in clearing the radioactive alpha daughters of $^{225}$Ac, including $^{213}$Bi. Therefore, groups of 5 mice were treated with DMPS orally 24 hours prior to $^{213}$Bi-DOTA-biotin injection. DMPS-treated mice displayed significantly lower levels of radioactivity in the kidneys compared to the untreated controls at all time points (Fig. 1C and 1D). For example, after 45 minutes the renal uptake of $^{213}$Bi was 1.5 ± 0.2 for mice treated with DMPS, compared to 4.5 ± 0.9 for mice in the PRIT group that did not receive DMPS ($p = 0.0006$). Mice treated without DMPS displayed tumor-to-kidney ratios of radioactivity of approximately 1:1 at all time points after injection of 800 µCi of $^{213}$Bi-DOTA-biotin. In contrast, mice that were treated with DMPS and 800 µCi of $^{213}$Bi-DOTA-biotin demonstrated tumor-to-kidney ratios up to 3.5 by 45 minutes after injection.

Radiation absorbed doses for each organ per unit of administered activity were calculated based on the biodistribution data using standard Medical Internal Radiation Dose methods. The mean absorbed doses in all tissues other than kidney and tumor
were similar either in the presence or absence of DMPS (data not shown due to space constraints). In DMPS-treated mice, however, the total absorbed dose of $^{213}$Bi per unit administered activity was found to be 0.7 cGy/µCi in the kidneys compared to 1.6 cGy/µCi for mice in the standard PRIT group without DMPS chelation. These data suggest a >60% reduction in the renal absorbed dose of $^{213}$Bi for DMPS-treated mice. While the total absorbed dose in tumor was slightly lower in the DMPS treated mice (2.0 cGy/µCi) compared to the untreated group (2.6 cGy/µCi), the tumor-to-kidney dose ratio remained approximately 2 times greater in the mice that received the renal protective agent.

**Alpha Camera Imaging and Quantitative Autoradiography**

Since alpha-particle emitters used in internal radiotherapy may exhibit heterogeneous distributions in tumors, a novel alpha camera was employed to detect alpha particles in tissues *ex vivo* and thus assess for non-uniform activity distribution causing a non-uniform dose distribution. We employed this quantitative imaging technique to obtain data on the $^{213}$Bi activity distribution on a sub-organ level in cryosections of human HEL xenograft tumors at various times after injection of radioactivity. Non-uniform intratumoral activity distributions were found for tumor-specific $^{213}$Bi-DOTA-biotin at the 10 minute time point, however 45 minutes after injection the distribution was more uniform. At this 45 minute time point the alpha camera disclosed that the radionuclide was well and deeply distributed within the tumor tissue. The blue areas seen in the periphery of Figure 2 represent areas with lower activity concentration, while areas of red higher activity were distributed throughout the central parts of the tumor. H&E staining verified a highly proliferative tumor cell population in the imaged area and corresponding IHC confirmed a vascular net well established within the same area. Based on these data combined with its short path-length and high linear
energy transfer (LET), we further investigated the biologic outcomes of PRIT using $^{213}$Bi in a series of *in vivo* therapeutic studies.

**PRIT of Human Leukemia Xenografts**

In light of favorable tumor biodistributions shown by both gamma counting and alpha camera methodologies, we performed therapy experiments to compare efficacy of $^{90}$Y and $^{213}$Bi in a minimal residual disease model of AML. Experimental groups of 10 mice were placed on a biotin-deficient diet and two days later were injected sc into the flank with $10 \times 10^6$ HEL cells. Two days after leukemia cell injection, the mice were treated with 1.4 nmol anti-CD45 BC8 Ab-SA, followed 21 hours later by 5.8 nmol CA, then 3 hours later with either 800 $\mu$Ci or 1200 $\mu$Ci of $^{90}$Y-DOTA-biotin or 400 $\mu$Ci or 800 $\mu$Ci of $^{213}$Bi-DOTA-biotin. Comparison groups were untreated mice that had the received sc injection of HEL cells alone and mice that were injected with 1200 $\mu$Ci $^{90}$Y-DOTA-biotin or 800 $\mu$Ci $^{213}$Bi-DOTA-biotin after treatment with a non-binding isotype-matched control Ab-SA conjugate and subsequent CA. Untreated mice and PRIT control mice that received 1200 $\mu$Ci $^{90}$Y-DOTA-biotin and control Ab-SA conjugate all had exponential growth of tumors requiring euthanasia by 14 days after injection of radiolabeled biotin (Fig. 3A and 3B). Mice that received BC8 Ab-SA conjugate and CA followed by 800 $\mu$Ci of $^{90}$Y-DOTA-biotin had significant reduction in tumor growth compared to control groups. All mice in control groups experienced rapid tumor growth resulting in euthanasia of all mice before day 22 after treatment (Fig. 3A). In the group that received 800 $\mu$Ci of $^{90}$Y-DOTA-biotin, the first mouse did not require euthanasia until day 26 after therapeutic injection (Fig. 3B). Mice from this treatment group displayed significantly improved survival ($p < 0.0001$) compared to control groups where two mice remained alive past 120 days following therapy. Eight of 10 mice in this treatment group died of progressive leukemia between 26 and 75 days after therapy while 2 mice remained
tumor-free and survived over 120 days (Fig. 3B). Mice that received 1200 µCi of $^{90}\text{Y}$-DOTA-biotin exhibited an even more impressive therapeutic effect compared to control mice with 60% of mice in this group disease-free 120 days after therapy ($p < 0.0001$). Three mice that received 1200 µCi of $^{90}\text{Y}$-DOTA-biotin developed tumors and were euthanized by day 64 and one mouse in this treatment group early exhibited regimen-related toxicity with huddling behavior and extensive loss of body weight necessitating euthanasia 14 days after therapy (Fig. 3).

All $^{213}\text{Bi}$ control groups exhibited anticipated growth of tumors requiring euthanasia by 16 days (untreated mice) or by 40 days (pretargeted isotype-matched negative control Ab-SA mice that received 800 µCi of $^{213}\text{Bi}$-DOTA-biotin; Fig. 4), similar to the control mice receiving $^{90}\text{Y}$-DOTA biotin. We postulate that delayed tumor growth seen in the isotype-matched control mice injected with radiolabeled $^{213}\text{Bi}$-DOTA-biotin may have been due to circulating $^{213}\text{Bi}$ in the bloodstream, affecting the highly-vascularized HEL xenografts. Overall, experimental mice pretargeted with anti-CD45 Ab-SA followed by either 400 or 800 µCi of $^{213}\text{Bi}$-DOTA-biotin experienced superior survival rates compared to similar mice treated with higher doses of $^{90}\text{Y}$-DOTA-biotin in our minimal residual leukemia model. Mice that received pretargeted anti-CD45 Ab-SA, CA, and 400 µCi of $^{213}\text{Bi}$-DOTA-biotin demonstrated a significant therapeutic benefit compared to the 800 µCi $^{213}\text{Bi}$-DOTA-biotin negative control mice ($p = 0.002$), as did anti-CD45 BC8 Ab-SA pretargeted mice that received 800 µCi of $^{213}\text{Bi}$-DOTA-biotin (Fig. 4; $p < 0.0001$). One mouse pretargeted with anti-CD45 Ab-SA followed by 400 µCi of $^{213}\text{Bi}$-DOTA-biotin died by day 8 due to toxicity while 2 other mice in this group were euthanized by day 64 due to progressive xenograft growth. In the group of mice that received anti-CD45 Ab-SA and 800 µCi of $^{213}\text{Bi}$-DOTA-biotin, 2 mice died from radiation-induced toxicity (one each on day 28 and day 49) and only 1 mouse developed overt leukemia leading to
euthanasia on day 63. Both the 400 and 800 µCi of $^{213}$Bi-DOTA-biotin anti-CD45 pretargeted treatment groups had 70% long term disease-free survival extending to >120 days post-therapy.

**Direct Comparison of Anti-CD45 PRIT using $^{90}$Y- versus $^{213}$Bi-DOTA-biotin at Equivalent Absorbed Dose**

In order to further determine the therapeutic difference between the pretargeted alpha- and beta-emitting radionuclides, equivalent absorbed doses to tissues were assessed (Table 1). These concentration ranges of radioactivity were determined based on equivalent absorbed doses over a specific organ volume. Thus, tissue dosimetry values for each radionuclide were normalized to an equivalent liver dose for comparative purposes. We estimated that the equivalent liver dose for mice bearing HEL tumors that received 800 µCi of $^{213}$Bi-DOTA-biotin would be a dose of 112 µCi $^{90}$Y-DOTA-biotin. Anti-CD45 Ab-SA PRIT was therefore subsequently performed using these respective doses in the model of minimal residual leukemia (Fig. 5). Results from this experiment demonstrated no significant difference in survival detected between untreated mice and the group that received 112 µCi $^{90}$Y-DOTA-biotin; mice that received 112 µCi $^{90}$Y-DOTA-biotin survived 17 or fewer days while mice that received no therapy survived a maximum of 14 days. There was a significant difference, however, between the group that was injected with 112 µCi $^{90}$Y-DOTA-biotin and the group that was injected with 800µCi $^{213}$Bi-DOTA-biotin ($p<0.0001$). Mice receiving 800 µCi $^{213}$Bi-DOTA-biotin had delayed tumor growth compared to control animals, with only one mouse requiring euthanasia (due to tumor growth on day 30 after therapy) and with 7 mice achieving long-term disease-free survival (at least 120 days).
Assessment of Delayed Radiation-induced Toxicity after Anti-CD45 Ab-SA and $^{213}$Bi-DOTA-biotin PRIT

Long-term radiotoxicity in mice surviving 120 days after PRIT with anti-CD45 Ab-SA followed by $^{213}$Bi-DOTA-biotin at 400 µCi or 800 µCi was assessed by evaluating hepatic and renal functions in addition to hematologic recovery (Table 2). Of at least 4 mice surviving in each group for 120 days, all anti-CD45 PRIT animals displayed normal body weights despite a slight non-significant increase in hepatic transaminase levels detected in the 400 µCi or 800 µCi $^{213}$Bi-DOTA-biotin groups compared to a group of untreated age-matched control mice. ALT, AST, and ALP levels were 389 ± 530, 214 ± 170, and 91 ± 41 in the mice that received 400 µCi $^{213}$Bi-DOTA-biotin, respectively, and 371 ± 480, 385 ± 290, and 119 ± 63 in those that were treated with 800 µCi $^{213}$Bi-DOTA-biotin, respectively. In contrast, untreated mice displayed ALT, AST, and ALP levels of 122 ± 84, 215 ± 94, and 69.7 ± 21 (p = ns, for all tests). Blood counts in pretargeted mice remained similar to those in untreated age-matched control mice. Mice that received 800 µCi $^{213}$Bi-DOTA-biotin had a white blood count (WBC, k/µL), platelet count (k/µL), hemoglobin (Hgb, g/dL), and neutrophil count (per µL) of 9.9 ± 2.0, 983 ± 400, 13.6 ± 0.7, and 3145 ± 1300, respectively, whereas mice that received 400 µCi $^{213}$Bi-DOTA-biotin had counts of 12 ± 2.0, 1152 ± 170, 15.0 ± 0.2, and 5835 ± 630, respectively. Age-matched control mice had a WBC of 14.2 ± 1.9, platelet count of 1732 ± 149, Hgb of 14.4 ± 1.2, and neutrophil count of 5820 ± 355 (p = ns, for all tests). No significant differences were seen in BUN or Cr levels between mice that received 800 µCi $^{213}$Bi-DOTA-biotin (34 ± 10 and 0.57 ± 0.1, respectively), 400 µCi $^{213}$Bi-DOTA-biotin (28 ± 3.0 and 0.48 ± 0.04, respectively), or age-matched control mice (26 ± 8.4 and 0.48 ± 0.01, respectively). Necropsies revealed no evidence of leukemia in any $^{213}$Bi treated mice surviving beyond day 120 and histologic sections obtained from these mice did not show
any evidence of disease or radiation-induced changes compared to age-matched controls (data not shown due to space constraints). We hypothesize that the minimal toxicity exhibited by anti-CD45 PRIT in this model may have been due to the very short path-length (50 μm) of the alpha particles from $^{213}$Bi compared to beta-emitting isotopes such as $^{90}$Y (~5,000 μm).

**DISCUSSION**

This report critically evaluates the merits of PRIT using an alpha-emitting radionuclide in a myeloid leukemia xenograft model. The results described in this manuscript demonstrated that anti-CD45 PRIT using $^{213}$Bi-DOTA-biotin provided rapid tumor localization and excellent biodistributions of radioactivity, with significant improvements in efficacy compared with a PRIT approach using a beta-emitting radionuclide ($^{90}$Y). *In vivo* images using a novel alpha camera displayed rapid tumor localization and favorable $^{213}$Bi radionuclide distribution at a micro-dosimetric level. Importantly, the pretargeted approach provided an excellent therapeutic index (target-to-non-target ratio) for each targeting radionuclide with tumor-to-blood ratios of up to 13:1 and tumor-to-normal organ ratios of as high as 24:1, thus ensuring that the toxic effects on normal tissues were balanced by the cytotoxic effects on tumor cells. Since the therapeutic index of an anti-neoplastic agent depends on the balance between toxic effects on normal tissues and cytotoxic effects on tumor cells, the pretargeted approach provided excellent tumor regressions and improved overall survival. The excellent tumor localization of the anti-CD45 Ab-SA conjugate using PRIT and $^{213}$Bi-DOTA-biotin translated into improved xenograft survival rates compared to animals receiving $^{90}$Y-DOTA-biotin.
Although these experiments were encouraging, we recognize that this mouse leukemia xenograft model differs significantly from the state of naturally occurring leukemias in patients. In this xenograft system the human target CD45$^+$ cells are confined to tumors, which are more similar to chloromas than to typical disseminated leukemia. In addition, all other tissues in the xenograft model lack expression of human CD45 and will not bind anti-human CD45 Ab. Therefore, toxicity profiles in the human may not be reliably mimicked in our xenograft system. To overcome this limitation, we previously tested anti-CD45 PRIT in a murine syngeneic system in which CD45 was present on normal hematolymphoid tissues. The results of these complementary studies showed suggested that PRIT supported an improvement in the therapeutic index over that currently achievable with conventional RIT methodologies.\textsuperscript{13}

Although several clinical trials have provided encouraging results using beta-emitting radionuclides (primarily $^{131}$I and $^{90}$Y) to treat malignancies, the relatively long path-lengths of beta emissions can produce dose-limiting myelosuppression at conventional doses\textsuperscript{25,26} and non-hematologic dose-limiting toxicities at myeloablative doses when used as part of a hematopoietic cell transplant conditioning regimen.\textsuperscript{27-29} While the longer path-length of these beta-emitting radionuclides may be best suited for diseases with enlarged tumor masses such as lymphomas, it has been postulated that alternative radionuclides with shorter path-lengths may be better suited for clinical scenarios of minimal residual disease or where circulating isolated tumor cells are present in order to maintain radiation-induced toxicities at a minimum.\textsuperscript{8,29} In particular, alpha-emitting radionuclides with their very short path-lengths may be particularly advantageous for leukemias where malignant cells are dispersed and the goal is rapid and efficient cell kill with minimal toxicities inflicted upon surrounding tissues.\textsuperscript{30-34} It has been estimated that up to twice as much energy is deposited outside a 200 $\mu$m tumor when employing an Ab
labeled with a beta-emitting radionuclide such as $^{90}$Y or $^{131}$I compared to an Ab conjugated to an alpha-emitting agent. In addition, the relatively low LET characteristics of beta-particles (~0.2 keV/μm) may result in suboptimal killing of tumor cells and may ultimately contribute to the relapse of aggressive malignancies.

Alpha-emitting radionuclides exhibit very high cytotoxicity, further making them attractive alternatives to the beta-emitting radionuclides for RIT. The higher cytotoxicity delivered by an alpha particle results from the large amount of energy that is emitted in a linear fashion within a few cell diameters (~50-90 μm). Moreover, the high LET energy transfer of alpha-emitters (~100 keV/μm) confers a high relative biological effectiveness (RBE) for cell killing compared to that for beta-emitters. This high RBE of alpha particles results in double-strand DNA breaks that are commonly so great that cell repair mechanisms may not be effective. Not surprisingly, alpha-emitters have shown greater therapeutic efficacy in cell culture and animal studies than beta-emitting radionuclides. Of particular relevance to our study, Friesen et al. showed that a $^{213}$Bi-labeled anti-CD45 Ab, but not a $^{90}$Y-labeled anti-CD45 Ab, could overcome resistance of AML cells to beta-irradiation and anthracyclines to achieve effective tumor cell killing. The $^{213}$Bi-labeled anti-CD45 Ab-induced DNA damage and apoptosis inflicted on AML cells could not be repaired, in contrast to DNA damage induced by either beta or gamma irradiation and doxorubicin. Taken together, these results suggest that alpha-emitting radionuclides may be superior to beta-emitting radionuclides for treatment of leukemia.

Only a small number of alpha-emitting radionuclides have been considered suitable for in vivo applications, however, primarily due to availability and decay properties. The alpha-emitting radionuclides $^{211}$At ($t_{1/2} = 7.2$ hours), $^{212}$Bi ($t_{1/2} = 60$ minutes) and $^{213}$Bi ($t_{1/2} = 45$ minutes) have been favored because these radionuclides do not produce daughter...
radionuclides that also decay by alpha-emission.\textsuperscript{30,31} More recently longer lived alpha-emitting radionuclides, such as \textsuperscript{225}Ac (\(t_{1/2} = 10\) days), \textsuperscript{223}Ra (\(t_{1/2} = 11.4\) days), and \textsuperscript{227}Th (\(t_{1/2} = 18.7\) days) have been investigated.\textsuperscript{40-44} These radionuclides do generate short-lived daughter therapeutic alpha-particles (\textsuperscript{221}Fr, \textsuperscript{217}At, \textsuperscript{213}Bi) from \textsuperscript{225}Ac inside a targeted leukemia cell, which largely account for the increased potency of \textsuperscript{225}Ac-atomic nanogenerators over \textsuperscript{213}Bi constructs.\textsuperscript{16} Importantly, however, studies in mice and cynomologous monkeys suggest that these alpha daughters also have significant potential to inflict renal damage due to uptake of bismuth radioisotopes from the decay chain.\textsuperscript{45,46} A recent study by our group confirmed the efficacy of DMPS in animals treated with PRIT using an anti-CD20 Ab-SA conjugate followed by \textsuperscript{213}Bi-DOTA-biotin with an >50% reduction of the total radiation absorbed dose delivered to the kidneys.\textsuperscript{17} In this study we demonstrate similar results when DMPS was administered to the animals' drinking water; serum BUN and Cr levels from long-term surviving mice were not different from levels determined from age-matched control animals, suggesting the absence of functional radiation-induced nephritis. Moreover, after delivering a 10 Gy dose to the liver, the dose to the kidneys when \textsuperscript{213}Bi-DOTA-biotin was employed was similar to the renal dose delivered by \textsuperscript{90}Y-DOTA-biotin (Table 1; 5.4 Gy and 5.7 Gy, respectively). This renal dose was considerably less than the 10 Gy delivered to the kidney using conventional alpha-particle RIT, which has been shown to result in an approximately 50% reduction in the glomerular filtration rate.\textsuperscript{47}

Finally, our study confirms the encouraging results from pilot PRIT studies using short-lived alpha-emitters for leukemia therapy.\textsuperscript{38,48} Our study demonstrated the curative potential of the PRIT approach for mice with myeloid leukemia and the superiority of \textsuperscript{213}Bi compared to \textsuperscript{90}Y in our model. While a number of challenges remain to achieve widespread applicability of this novel anti-CD45 PRIT approach using alpha-emitting
radionuclides, further improvements targeting leukemic cells have the potential to increase the therapeutic index and form a basis for the treatment of a wide variety of malignant and life-threatening nonmalignant diseases, including hematological as well as solid tumors.

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JM Pagel contributed to the conception, design, analysis and interpretation of the research and wrote the manuscript. A Kenoyer and SI Park contributed to the design of the research and performed research and analyzed data. T Bäck performed research and collected data. DK Hamlin contributed vital reagents. DS Wilbur, Y Lin, and DR Fisher contributed to the conception and interpretation of research. S Frayo, A Axtman, J Shenoi, and J Orozoco performed research and collected data. AK Gopal and DJ Green contributed to the interpretation of data. FR Appelbaum contributed to the conception and interpretation of research and revised the manuscript. OW Press contributed to the conception, design, analysis and interpretation of the research and revised the manuscript. There are no conflicts of interest for any of the authors.

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REFERENCES


TABLES

Table 1: Comparative Radiation Absorbed Doses after Anti-CD45 PRIT using $^{90}$Y- or $^{213}$Bi-DOTA-biotin. Absorbed doses (Gy) to blood, spleen, kidney, and tumor 10 minutes after delivery of $^{213}$Bi-DOTA-biotin and 1 hour after $^{90}$Y-DOTA-biotin were calculated for each radionuclide to deliver 5 Gy in the liver.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$^{90}$Y-DOTA-biotin (Gy)</th>
<th>$^{213}$Bi-DOTA-biotin (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Tumor</td>
<td>13.1</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Table 2: Late Toxicities after Anti-CD45 PRIT using 400 μCi or 800 μCi $^{213}$Bi-DOTA-biotin.

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC, k/μl</th>
<th>Platelets, k/μl</th>
<th>Hgb, g/dl</th>
<th>HCT, %</th>
<th>Neutrophil, /μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.2 ± 1.9</td>
<td>1732 ± 149</td>
<td>14.4 ± 1.2</td>
<td>48.0 ± 2.4</td>
<td>5820 ± 355</td>
</tr>
<tr>
<td>400 μCi</td>
<td>12.2 ± 2.0</td>
<td>1152 ± 171</td>
<td>15.0 ± 0.2</td>
<td>48.8 ± 0.8</td>
<td>5835 ± 630</td>
</tr>
<tr>
<td>800 μCi</td>
<td>9.9 ± 2.0</td>
<td>983 ± 407</td>
<td>13.6 ± 0.7</td>
<td>45.7 ± 2.0</td>
<td>3185 ± 1280</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>BUN, mg/dl</th>
<th>Cr, mg/dl</th>
<th>ALP, U/L</th>
<th>ALT, U/L</th>
<th>AST, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.7 ± 8.4</td>
<td>0.48 ± 0.01</td>
<td>69.7 ± 21</td>
<td>122 ± 84</td>
<td>215 ± 94</td>
</tr>
<tr>
<td>400 μCi</td>
<td>27.7 ± 3.3</td>
<td>0.48 ± 0.04</td>
<td>90.8 ± 41</td>
<td>389 ± 530</td>
<td>214 ± 170</td>
</tr>
<tr>
<td>800 μCi</td>
<td>33.5 ± 9.7</td>
<td>0.57 ± 0.10</td>
<td>119 ± 63</td>
<td>371 ± 480</td>
<td>385 ± 290</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Fig. 1: Biodistributions of radioactivity in HEL xenograft-bearing athymic mice injected with $^{90}$Y-DOTA-biotin or $^{213}$Bi-DOTA-biotin after anti-CD45 BC8 Ab-SA conjugate and CA. HEL-xenograft-bearing mice were injected iv via the tail vein with 1.4 nmol of anti-CD45 BC8 Ab-SA conjugate followed 22 hours later with 5.8 nmol of CA and subsequently 2 hours later with 1.2 nmol of radiolabeled DOTA-biotin. Groups of 5 mice were euthanized at 3 time points post-injection. The radioactivity in blood, tumor, and normal organs was measured by gamma counting, corrected for decay, and expressed as %ID/g of tissue. (A) $^{90}$Y-DOTA-biotin uptake at 1 (☐), 24 (■), and 72 (☒) hours post-injection; (B and C) $^{213}$Bi-DOTA-biotin uptake at 10 (☐), 45 (■), and 90 (☒) minutes post-injection with anti-CD45 Ab-SA without (B) or with DMPS (C); (D) $^{213}$Bi-DOTA-biotin uptake at 10 (☐), 45 (■), and 90 (☒) minutes post-injection following delivery of non-binding control Ab-SA conjugate.

Fig. 2: Quantitative alpha camera autoradiography. The intratumoral distribution of $^{213}$Bi-DOTA-biotin after anti-CD45 PRIT was analyzed using a novel digital autoradiography bio-imaging system. This alpha camera system is dedicated for the ex-vivo detection of alpha particles in tissue and is employing a scintillation setup. The technique is quantitative and fully linear towards activity content in the imaged sections. At serial times after injection (10, 45 and 90 minutes) tumors were cryosectioned (thickness 12 - 16 μm). Three serial, immediately adjacent, sections were obtained; the first for alpha imaging, the second for H&E staining, and the third for IHC of blood vessels using CD34. This representative digitally collected alpha camera image was obtained 10 minutes after $^{213}$Bi-DOTA-biotin injection and was color coded to express the different levels of activity concentration of $^{213}$Bi.
Fig. 3: Leukemia xenograft regression and survival after treatment with anti-CD45 PRIT using $^{90}$Y-DOTA-biotin. Athymic mice bearing HEL xenografts were injected iv via tail vein two days after tumor implantation with 1.4 nmol of anti-CD45 Ab-SA conjugate followed 22 hours later with 5.8 nmol of CA and then with 1.2 nmol of $^{90}$Y-DOTA-biotin (800 µCi and 1200 µCi) 3 hours later. Tumor-bearing control mice were either untreated or treated with a non-binding control Ab-SA conjugate prior to 1200 µCi radiolabeled DOTA-biotin injection. (A) Tumor volume curves are truncated at the time of euthanasia due to excessive tumor growth in the first mouse in each group. (B) Mice were analyzed for survival as a function of time. Deaths due to toxicity are marked (†).

Fig. 4: Leukemia xenograft regression and survival after treatment with anti-CD45 PRIT using $^{213}$Bi-DOTA-biotin. Athymic mice bearing HEL xenografts were injected iv via tail vein two days after tumor implantation with 1.4 nmol of anti-CD45 Ab-SA conjugate followed 22 hours later with 5.8 nmol of CA and subsequently with 1.2 nmol of $^{213}$Bi-DOTA-biotin (400 µCi and 800 µCi) 3 hours later. Tumor-bearing control mice were either untreated or treated with a non-binding control Ab-SA conjugate prior to 800 µCi radiolabeled DOTA-biotin injection. (A) Tumor volume curves are truncated at the time of euthanasia due to excessive tumor growth in the first mouse in each group. (B) Mice were analyzed for survival as a function of time. Deaths due to toxicity are marked (†).

Fig. 5: Leukemia xenograft regression and survival after PRIT with $^{213}$Bi- or $^{90}$Y-DOTA-biotin at equivalent absorbed doses. Athymic mice bearing HEL xenografts were injected iv via tail vein two days after tumor cell implantation with 1.4 nmol of anti-CD45 Ab-SA conjugate followed 22 hours later with 5.8 nmol of CA and subsequently
with 1.2 nmol of radiolabeled DOTA-biotin 3 hours later to deliver an absorbed dose of 10 Gy from each radionuclide delivered to the liver (112 µCi \(^{90}\)Y-DOTA-biotin and 800 µCi \(^{213}\)Bi-DOTA-biotin). Control tumor-bearing control mice were untreated. (A) Tumor volume curves are not truncated. (B) Mice were also analyzed for survival as a function of time.
Fig 1

A

\[ ^{90}Y \text{-DOTA-} \text{biotin} \]

\[
\begin{array}{c}
\text{Blood} & \text{Lung} & \text{Liver} & \text{Spleen} & \text{Stomach} & \text{Kidneys} & \text{S. Int.} & \text{Colon} & \text{Tumor} & \text{Muscle} \\
\%
\end{array}
\]

B

\[ ^{213}\text{Bi-DOTA-biotin} \]

\[
\begin{array}{c}
\text{Blood} & \text{Lung} & \text{Liver} & \text{Spleen} & \text{Stomach} & \text{Kidneys} & \text{S. Int.} & \text{Colon} & \text{Tumor} & \text{Muscle} \\
\%
\end{array}
\]

C

\[ ^{213}\text{Bi-DOTA-biotin + DMPS} \]

\[
\begin{array}{c}
\text{Blood} & \text{Lung} & \text{Liver} & \text{Spleen} & \text{Stomach} & \text{Kidneys} & \text{S. Int.} & \text{Colon} & \text{Tumor} & \text{Muscle} \\
\%
\end{array}
\]

D

\[ ^{213}\text{Bi-DOTA-biotin, control} \]

\[
\begin{array}{c}
\text{Blood} & \text{Lung} & \text{Liver} & \text{Spleen} & \text{Stomach} & \text{Kidneys} & \text{S. Int.} & \text{Colon} & \text{Tumor} & \text{Muscle} \\
\%
\end{array}
\]
Fig 4

**A**

- Tumor volume, mm^3
- Days posttherapy
- No treatment
- Control Ab 800 μCi
- 800 μCi ^213^Bi-DOTA-biotin
- 400 μCi ^213^Bi-DOTA-biotin

**B**

- % Survival
- Days posttherapy
- 800 μCi ^213^Bi-DOTA-biotin
- 400 μCi ^213^Bi-DOTA-biotin
- Control Ab 800 μCi
- No treatment
Fig 5

A

Tumor volume, mm$^3$

- 112μCi $^{90}$Y-DOTA-biotin
- No treatment
- $\pm$ 800μCi $^{213}$Bi-DOTA-biotin

Days posttherapy

B

% Survival

- 112μCi $^{90}$Y-DOTA-biotin
- $\pm$ 800μCi $^{213}$Bi-DOTA-biotin
- No treatment

Days posttherapy
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