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Theranostic pretargeted radioimmunotherapy of colorectal cancer xenografts in mice using picomolar affinity $^{86}$Y- or $^{177}$Lu-DOTA-Bn binding scFv C825/GPA33 IgG bispecific immunoconjugates

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Abstract Purpose GPA33 is a colorectal cancer (CRC) antigen with unique retention properties after huA33-mediated tumor targeting. We tested a pretargeted radioimmunotherapy (PRIT) approach for CRC using a tetravalent bispecific antibody with dual specificity for GPA33 tumor antigen and DOTA-Bn–(radiolanthanide metal) complex.

Methods PRIT was optimized in vivo by titrating sequential intravenous doses of huA33-C825, the dextran-based clearing agent, and the C825 hapten $^{177}$Lu- or $^{86}$Y-DOTA-Bn in mice bearing the SW1222 subcutaneous (s.c.) CRC xenograft model.

Results Using optimized PRIT, therapeutic indices (TIs) for tumor radiation-absorbed dose of 73 (tumor/blood) and 12 (tumor/kidney) were achieved. Estimated absorbed doses (cGy/MBq) to tumor, blood, liver, spleen, and kidney for single-cycle PRIT were 65.8, 0.9 (TI 73), 6.3 (TI 10), 6.6 (TI 10), and 5.3 (TI 12), respectively. Two cycles of PRIT (66.6 or 111 MBq $^{177}$Lu-DOTA-Bn) were safe and effective, with a complete response of established s.c. tumors (100 – 700 mm$^3$) in nine of nine mice, with two mice alive without recurrence at >140 days. Tumor log kill in this model was estimated to be 2.1 – 3.0 based on time to 500-mm$^3$ tumor recurrence. In addition, PRIT dosimetry/diagnosis was performed by PET imaging of the positron-emitting DOTA hapten $^{86}$Y-DOTA-Bn.

Conclusion We have developed anti-GPA33 PRIT as a triple-step theranostic strategy for preclinical detection, dosimetry,
and safe targeted radiotherapy of established human colorectal mouse xenografts.

**Keywords** Multistep targeting · Bispecific antibodies · GPA33 · Radioimmunotherapy · Pretargeting

**Introduction**

Despite therapeutic advances in the last decade, colorectal cancer (CRC) is still the fourth leading cause of death worldwide, and 49,700 CRC deaths are projected for 2015 in the US alone [1]. Although locoregional therapies such as surgery and radiation can be curative in many cases, these approaches do not control tumors that have metastasized at diagnosis, for which chemotherapy and targeted small molecules are most often palliative, with rapid development of resistance. Clearly, there is an unmet need for improved therapeutics, particularly for the treatment of unresectable metastatic disease. In principle, targeted radiation therapy strategies such as radioimmunotherapy (RIT) could potentially address this unmet need, because they enable delivery of tumoricidal radiation doses to multiple systemically dispersed sites simultaneously (e.g., ranging from macroscopic nodal or hepatic oligometastases to occult micrometastatic disease) as long as the therapeutic indices (TIs) are favorable [2]. Notably, the majority of RIT trials in CRC have been with IgGs directed at carcinoembryonic antigen (CEA) (e.g., 131I-hMN-14 [3] and 90Y-cT84.66 [4]). So far, these studies have had only limited clinical success because of suboptimal TIs of only 5–10:1 for macroscopic tumors, which is inadequate for curative therapy without dose-limiting hematologic toxicity.

Since particularly radioresistant solid tumors such as CRC may require a total absorbed dose of 7,000–10,000 cGy for cure, we need highly selective tumor targeting with respect to radiosensitive normal tissues (typically bone marrow, which may be suppressed by doses as low as 150 cGy). The RIT approach that offers the greatest potential for improving TI is the pretargeted approach (PRIT) utilizing bispecific antibodies (BsAb) [5]. For small-volume or microscopic disease, there is evidence that uptake, and consequently TI, may be higher in both animal models [6, 7] and human patients [8, 9]. This, taken in conjunction with the reduced tumor cell number in such small tumors, means that the likelihood of achieving complete ablation can be expected to be greater than in macroscopic disease, as long as a radionuclide appropriate for small-volume disease is used [10]. However, in the most general case, small-volume disease will coexist with macroscopic disease in the same patient and treatment approaches are required that address the entire disease spectrum. Moreover, if PRIT can improve the treatment of macroscopic disease, it will concomitantly improve that of microscopic disease by the same mechanisms and at no additional cost.

PRIT, a concept that separates the delivery of the targeting antibody to tumor from the delivery of the radioactivity, was initially developed by Reardan et al. [11], Goodwin et al. [12], and Feng et al. [13] using anti-radiometal chelate antibodies. The initial uptake studies demonstrated the proof of principle for this approach, showing improved tumor targeting compared to conventional RIT, but TIs were still suboptimal, partly because of the insufficient retention (on the order of several hours) of the radioactivity when carried by anti-chelate antibodies of only nanomolar affinity [14, 15]. The BsAb/radiometal binding approach took a major step forward with the development of ultra-high affinity antibodies for the radiometal–chelate binding step, based on the work of Orcutt et al. [16] at the Massachusetts Institute of Technology (MIT). Guided by a mathematical modeling approach that predicted the optimal required affinity of the hapten antibody binding step in PRIT, the MIT team, using directed evolution and yeast surface display, was able to affinity mature by more than 1000-fold to the low picomolar range the original 2D12.5 antibody specific for the benzyl(Bn)-DOTA–metal complex, now reformatted as the “C825” single-chain variable fragment (scFv) [16]. Next, the MIT team engineered BsAbs incorporating the C825 sequence utilizing a highly modular IgG-scFv scaffold (e.g., anti-CEA/C825) [17]. These IgG-scFv antibody compositions are tetravalent molecules, consisting of two binding sites for tumor antigen (typically, about 10 nM), and two binding sites for the Bn-DOTA-radiometal hapten (about 10–20 pM).

We recently adopted this methodology for PRIT by focusing on anti-GD2 antibodies with a documented record of safe use and proven tumor localization in humans [18]. For CRC, we reasoned that the GPA33 target could be ideal for PRIT. GPA33 is a transmembrane glycoprotein abundantly expressed in over 95% of CRCs, with restricted expression in normal tissue (colon and bowel epithelium) [19]. It exhibits long-term residence in cell membrane in tumors, relative to intestine, with minimal internalization and minimal vascular shedding. Using a variety of radiolabeled antibody forms, including initially a murine monoclonal antibody (A33 [20]) and subsequently a humanized version (huA33 [21]), this antigen is one of the most extensively studied targets in vivo. For example, we have performed 124I-huA33 imaging in patients with CRC and confirmed through kinetic modeling that the differential clearance between antigen-positive tumor and antigen-positive intestine would yield TIs sufficient for tumor response to PRIT [8, 22, 23].

As we developed PRIT, the need for radiation dosimetry based on quantitative imaging for measurement of time-dependent activity distribution in tumor and normal tissues for optimal translation of PRIT to humans became apparent. Combined therapeutic and diagnostic, or “theranostic,” PRIT refers to the use of reagents with virtually identical chemistries as vehicles for both diagnostic and therapeutic applications.
We have previously described successful applications of this approach using PET imaging of $^{124}$I as a surrogate for $^{131}$I therapy in thyroid cancer [24–26]. In the present studies, we applied $^{177}$Lu as both the therapeutic ($\beta$-emission for RIT) and diagnostic ($\gamma$-emission for imaging) radionuclide, and $^{177}$Lu itself, and $^{86}$Y as its diagnostic surrogate. Furthermore, we emphasized $^{177}$Lu because the lower $\beta$-emission energy and short path length make it more likely to eradicate small tumors, in comparison to a longer $\beta$-emission path length radionuclide such as $^{90}$Y.

In the study reported here, we tested the principal hypothesis that optimized theranostic PRIT based on GPA33 antigen tumor targeting and a BsAb-huA33 construct can be used to achieve the high TIs required for effective and safe CRC therapy in xenografts in mice and, ultimately, in humans.

**Materials and methods**

**Cloning and expression of huA33-C825**

HuA33-C825 was prepared using the sequences for huA33 [21] and C825 [16], a murine scFv antibody with high affinity for DOTA–radiometal complexes (Fig. 1a). The BsAb was made using the same platform as control hu3F8-C825 [18], only replacing variable regions (VH and VL) of hu3F8 with those of huA33 [21]. The IgG-scFv BsAb (molecular weight about 210 kDa) was produced in CHO cells and purified by protein A affinity chromatography as previously described [18].

**Surface plasmon resonance studies**

A Biacore T100 biosensor, a CM5 sensor chip, and related reagents were purchased from GE Healthcare. The human GPA33 recombinant protein was purchased from Novoprotein. A BSA-(Y)-DOTA-Bn conjugate was prepared as previously described [18]. Both antigens were immobilized using the amine coupling kit (GE Healthcare). Purified BsAbs and control antibodies were analyzed and data fit to a bivalent analyte model using the Biacore T100 evaluation software as previously described [18].

**Anti-GPA33 PRIT reagents, protocol, and xenograft studies**

The GPA33(+) human CRC cell line SW1222 was obtained from the Ludwig Institute for Cancer Immunotherapy (New York, NY). SW1222 is notable among commonly investigated human CRC cell lines for preclinical RIT [27–30] in that it forms relatively well-differentiated and highly vascular subcutaneous (s.c.) tumors and is thus well-suited to antibody targeting [31]. $^{131}$I-A33 was used to demonstrate that a dose of 68 Gy to SW1222 tumors resulted in cures [29]. Details regarding the culturing of SW1222 and inoculation into immunocompromised mice are provided in the Supplementary material. Established tumors (50 – 700 mm$^3$) were observed in 7 – 10 days; tumor volumes (TVs) were estimated using the formula for the volume ($V$) of an ellipsoid: $V = 4/3\pi (a \times b \times c)$, with dimensions in millimeters. All reagents were administered intravenously via a lateral tail vein.

The anti-GPA33 PRIT protocol included injections of the following three reagents. HuA33-C825 BsAb (0.25 mg, 1.19 nmol) was injected first to allow localization in the tumor ($t = −28$ h). At $t = −4$ h 24 h later circulating BsAb was cleared by injection of a clearing agent (CA; 62.5 µg, 0.125 nmol) comprising a 500-kDa dextran-(Y)-DOTA-Bn conjugate prepared as previously described by Orcutt et al. [32] and formulated in saline for injection (7.625 nmol of (Y)-DOTA-Bn). Finally, 4 h after injection of CA, the $^{177}$Lu-DOTA-Bn was injected ($t = 0$ h). Radiolabeled DOTA-Bn was prepared as previously described [18] by incubating DOTA-Bn (p-NH$_2$-Bn-DOTA, molecular weight 655 Da; Macrocyclics) and $^{177}$Lu (carrier-added, specific activity about 1,110 GBq/mg; Perkin Elmer) or $^{86}$Y (carrier-free, specific activity about 1,110 GBq/mg; Radiological Chemistry and Imaging Laboratory at Washington University in St. Louis) at 80 °C for 1 h, and formulated in saline for injection (the PRIT strategy is shown in Supplementary Fig. 1).

Micro single-photon emission computed tomography integrated with X-ray computed tomography (X-SPECT/CT) imaging was performed on selected mice during PRIT optimization. In addition, separate biodistribution studies were conducted with huA33-C825 trace radiolabeled with $^{131}$I (Nordion) to estimate tumor uptake during PRIT (i.e., at 24 h post-injection, p.i.). Details regarding X-SPECT/CT imaging, tracer preparation and quality control, as well as the ex vivo biodistribution analysis of radioactivity following radiotracer injection are provided in the Supplementary material.

**PET imaging of anti-GPA33 PRIT + $^{86}$Y-DOTA-Bn**

A single group of five mice bearing GPA33(+) SW1222 tumors in the shoulder were given PRIT (as described above) using 8.6 – 8.8 MBq (about 50 pmol) of $^{86}$Y-DOTA-Bn, and noninvasively imaged at approximately 2 and 20 h p.i. using a microPET Focus 120 (CTI Molecular Imaging, Inc., Knoxville, TN) and previously described methods [33]. A detailed description of the imaging protocol is provided in the Supplementary material.

**Estimation of absorbed doses**

Groups of GPA33(+) SW1222 tumor-bearing mice ($n=4$ or 5) were given PRIT + 1.85 – 2.0 MBq (about 10 pmol) of $^{177}$Lu-
DOTA-Bn and sacrificed at 2 h \((n=5)\), 24 h \((n=4)\), and 120 h \((n=5)\) p.i. for biodistribution analysis of 177Lu activity in tumor and selected normal tissues. Details regarding calculation of estimated absorbed doses are provided in the Supplementary material.

**Titration of administered 177Lu-DOTA-Bn activity following PRIT with optimized BsAb and CA doses**

To determine the effect of the 177Lu-DOTA-Bn dose on the relative uptake of 177Lu-DOTA-Bn in tumor and kidney during PRIT, groups of tumor-bearing mice \((n=5 \text{ per group})\) were given PRIT + 11.1 MBq (11.1 – 11.4; 60 pmol), 55.5 MBq (54.6 – 55.1; 300 pmol), or 111.0 MBq (109.5 – 112.5 MBq; 600 pmol) of 177Lu-DOTA-Bn. All mice were sacrificed at 24 h p.i. of 177Lu-DOTA-Bn for biodistribution analysis of 177Lu activity. These data were plotted in combination with the PRIT + 1.85 – 2.0 MBq (about 10 pmol) biodistribution data following huA33-C825 PRIT + 177Lu-DOTA-Bn (1.85 – 2.0 MBq, about 10 pmol). The kidneys were collected from animals given PRIT + 11.1 – 111.0 MBq and frozen at –80 °C in OCT for autoradiography and histochemistry analysis. A detailed description of the autoradiography and histochemistry protocol is provided in the Supplementary material.

**Theranostic 177Lu-DOTA-Bn treatment using optimized PRIT strategy**

Groups of SW1222 tumor-bearing mice \((50 – 150 \text{ mm}^3)\) were injected with either huA33-C825 or nonspecific IgG-C825 PRIT \(\text{(i.e., single-cycle treatment; BsAb injection on day 6 and CA/177Lu-DOTA-Bn injections on day 7 after tumor inoculation) or two cycles of PRIT (i.e., fractionated treatment; BsAb injections on days 9 and 16 and CA/177Lu-DOTA-Bn}})

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![Structure of M-DOTA-Bn and C825 scFv affinities for either Y-DOTA-Bn or Lu-DOTA-Bn as reported by Orcutt et al. [16].](image1.png)

**Fig. 1** a Structure of M-DOTA-Bn and C825 scFv affinities for either Y-DOTA-Bn or Lu-DOTA-Bn as reported by Orcutt et al. [16]. b X-SPECT/CT maximum intensity projection images of the same mouse bearing a SW1222 tumor 2 h (left) and 20 h (right) following anti-GPA33 PRIT with 177Lu-DOTA-Bn. The following were administered for PRIT: 0.1 mg of BsAb, 40 μg of clearing agent, and 7.4 MBq 177Lu-DOTA-Bn. c Decay-corrected 177Lu activity biodistribution curves for SW1222 tumor as well as selected normal tissues from 2 to 120 h after injection following huA33-C825 PRIT + 177Lu-DOTA-Bn (1.85 – 2.0 MBq, about 10 pmol). d Representative noninvasive serial PET images of the same nude mouse bearing a subcutaneous SW1222 tumor in the shoulder (arrows) at 2 and 20 h after injection of huA33-C825 PRIT + 86Y-DOTA-Bn (8.6 – 8.8 MBq, about 50 pmol). Transverse and coronal slices through the tumor are shown. The images have been corrected for decay to the time of injection and calibrated for quantitation of activity in regions of interest and scaled to the same intensity.
injections on days 10 and 17 after tumor inoculation). For PRIT with nonspecific IgG-C825, an equivalent milligram dose of the GD2-targeted BsAb hu3F8-C825 [18] was used in place of huA33-C825, since it does not cross-react with SW1222 tumor. TVs were measured three times a week and the following definitions were used to describe treatment response: a complete response (CR) was defined as tumor shrinkage to 20 % of initial TV during treatment; a partial response (PR) was defined as a tumor showing no change in growth between successive measurements or any other tumor shrinkage not considered a CR; and an excessive tumor burden was defined as a final TV of >2,000 mm³.

Treated mice underwent noninvasive planar scintigraphy at 20 h p.i. of 177Lu-DOTA-Bn (details of the imaging method are provided in the Supplementary material) to verify tumor-specific versus nonspecific targeting as well as to assess whole-body clearance in mice receiving 177Lu-DOTA-Bn alone [34]. Animals were observed until they were sacrificed due to excessive tumor burden (unless otherwise noted). Animals showing a weight loss greater than 15 % of their initial body weight in 1 or 2 days, or 20 % or more of their starting weight, were removed from the group at that time and sacrificed. To further evaluate toxicity, a total of eight randomly selected animals undergoing treatment were submitted for histopathologic assessment of the site of s.c. tumor, and assessment of the kidney, bone marrow (sternum, vertebrae, femur, and tibia), liver, and spleen by board-certified pathologists at the Memorial Sloan Kettering Cancer Center Laboratory of Comparative Pathology. Immunohistochemical (IHC) staining for the presence of GPA33 was also carried out in these mice using previously described protocols [8]. The maximum tolerated dose (MTD) was not determined in this study.

Results

In vitro characterization of huA33-C825

Biochemical purity analysis of huA33-C825 by size-exclusion high-pressure liquid chromatography (SE-HPLC) is shown in Supplementary Fig. 2a. SE-HPLC showed a major peak (90 % by UV analysis) with an approximate molecular weight of 210 kDa, as well as some minor peaks assumed to be aggregates removable by gel filtration. The BsAb remained stable by SE-HPLC and Biacore after multiple freeze and thaw cycles (data not shown). The binding affinity was measured by Biacore T100. For GPA33, huA33-C825 had a $k_{on}$ of $9.15 \times 10^4$ M$^{-1}$s$^{-1}$, a $k_{off}$ of $5.81 \times 10^{-3}$ s$^{-1}$, and an overall $K_D$ of 63.5 nM—less than the parental huA33-IgG1 values ($k_{on}$ 6.14$ \times 10^5$ M$^{-1}$s$^{-1}$, $k_{off}$ 1.05$ \times 10^{-3}$ s$^{-1}$, and overall $K_D$ 1.71 nM; Supplementary Fig. 2b). For antigen BSA-(Y)-DOTA-Bn, huA33-C825 had a $k_{on}$ of $1.90 \times 10^6$ M$^{-1}$s$^{-1}$, a $k_{off}$ of 2.20$ \times 10^{-4}$ s$^{-1}$, and an overall $K_D$ of 11.6 nM—comparable to the control IgG-C825 values (the anti-GD2 BsAb hu3F8-C825; $k_{on}$ 1.60$ \times 10^4$ M$^{-1}$s$^{-1}$, $k_{off}$ 3.37$ \times 10^{-4}$ s$^{-1}$, and overall $K_D$ 21.2 nM; Supplementary Fig. 2c). In summary, huA33-C825 retained high binding affinity to BSA-(Y)-DOTA-Bn, but lost considerable affinity to antigen GPA33.

Optimization of PRIT with huA33-C825, CA, and 177Lu-DOTA-Bn

A combination of X-SPECT/CT imaging and ex vivo biodistribution studies of huA33-C825 PRIT + 177Lu-DOTA-Bn were used to optimize huA33-C825 and CA doses during PRIT with the aims of achieving high uptake by the tumor while simultaneously minimizing exposure of radiosensitive normal organs such as the blood and kidney (see Supplementary Fig. 3). A representative X-SPECT/CT image following PRIT + 177Lu-DOTA-Bn targeting of GPA33(+) to a SW1222 xenograft in vivo is shown in Fig. 1b. The 131I-huA33-C825 uptake in tumor at 24 h p.i. (average±standard deviation, SD) was 3.71±1.00 %ID/g (activity values are shown in Supplementary Table 2). Taking into account the tracer-specific activity and dose, this corresponds to an absolute huA33-C825 uptake of 44 pmol/g (with about 50 % IR, then 88 pmol/g).

Absorbed dose estimates for optimized PRIT + 177Lu-DOTA-Bn

Decay-corrected time-activity curves up to 120 h p.i. of 177Lu-DOTA-Bn for tumor, kidneys, liver, spleen, and blood are shown in Fig. 1c (activity values are shown in Supplementary Table 1). The estimated absorbed doses of 177Lu-DOTA-Bn for blood, tumor, liver, spleen, and kidneys were 0.9, 65.8, 6.3, 6.6, and 5.3 Gy/MBq, respectively (Table 1). The ratio of absorbed dose estimates for tumor to those for selected normal tissues (i.e., TI) ranged from about 10 (e.g., for liver, spleen, and kidney) to about 220 for muscle (Table 1).

Serial noninvasive PET imaging of GPA33(+) xenografts with PRIT + 86Y-DOTA-Bn

Representative PET images of SW1222 tumor-bearing mice at 2 and 20 h p.i. of PRIT + 86Y-DOTA-Bn are shown in Fig. 1d. The corresponding maximum intensity projection PET images are provided in Supplementary Fig. 4. The CRC xenograft in the shoulder can be clearly delineated with minimal uptake in normal tissue and thus high overall contrast. The uptake in tumor was quantified by region-of-interest analysis of each of the calibrated PET images, and was determined to be 8.75±0.91 and 8.44±1.01 %ID/g (average±standard error of the mean, SEM; n=5) at 2 and 20 h p.i., respectively, indicating rapid uptake of 86Y-DOTA-Bn (presumably by tumor-
associated huA33-C825) and slow subsequent clearance of the huA33-C825/86Y-DOTA-Bn complex from the tumor. The tumor uptake determined by ex vivo biodistribution at 24 h p.i. was 7.85±0.65 %ID/g. A comparison of the tumor uptake determined by either PET imaging or biodistribution of PRIT with either 86Y-DOTA-Bn or 177Lu-DOTA-Bn at 2 and 24 h p.i. is shown in Supplementary Fig. 5. No significant difference (\(P>0.05\)) was observed between the two isotopes at either time point, suggesting good correlation between the in vivo fates of the two distinct M-DOTA-Bn haptens following PRIT.

**Titration of 177Lu-DOTA-Bn administered activity during PRIT**

The uptake in tumor and kidney at 24 h p.i. (as %ID/g, average±SD) as a function of administered 177Lu-DOTA-Bn activity ranging from about 2.0 to 111.0 MBq is shown in Fig. 2a. While the relative 177Lu activity decreased about four-fold for tumor with increasing administered activity (e.g., about 8.5 to 2 % ID/g for 2.0 MBq and 111.0 MBq, respectively), the relative 177Lu activity in kidney remained <1 %ID/g and relatively constant (average range 0.46 – 0.96 %ID/g), suggesting that saturation of available specific M-DOTA-Bn receptors by hapten was occurring in the tumor but not in the kidney compartments. The absolute 177Lu activity uptake in tumor is shown in Fig. 2b, and was fitted to a one-phase association equation (GraphPad Prism version 6.00), with saturation apparent at a 177Lu-DOTA-Bn activity of about 11 pmol/g of tumor (\(R^2=0.89\)). Thus, during PRIT studies, 177Lu-DOTA-Bn activities ≤55.0 MBq were considered subsaturating. Representative autoradiography and histochemistry data for kidney for three different 177Lu-DOTA-Bn activity levels (11.1, 55.5, and 111.0 MBq) are shown in Fig. 2c. The 177Lu activity appeared to be distributed predominantly in the renal cortex, with the cortex-to-medulla activity concentration ratios increasing with increases in administered 177Lu-DOTA-Bn activity.

**PRIT studies**

A total of three separate therapy studies with 177Lu-DOTA-Bn were conducted in SW1222 tumor-bearing mice to evaluate efficacy. A summary of all tumor response data as well as estimated absorbed doses for tumor, blood, and kidney for all PRIT groups is provided in Table 2. No significant weight loss was observed in any of the control or treatment groups (data not shown).
First, five groups of tumor-bearing mice (n=6 – 8 per group; 7-day old tumors, TV 100 – 200 mm³) were treated with: either vehicle alone (i.e., untreated, n=8), 33.3 MBq ¹⁷⁷Lu-DOTA-Bn alone (vehicle given during BsAb and CA injections, n=6), nonspecific hu3F8-C825 PRIT + 33.3 MBq ¹⁷⁷Lu-DOTA-Bn (control antibody, n=8), or huA33-C825 PRIT + either 11.1 MBq or 33.3 MBq ¹⁷⁷Lu-DOTA-Bn (antibody of interest, both n=8). Tumor response data from treatment groups are shown in Fig. 3 and Supplementary Fig. 6. Representative scintigraphy images of selected treated mice are shown in Supplementary Fig. 7 and confirm specific tumor targeting during PRIT including huA33-C825 and ¹⁷⁷Lu-DOTA-Bn compared with controls and nonspecific PRIT. To summarize efficacy, no CRs were seen in any of the GPA33-targeted PRIT or control groups. Also, unremarkable PRs (slight tumor growth delays) were seen only in groups treated with huA33-C825 PRIT + ¹⁷⁷Lu-DOTA-Bn administered activity up to 33.3 MBq.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Absorbed dose (cGy/MBq)</th>
<th>Therapeutic index</th>
</tr>
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<tbody>
<tr>
<td>Blood</td>
<td>0.9</td>
<td>73</td>
</tr>
<tr>
<td>Tumor</td>
<td>65.8</td>
<td>–</td>
</tr>
<tr>
<td>Heart</td>
<td>1.4</td>
<td>47</td>
</tr>
<tr>
<td>Lung</td>
<td>1.8</td>
<td>37</td>
</tr>
<tr>
<td>Liver</td>
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<td>10</td>
</tr>
<tr>
<td>Spleen</td>
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<td>10</td>
</tr>
<tr>
<td>Stomach</td>
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<td>Small Intestine</td>
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<td>Large Intestine</td>
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<td>219</td>
</tr>
<tr>
<td>Bone</td>
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</table>
In the second therapy study, a two-cycle regimen of huA33-C825 PRIT was investigated ($n=4$ or $5$ per group; 10-day old tumors, TV $200–700$ mm$^3$). Similar to the first treatment study, the five untreated mice had to be sacrificed within 30 days due to excessive tumor burden, and the time to reach 500 mm$^3$ was $13\pm2$ days (data not shown). Two of five animals treated with $2\times$ huA33-C825 PRIT + $11.1$ MBq $^{177}$Lu-DOTA-Bn (total $^{177}$Lu-DOTA-Bn administered activity $22.2$ MBq; estimated tumor absorbed dose $15$ Gy) showed CR (Fig. 4a). In the animals with recurrent tumors, the time to reach 500 mm$^3$ was $9$ or $36$ days. Five of five animals treated with $2\times$ huA33-C825 PRIT + $33.3$ MBq $^{177}$Lu-DOTA-Bn (total $^{177}$Lu-DOTA-Bn administered activity $66.6$ MBq; estimated tumor absorbed dose $44$ Gy) showed CR (Fig. 4b). In the animals with recurrent tumors, the time to reach $500$ mm$^3$ was $7, 12, 23$, and $65$ days (average $27\pm26$ days), and a single mouse showed a tumor size of $<10$ mm$^3$ at 140 days. Four of four mice treated with $2\times$ huA33-C825 PRIT + $55.5$ MBq $^{177}$Lu-DOTA-Bn (total $^{177}$Lu-DOTA-Bn administered activity $111.0$ MBq; estimated tumor absorbed dose $73$ Gy) showed CR (Fig. 4c, left). In the animals with recurrent tumors, the time to reach $500$ mm$^3$ was $34, 42$, and $45$ days (average $40\pm6$ days), and a single mouse had a tumor size of $44$ mm$^3$ at 140 days. No major treatment-related toxicities were observed in eight of eight mice randomly selected for necropsy and pathologic assessment (pathologist summary provided in the Supplementary material). In addition, IHC staining of the tumor sections revealed that the residual tumor was GPA33-positive (Fig. 4d).

Following these PRIT studies, we made some very approximate estimates of the number of clonogenic tumor cells remaining per mouse after dual-cycle treatment with huA33-C825 PRIT + $55.5$ MBq $^{177}$Lu-DOTA-Bn. We noted that the individual tumors that responded to treatment with a CR or greater dropped below the detection (palpation) threshold at about $12$ days after the second treatment dose, or at about $30$ days after inoculation of tumor cells (Fig. 4c). This was used as time zero, from which regrowth of clonogenic cells would begin, to a volume of $500$ mm$^3$ (i.e., recurrence). The estimated tumor-absorbed dose of $73$ Gy resulted in CRs in four of four mice, including a single survivor without recurrence at 140 days. Based on an observed tumor doubling time of $5$ days, the three tumors that recurred at approximately $35, 40$, and $45$ days (seven to nine doublings) after the nadir had an implied cell kill of $2$ – $3$ logs. For the tumor without visible regrowth during the 140-day observation period, it was more difficult to estimate a cell kill because it may or may not have been cured. If the tumor had recurred at $140$ days ($28$ doublings) after the nadir, the implied cell kill would have been approximately $8$ logs. If we assume some numerical value for clonogenic cell density, it is possible to calculate how many logs of cell kill would be required to produce a significant probability of cure. For example, a clonogenic cell density of $10^9$ cells per gram would imply that approximately $9$ logs of kill are required to reduce a $500$-mg tumor to a single surviving clonogenic cell. However, if the true number was $10^6$ cells per gram, then only $6$ logs of kill would be required. These values (and their associated uncertainties) translate into the amount of radiation dose that would be required to achieve a significant level of tumor cure. If an estimated tumor dose of $73$ Gy gives approximately $3$ logs of kill but $9$ logs are needed (i.e., $10^9$ cells/g), then the dose would have to be three times greater (i.e., $220$ Gy). In contrast, if only $6$ logs are needed (i.e., $10^6$ cells/g) then the dose would need to be two times greater (i.e., $150$ Gy). However, if the true number of clonogenic cells per gram was only $10^5$ (e.g., a cancer stem cell-like scenario), doses similar to the current estimated value of $73$ Gy would already be sufficiently high to produce a significant likelihood of tumor cure. Our experimental observation of a $25\%$ probability of cure seems to most closely resemble the last of these scenarios. Of course,

### Table 2. Anti-GPA33 PRIT studies with either single-cycle or dual-cycle anti-GPA33 PRIT + $^{177}$Lu-DOTA-Bn administered activities up to $111.0$ MBq

<table>
<thead>
<tr>
<th>Anti-GPA33 PRIT</th>
<th>$^{177}$Lu-DOTA-Bn administered activity (MBq)</th>
<th>Tumor-absorbed dose (cGy)</th>
<th>Blood-absorbed dose (cGy)</th>
<th>Kidney-absorbed dose (cGy)</th>
<th>Complete response</th>
<th>Survival at 140 days</th>
<th>Time to tumor recurrence 500 mm$^2$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$11.1$</td>
<td></td>
<td>$730$</td>
<td>$10$</td>
<td>$59$</td>
<td>$0/8$</td>
<td>$0/8$</td>
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<tr>
<td>$33.3$</td>
<td></td>
<td>$2,191$</td>
<td>$30$</td>
<td>$176$</td>
<td>$0/8$</td>
<td>$0/8$</td>
<td>–</td>
</tr>
<tr>
<td>$111.0$</td>
<td></td>
<td>$2,580$</td>
<td>$100$</td>
<td>$588$</td>
<td>$2/5$</td>
<td>$1/5$</td>
<td>65</td>
</tr>
<tr>
<td>Dual-cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2\times11.1$</td>
<td>$22.2$</td>
<td>$1,460$</td>
<td>$20$</td>
<td>$118$</td>
<td>$2/5$</td>
<td>$0/5$</td>
<td>9, 36; median 22.5</td>
</tr>
<tr>
<td>$2\times33.3$</td>
<td>$66.6$</td>
<td>$4,382$</td>
<td>$60$</td>
<td>$354$</td>
<td>$5/5$</td>
<td>$1/5$</td>
<td>7, 12, 23, 65; median 17.5</td>
</tr>
<tr>
<td>$2\times55.5$</td>
<td>$111.0$</td>
<td>$7,304$</td>
<td>$100$</td>
<td>$588$</td>
<td>$4/4$</td>
<td>$1/4$</td>
<td>34, 42, 45; median 42</td>
</tr>
</tbody>
</table>

* Adjusted for average tumor uptake of $3\%$ ID/g at 24 h p.i. to account for saturating dose

$^{b}$ 65.8 cGy/MBq based on average tumor uptake of $8.5\%$ ID/g at 24 h p.i.

$^{c}$ 0.9 cGy/MBq

$^{d}$ 5.3 cGy/MBq
in reality, we do not know what the clonogenic tumor cell density is, and tumor regression and regrowth are likely to be more complex than a simple monoexponential function, so these speculative calculations should be considered with caution.

Finally, a third PRIT study was performed at a $^{177}$Lu-DOTA-Bn dose of 111.0 MBq, administered as a single cycle to compare treatment with single versus fractionated PRIT dosing ($n$ = 5 per group; 10-day old tumors, TV $50 - 300$ mm$^3$). All untreated mice showed similar tumor growth kinetics to those seen in previous studies (tumor doubling time about 5 days, data not shown). PRIT produced PR in three of the five mice as well as CR in two mice with delayed recurrence in one mouse (the time to reach 500 mm$^3$ was 65 days) and a single survivor without recurrence at 140 days (at 140 days after tumor inoculation, no measurable tumor at the site of s.c. injection; 10-day TV 121 mm$^3$; Fig. 4c, right). A single treated mouse showing a

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**Fig 4** Tumor response data for dual-cycle PRIT + (a) 11.1 MBq $^{177}$Lu-DOTA-Bn (total 22.2 MBq), + (b) 33.3 MBq $^{177}$Lu-DOTA-Bn (total 66.6 MBq) or + (c, left) 55.5 MBq $^{177}$Lu-DOTA-Bn (total 111.0 MBq). Tumor response data for single-cycle PRIT + 111.0 MBq of $^{177}$Lu-DOTA-Bn is also shown (c, right). d Morphologic assessment and IHC detection of GPA33 antigen in formalin-fixed, paraffin-embedded sections of tumors from selected GPA33(+) PRIT-treated mice: left nonresponding (NR) tumor (tumor size at the time of sacrifice $>1,500$ mm$^3$) from a mouse treated with 2 x huA33-C825 PRIT + 11.1 MBq (total 22.2 MBq); right tumor with CR showing senescence at 140 days from a mouse treated with 2 x huA33-C825 PRIT + 55.5 MBq (total 111.0 MBq). Sections of thickness 5 μm were stained with hematoxylin and eosin (H&E) and immunohistochemically analyzed with murine monoclonal antibody A33 for the presence of GPA33 antigen (GPA33+). The NR tumor appears as a bulky mass displaying little to no necrosis in the solid antigen-positive tumor areas. High magnification shows large intact tumor cells without necrotic changes. The CR senescent tumor is considerably smaller (about 40 mm$^3$ at the time of sacrifice at 140 days) than the NR tumor and shows large necrotic areas (asterisk) in the center with surrounding partially papillary-shaped vital tumor formations. High magnification shows a papillary tumor area with a central stroma core and surrounding GPA33-positive necrotic areas.
PR had to be sacrificed due to mobility concerns (unrelated to treatment) 18 days after treatment.

**Discussion**

We achieved our goal of demonstrating that a dosimetry-based TI could be used as a benchmark for successful therapeutic targeting by PRIT in CRC. We demonstrated mean TIs of about 73 for blood and 12 for kidney, and an estimated tumor dose of 43 – 73 Gy with an estimated 2.1 – 3.0 log kill at a 73-Gy tumor-absorbed dose, without detectable bone marrow and renal toxicity. We used \(^{177}\)Lu-DOTA-Bn for theranostic RIT with administered activities up to 111.0 MBq delivered in a fractionated dose strategy (e.g., \(2 \times \) huA33-C825 PRIT + 55.5 MBq) to produce CR with 100 % frequency, including survival beyond 140 days in two of nine mice, with no major treatment-related toxicities observed. IHC analysis of the residual tumors revealed a GPA33(+) phenotype, suggesting that the reason for recurrence of some tumors was insufficient tumor-absorbed dose rather than escape by genetic mutation or some other mechanism. We also found that PRIT is a highly flexible approach that is readily adaptable to multiple dosing regimens, and in fact divided doses were more effective than equivalent single doses in inducing CR. Based on the absence of any treatment-related toxicities, all of our therapeutic regimens delivered normal-tissue absorbed doses well below the respective normal-tissue MTDs. Dose escalation could therefore safely proceed in this setting.

At the MTD, we anticipate that the normal tissues most sensitive to off-target radiotoxicity during anti-GPA33 PRIT would be bone marrow (using blood as a dosimetric surrogate for bone marrow), lung, liver, and kidneys (estimated absorbed doses 0.9, 1.8, 6.3, and 5.3 cGy/MBq, respectively; Table 1). Based on human normal-tissue radiation dose tolerance estimates derived from clinical observations [35], the MTDs are 250 cGy for bone marrow, 1,500 cGy for lung, 3,000 cGy for liver, and 2,000 cGy for kidney. Therefore, we estimate that the maximum tolerated pretargeted \(^{177}\)Lu-DOTA-Bn activity is 278 MBq, with the bone marrow as the dose-limiting organ. At this activity, the estimated absorbed dose delivered to tumor would be 18,292 cGy (183 Gy), with 250 cGy to blood (marrow) and 1,473 cGy to kidney.

We demonstrated that serial, noninvasive X-SPECT/CT imaging of s.c. GPA33(+) tumors was possible for PRIT with \(^{177}\)Lu-DOTA-Bn, suggesting that quantitative imaging combined with therapy is possible (e.g., with QSPECT [36]). In addition, we demonstrated that serial, noninvasive PET imaging of s.c. GPA33(+) tumors was possible for PRIT with \(^{86}\)Y-DOTA-Bn. It should be noted that the affinities for C825 binding of yttrium and lutetium are virtually identical and in the picomolar range. \(^{177}\)Lu has a considerably longer half-life than \(^{86}\)Y (6.73 days and 14.7 h, respectively). Even so, the kinetics of rapid localization and long-term retention of the M-DOTA-Bn favors the use of \(^{86}\)Y because the half-life is long enough to allow accurate determination of the time course of uptake and subsequent prolonged clearance of a targeted therapeutic radiolanthanide. Furthermore, the half-life of \(^{86}\)Y is sufficiently short to not appreciably increase the absorbed doses to normal organs during \(^{177}\)Lu-based PRIT and could also allow repeated measurements before each cycle of a fractionated approach. Thus, \(^{86}\)Y is a suitable surrogate diagnostic for \(^{177}\)Lu. Further, PET imaging is more quantitatively accurate than other nuclear imaging methods such as SPECT with \(^{177}\)Lu and in general. Finally, \(^{86}\)Y could be used as a pretherapy tracer for RIT dosimetry with either \(^{90}\)Y or \(^{177}\)Lu during anti-GPA33 PRIT. \(^{86}\)Y has a complex spectrum, and radiation exposure is not insignificant. However, in a theranostic application, in which much larger treatment doses are anticipated, the gain from its use would outweigh the risk, because of the potential for improving patient selection and treatment planning. Also, corrections must be made for optimal reconstruction; notably, in addition to positrons, the large number of prompt gamma rays emitted by \(^{86}\)Y can interfere with PET image quantitation, but accuracy can be significantly improved with correction algorithms [37, 38].

During our investigation of anti-GPA33 PRIT, we made some notable observations regarding opportunities for improvement of current targeting reagents. First, the binding affinity of huA33 for its cognate antigen was reduced when the C825 was attached. Although we were able to achieve high TIs and apparently prolonged tumor retention during in vivo PRIT, the in vitro binding affinity data (Biacore) showed that huA33-C825 showed considerably lower affinity for antigen GPA33 as compared to parental huA33-IgG. This is consistent with modeling predictions of the affinity-dependence of tumor uptake, which indicate that large molecules (e.g., IgGs) are able to achieve similar uptake levels at much lower affinities in the 10\(^{-8}\) to 10\(^{-6}\) mol/L \(K_d\) range, presumably due to the slow rate of intravasation allowing repeated binding within the tumor [39, 40]. The C825 affinity for radiolanthanide Bn DOTA was unaffected. Nonetheless, we do not rule out the possibility that tumor targeting could be improved by affinity recovery, as well as by addressing other issues related to the pharmacokinetics and biodistribution of the BsAb [41]. These issues are currently under study. Second, uptake of the M-DOTA-Bn by antibody prelocalized to tumor was only 10 % of that predicted on a picomolar basis. We speculate that the low maximum capacity is likely due to a combination of factors, including the metabolism of the dextran CA and subsequent leakage of small DOTA-dextran fragments into the circulation, and the in vivo stability (e.g., proteolytic) of the DOTA-binding scFv C825. Future studies will include alternative CA with improved serum stability,
to reduce the likelihood of small DOTA hapten fragment leakage into the circulation following hepatic metabolism as well as using no-carrier-added formulations of $^{177}$Lu-DOTA-Bn, which could offer an improvement of about three-fold in $^{177}$Lu-specific activity (from about 1, 110 GBq/mg to a theoretical specific activity of 4, 033 GBq/mg Lu).

Successful development of a theranostic PRIT approach in CRC would meet an unmet need for cancer detection and management, and a platform-based PRIT paradigm may indeed be feasible using a variety of IgG-C825 antibodies targeting human cancers. Our previous investigations of PRIT, including PRIT of neuroblastoma based on hu3F8-C825 [18], support the practicality of the platform concept. For one thing, once optimal clearing reagents and C825 haptenes have been developed, these same reagents can be used as a component of theranostic approaches in combination with bifunctional antibody forms of antigen specificities for a broad spectrum of human tumors. In addition, direct applicability to most human tumors provides several possible advantages. First, radiation effects at high absorbed doses have wholly distinct patterns of resistance from that of current drugs and (nonradioactive) antibodies, and we therefore expect complementary effects to current drugs. Second, utilizing a theranostic approach, both diagnostic and therapeutic selection information can be obtained at “tracer doses.” This permits optimized patient dosing and the avoidance of treatment in patients whose tumors are not targeted by the antibody and who are therefore not likely to benefit from this treatment because of low tumor radiation dose or high dose to critical organs such as the kidney and gut. Furthermore, in this study we characterized the importance of the mass used in the three targeting steps and their effect on tumor localization and normal organ uptake. Similar data will need to be obtained in patients. To optimize these dose-seeking studies a modeling approach may be utilized [42]. Finally, a modular therapy in which the radioactivity is separated from the antibody infusion is appealing in a clinical setting, leading to an inherently multidisciplinary approach. For example, patient management and radiation safety measures are simplified.

Conclusion

Using a three-step PRIT protocol with the anti-GPA33/anti-DOTA(metal) BsAb huA33-C825, a dextran-based CA, and $^{177}$Lu-DOTA-Bn, mice with established s.c. SW1222 human CRC xenografts were treated with $^{177}$Lu-DOTA-Bn administered activities as high as 111.0 MBq without demonstrable radiotoxicity to normal tissues including bone marrow and kidney. Fractionated administered activities were significantly more effective than equivalent single activities in terms of tumor response. In addition, serial noninvasive PET imaging with pretargeted $^{86}$Y-DOTA-Bn was successfully demonstrated to be a basis for optimizing diagnosis and dosimetry of pretargeted $^{177}$Lu-DOTA-Bn and $^{86}$Y-DOTA-Bn for PRIT in a murine model of human CRC.

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Compliance with ethical standards

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Conflicts of interest None.

Ethical approval All animal experiments were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center, and institutional guidelines for the proper and humane use of animals in research were followed.

References


