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(Instructions) Pretargeted Radioimmunotherapy Based on the Inverse Electron Demand Diels-Alder Reaction

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TITLE:
Pretargeted Radioimmunotherapy based on the Inverse Electron Demand Diels-Alder Reaction

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radioimmunotherapy, pretargeted radioimmunotherapy, pretargeting, inverse electron demand Diels-Alder reaction, tetrazine, *trans*-cyclooctene, huA33, A33 antigen, lutetium-177

SUMMARY:
This protocol describes the synthesis and characterization of a *trans*-cyclooctene (TCO)-modified antibody and a ^{177}Lu-labeled tetrazine (Tz) radioligand for pretargeted radioimmunotherapy (PRIT). In addition, it details the use of these two constructs for *in vivo* biodistribution and longitudinal therapy studies in a murine model of colorectal cancer.

ABSTRACT:
While radioimmunotherapy (RIT) is a promising approach for the treatment of cancer, the long pharmacokinetic half-life of radiolabeled antibodies can result in high radiation doses to healthy tissues. Perhaps not surprisingly, several different strategies have been developed to circumvent this troubling limitation. One of the most promising of these approaches is pretargeted radioimmunotherapy (PRIT). PRIT is predicated on decoupling the radionuclide from the immunoglobulin, injecting them separately, and then allowing them to combine *in vivo* at the target tissue. This approach harnesses the exceptional tumor-targeting properties of antibodies while skirting their pharmacokinetic drawbacks, thereby lowering radiation doses to non-target tissues and facilitating the use of radionuclides with half-lives that are considered too short for use in traditional radioimmunoconjugates. Over the last five years, our laboratory and others have developed an approach to *in vivo* pretargeting based on the inverse electron-demand Diels-Alder (IEDDA) reaction between *trans*-cyclooctene (TCO) and tetrazine (Tz). This

45 strategy has been successfully applied to pretargeted positron emission tomography (PET) and
46 single-photon emission computed tomography (SPECT) imaging with a variety of antibody-
47 antigen systems. In a pair of recent publications, we have demonstrated the efficacy of IEDDA-
48 based PRIT in murine models of pancreatic ductal adenocarcinoma and colorectal carcinoma. In
49 this protocol, we describe protocols for PRIT using a ^{177}Lu-DOTA-labeled tetrazine radioligand
50 ([^{177}Lu]Lu-DOTA-PEG$_7$-Tz) and a TCO-modified variant of the colorectal cancer targeting huA33
51 antibody (huA33-TCO). More specifically, we will describe the construction of huA33-TCO, the
52 synthesis and radiolabeling of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz, and the performance of *in vivo*
53 biodistribution and longitudinal therapy studies in murine models of colorectal carcinoma.
54
55 **INTRODUCTION:**
56 Radioimmunotherapy (RIT) — the use of antibodies for the delivery of therapeutic
57 radionuclides to tumors — has long been an enticing approach to the treatment of cancer[1,2].
58 Indeed, this promise has been underscored by the United States Food and Drug
59 Administration's approval of two radioimmunoconjugates for the treatment of Non-Hodgkin's
60 Lymphoma: ^{90}Y-ibritumomab tiuxetan and ^{131}I-tositumomab[3,4]. Yet even from its earliest days,
61 the clinical prospects of RIT have been hampered by a critical complication: high radiation dose
62 rates to healthy tissues[5,6]. Generally speaking, radioimmunoconjugates for RIT are labeled with
63 long-lived radionuclides (*e.g.,* ^{131}I [$t_{1/2}$ = 8.0 days] and ^{90}Y [$t_{1/2}$ = 2.7 days]) with physical half-lives
64 that dovetail well with the long pharmacokinetic half-lives of immunoglobulins. This is essential,
65 as it ensures that sufficient radioactivity remains once the antibody has reached its optimal
66 biodistribution after several days of circulation. However, this combination of long residence
67 times in the blood and long physical half-lives inevitably results in the irradiation of healthy
68 tissues, thereby reducing therapeutic ratios and limiting the efficacy of therapy[7]. Several
69 strategies have been explored to circumvent this problem, including the use of truncated
70 antibody fragments such as Fab, Fab', F(ab')$_2$, minibodies, and nanobodies[8-10]. One of the most
71 promising and fascinating, yet undeniably complex, alternative approaches is *in vivo*
72 pretargeting[11].
73
74 *In vivo* pretargeting is an approach to nuclear imaging and therapy that seeks to harness the
75 exquisite affinity and selectivity of antibodies while skirting their pharmacokinetic drawbacks[11-
76 13]. To this end, the radiolabeled antibody used in traditional radioimmunotherapy is
77 deconstructed into two components: a small molecule radioligand and an immunoconjugate
78 that can bind *both* a tumor antigen and the aforementioned radioligand. The immunoconjugate
79 is injected first and given a 'head start', often several days, during which it accumulates in the
80 target tissue and clears from the blood. Subsequently, the small molecule radioligand is
81 administered and either combines with the immunoconjugate at the tumor or rapidly clears
82 from the body. In essence, *in vivo* pretargeting relies upon performing radiochemistry within
83 the body itself. By reducing the circulation of the radioactivity, this approach simultaneously
84 reduces radiation doses to healthy tissues and facilitates the use of radionuclides (*e.g.,* ^{68}Ga, $t_{1/2}$
85 = 68 min[211]; As, $t_{1/2}$ = 7.2 h) with half-lives that are typically considered incompatible with
86 antibody-based vectors.
87

88   Starting in the late 1980s, a handful of different approaches to *in vivo* pretargeting have been
89   developed, including strategies based on bispecific antibodies, the interaction between
90   streptavidin and biotin, and the hybridization of complementary oligonucleotides[14-18]. Yet each
91   has been held back to varying degrees by complications, most famously the potent
92   immunogenicity of streptavidin-modified antibodies[19,20]. Over the last five years, our group and
93   others have developed an approach to *in vivo* pretargeting based on the rapid and
94   bioorthogonal inverse electron demand Diels-Alder ligation between *trans*-cyclooctene (TCO)
95   and tetrazine (Tz)[21-24]. The most successful of these strategies have employed a TCO-modified
96   antibody and a Tz-bearing radioligand, as TCO is typically more stable *in vivo* than its Tz partner
97   (**Figure 1**)[25,26]. As in other pretargeting methodologies, the mAb-TCO immunoconjugate is
98   administered first and given time to clear from circulation and accumulate in tumor tissue.
99   Subsequently, the small molecule Tz radioligand is injected, after which it either clicks with the
100 immunoconjugate within the target tissue or clears rapidly from the body. This *in vivo*
101 pretargeting strategy has proven highly effective for PET and SPECT imaging with several
102 different antibody/antigen systems, consistently producing images with high contrast and
103 enabling the use of short-lived radionuclides such as ^{18}F ($t_{1/2}$ = 109 min) and ^{64}Cu ($t_{1/2}$ = 12.7
104 h)[21,22,24]. More recently, the efficacy of click-based pretargeted radioimmunotherapy (PRIT) has
105 been demonstrated in murine models of pancreatic ductal adenocarcinoma (PDAC) and
106 colorectal carcinoma[27,28]. To this end, the therapeutic radionuclide ^{177}Lu (β_{max} = 498 keV, $t_{1/2}$ =
107 6.7 days) was employed in conjunction with two different antibodies: 5B1, which targets
108 carbohydrate antigen 19.9 (CA19.9) ubiquitously expressed in PDAC, and huA33, which targets
109 A33, a transmembrane glycoprotein expressed in >95% of colorectal cancers. In both cases, this
110 approach to ^{177}Lu-PRIT yielded high activity concentrations in tumor tissue, created a dose-
111 dependent therapeutic effect, and simultaneously reduced activity concentrations in healthy
112 tissues compared to traditional directly-labeled radioimmunoconjugates.
113
114 In this article, we describe protocols for PRIT using a ^{177}Lu-DOTA-labeled tetrazine radioligand
115 ([^{177}Lu]Lu-DOTA-PEG$_7$-Tz) and a TCO-modified variant of the huA33 antibody (huA33-TCO).
116 More specifically, we describe the construction of huA33-TCO (**Figure 2**), the synthesis and
117 radiolabeling of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (**Figures 3** and **4**), and the performance of *in vivo*
118 biodistribution and longitudinal therapy studies in murine models of colorectal carcinoma.
119 Furthermore, in the representative results and discussion, we present a sample data set,
120 address possible strategies for the optimization of this approach, and consider this strategy in
121 the wider context of *in vivo* pretargeting and PRIT. Finally, it is important to note that while we
122 have chosen to focus on pretargeting using huA33-TCO and [^{177}Lu]Lu-DOTA-PEG$_7$-Tz in this
123 protocol, this strategy is highly modular and can be adapted to suit a wide range of antibodies
124 and radionuclides.
125
126 **PROTOCOL:**
127
128 All *in vivo* animal experiments described in this work were performed according to approved
129 protocols and executed under the ethical guidelines of the Memorial Sloan Kettering Cancer
130 Center, Weill Cornell Medical Center, and Hunter College Institutional Animal Care and Use
131 Committees (IACUC).

1. The preparation of huA33-TCO

NOTE: The synthesis of huA33-TCO has been previously reported[29]. However, for the ease of the reader, it is replicated here with adjustments for optimal conditions.

1.1   In a 1.7 mL microcentrifuge tube, prepare a 125 µL solution of (E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS) in dry dimethyl formamide (DMF) at a concentration of 40 mg/mL (0.15 M). This solution can be aliquoted and frozen at -80 °C for use in future experiments.

1.2   In a separate 1.7 mL microcentrifuge tube, prepare a 5 mg/mL solution of huA33 in 1 mL of phosphate buffered saline (PBS; 2.7 mM potassium chloride, 137 mM sodium chloride, and 11.9 mM potassium phosphate, pH 7.4).

1.3   Using small aliquots (<5 µL) of 0.1 M Na_2CO_3, adjust the pH of the antibody solution from Step 1.2 to 8.8-9.0. Use either pH paper or a pH meter with a microelectrode to monitor the pH and exercise care that the pH does not exceed 9.0.

1.4   To the antibody solution described in Step 1.3, slowly add a volume corresponding to 40 molar equivalents of TCO-NHS relative to the amount of antibody. For example, if 5 mg (30 nmol) of huA33 is in the solution, add 9.0 µL (1.20 µmol) of the 40 mg/mL (0.15 M) solution of TCO-NHS.

NOTE: TCO-NHS is hydrophobic. When adding it to the solution of antibody, add it slowly and with agitation to prevent precipitation. Do not exceed 10% DMF by volume in the final reaction solution.

1.5   Allow the reaction to incubate at 25 °C on a thermomixer for 1 h with mild agitation (500 rpm).

1.6   After 1 h, purify the huA33-TCO immunoconjugate using a pre-packed disposable size exclusion desalting column.

1.6.1   Equilibrate the size exclusion column as described by the supplier to remove any preservatives present in the column during storage. A typical procedure involves washing the column 5x with a volume of PBS that corresponds to the volume of the column: 5 x 2.5 mL of PBS.

1.6.2   Add the reaction mixture to the size exclusion column noting the volume of the reaction mixture.

1.6.3   After the reaction mixture has entered the column, add an appropriate amount of PBS to bring the total volume of solution added to the column up to 2.5 mL. For example, if the

conjugation reaction resulted in a total volume of 1.3 mL, add 1.2 mL of additional PBS to the column.

1.6.4   Collect the product using 2 mL of PBS as the eluent.

NOTE: This step will yield the final construct huA33-TCO in 2 mL of PBS, pH 7.4.

1.7   Measure the concentration of the huA33-TCO using a UV-Vis spectrophotometer monitoring the 280 nm wavelength. The molar extinction coefficient for most IgGs (ε_{280}) is 210,000 $M^{-1}cm^{-1}$.

1.8   If a solution with a higher concentration of immunoconjugate is desired, concentrate the huA33-TCO solution using a centrifugal filter unit with a 50,000 molecular weight cut-off following manufacturer instructions.

NOTE: Many antibodies have been known to aggregate or precipitate during concentration. When attempting this procedure with a new antibody, researchers should defer to the literature or their own experience handling the antibody in question.

1.9   Store the completed huA33-TCO immunoconjugate at 4 °C in the dark if it is needed immediately. If it is to be used more than 4 days in the future, store it at -80 °C in the dark.

NOTE: This is an acceptable stopping point in the procedure. The completed huA33-TCO immunoconjugate should be stable for at least 6 months storage at -80 °C in the dark.

2.   The Synthesis of Tz-PEG$_7$-NHBoc

2.1. In a 1.7 mL microcentrifuge tube, dissolve 5 mg of tetrazine *N*-hydroxysuccinimidyl ester (Tz-NHS; 12.6 µmol) in 0.15 mL of anhydrous dimethyl sulfoxide (DMSO).

2.2. In a separate 1.7 mL microcentrifuge tube, dissolve 8 mg of Boc-protected amino PEG polymer (Boc-NH-PEG$_7$-NH$_2$; 17.1 µmol) in 0.15 mL of anhydrous DMSO.

2.3. To the solution of Tz-NHS, add 3 µL (21.3 µmol, 1.7 molar equivalents) of triethylamine (TEA) and mix thoroughly.

2.4. Add the pink tetrazine solution to the NHBoc-PEG$_7$-NH$_2$ solution from Step 2.2 and incubate the reaction mixture for 30 min at room temperature (RT) with mild agitation.

2.5. After 30 minutes, dilute the reaction 1:1 in H$_2$O and purify the reaction using reversed-phase C$_{18}$ high-performance liquid chromatography (HPLC) to separate any unreacted Tz-NHS from the Tz-PEG$_7$-NHBoc. Use solvents without acid to prevent the premature removal of the Boc protecting group. Monitor both Tz-PEG$_7$-NHBoc and Tz-NHS at a wavelength of 525 nm.

220 NOTE: Retention times are obviously highly dependent on the HPLC equipment of each
221 laboratory (pumps, columns, tubing, *etc*.), and appropriate controls should be run prior to
222 purification. However, to present an example, if a gradient of 5:95 MeCN/H_2O (both **without**
223 any additive) to 95:5 MeCN/H_2O over 30 min, a flow rate of 1 mL/min, and an analytical 4.6 mm
224 x 250 mm C_{18} column are used, the retention times of Tz-NHS and Tz-PEG$_7$-NHBoc are around
225 16 min and 18 min, respectively.
226
227 2.6.   Freeze the collected HPLC eluent using liquid nitrogen and wrap the now-frozen
228 collection tube in aluminum foil. Place the frozen collection tube on a lyophilizer overnight to
229 remove the HPLC mobile phase. The product will be a characteristic bright pink solid.
230
231 NOTE: If liquid nitrogen is not available, freeze the collected HPLC eluent in dry ice or overnight
232 in a -80 °C freezer.
233
234 **3.   The Synthesis of Tz-PEG$_7$-NH$_2$**
235
236 3.1. To the product from Step 2.6, Tz-PEG$_7$-NHBoc, add 1.5 mL of dichloromethane (DCM) and
237 transfer this solution to a small round-bottom flask.
238
239 3.2. Add 0.25 mL of trifluoroacetic acid (TFA) dropwise to the pink solution from Step 3.1.
240
241 3.3. Allow the reaction to incubate for 30 min at RT with mild agitation.
242
243 3.4. After 30 min, evaporate the solvent *via* rotary evaporation. Do not exceed a water bath
244 temperature of 37 °C.
245
246 3.5. Reconstitute the pink viscous product in 0.5 mL of water.
247
248 3.6. Purify the product using reversed-phase C_{18} HPLC to separate Tz-PEG$_7$-NH$_2$ from the Boc-
249 protected precursor. Monitor both Tz-PEG$_7$-NHBoc and Tz-PEG$_7$-NH$_2$ at a wavelength of 525 nm.
250
251 NOTE: Retention times are obviously highly dependent on the HPLC equipment of each
252 laboratory (pumps, columns, tubing, *etc*.), and appropriate controls should be run prior to
253 purification. However, to present an example, if a gradient of 5:95 MeCN/H_2O (both **without**
254 any additive) to 95:5 MeCN/H_2O over 30 min, a flow rate of 1 mL/min, and an analytical 4.6 mm
255 x 250 mm C_{18} column are used, the retention times of Tz-PEG$_7$-NHBoc and Tz-PEG$_7$-NH$_2$ are
256 around 18 min and 13 min, respectively.
257
258 3.7. Freeze the collected HPLC eluent using liquid nitrogen and wrap the now-frozen collection
259 tube in aluminum foil. Place the frozen collection tube on a lyophilizer overnight to remove the
260 HPLC mobile phase. The product will be a bright pink solid.
261

262   3.8. Reconstitute the product from Step 3.7, Tz-PEG$_7$-NH$_2$, with 150 µL of DMSO and transfer to
263   a 1.7 mL microcentrifuge tube.

264

265   3.9. Measure the concentration of Tz-PEG$_7$-NH$_2$ using a UV-Vis spectrophotometer monitoring
266   the 525 nm wavelength. The molar extinction coefficient for Tz-PEG$_7$-NH$_2$ (ε_{525}) is 535 M^{-1}cm^{-1}.

267

268   **4.   The Synthesis of Tz-PEG$_7$-DOTA**

269

270   4.1. Dissolve Tz-PEG$_7$-NH$_2$ (4.5 mg, 6.9 µmol) in 0.15 mL of DMSO (or simply proceed with the
271   solution created in Step 3.8).

272

273   4.2. Add 22 molar equivalents of TEA (21 µL, 0.15 mmol) to the tetrazine-containing solution
274   from Step 4.1.

275

276   4.3. Add 10 mg (14.2 µmol) of p-SCN-Bn-DOTA as a solid and vortex the solution for about 2
277   min or until the material is fully dissolved.

278

279   4.4. Allow the reaction to incubate for 30 min at RT with mild agitation.

280

281   4.5. After 30 min, dilute the reaction 1:1 in H$_2$O and purify the product using reversed-phase C$_{18}$
282   HPLC to remove unreacted p-SCN-Bn-DOTA. The p-SCN-Bn-DOTA can be monitored at a
283   wavelength of 254 nm, while the Tz-PEG$_7$-DOTA is best monitored at a wavelength of 525 nm.

284

285   NOTE: Retention times are obviously highly dependent on the HPLC equipment of each
286   laboratory (pumps, columns, tubing, etc.), and appropriate controls should be run prior to
287   purification. However, to present an example, if a gradient of 5:95 MeCN/H$_2$O (both **without**
288   any additive) to 95:5 MeCN/H$_2$O over 30 min and an analytical 4.6 x 250 mm C$_{18}$ column are
289   used, Tz-PEG$_7$-DOTA and p-SCN-Bn-DOTA have retention times of around 15.6 min and 16.1
290   min, respectively.

291

292   4.6.   Freeze the collected HPLC eluent using liquid nitrogen and wrap the now-frozen
293   collection tube in aluminum foil. Place the frozen collection tube on a lyophilizer overnight to
294   remove the HPLC mobile phase. The product will be a bright pink powder.

295

296   4.7. Reconstitute the product in 0.15 mL of DMSO and measure the concentration using a UV-
297   Vis spectrophotometer monitoring the 525 nm wavelength. The molar extinction coefficient for
298   Tz-PEG$_7$-DOTA (ε_{525}) is 535 M^{-1}cm^{-1}.

299

300   4.8. Analyze the final compound by nuclear magnetic resonance (NMR) and high-resolution
301   mass spectrometry (HRMS) to verify that synthesis was successful. See **Table 1** for the
302   experimentally determined chemical shifts and molecular weights of all the compounds
303   discussed in this work.

304

305   4.9.   Store the purified Tz-PEG$_7$-DOTA solution in the dark at -80 °C.

306

307   NOTE: This is an acceptable stopping point in the procedure. The completed Tz-PEG$_7$-DOTA
308   precursor is stable for at least 1 year under these conditions.

309

310   **5. ^{177}Lu Radiolabeling of Tz-PEG$_7$-DOTA**

311

312   CAUTION: This step of the protocol involves the handling and manipulation of radioactivity.
313   Before performing these steps — or performing any other work with radioactivity —
314   researchers should consult with their home institution's Radiation Safety Department. Take all
315   possible steps to minimize exposure to ionizing radiation.

316

317   NOTE: When working with small amounts of radiometals it is recommended that all buffers be
318   free from trace metals to prevent interference in coordination site binding.

319

320   5.1.   In a 1.7 mL centrifuge tube, add 200 µL of 0.25 M ammonium acetate buffer adjusted
321   with aliquots of 1 M HCl to pH 5.5.

322

323   NOTE: If using less than 370 MBq of activity for the labeling, the volume of buffer used should
324   be reduced to 100 µL.

325

326   5.2.   Add the desired amount of [^{177}Lu]LuCl$_3$ to the buffer solution. The amount added will be
327   dependent on the number of subjects in the experiment and the radioactive doses being
328   administered. It is recommended that 1-2 extra doses worth of radioactivity be added as a
329   precaution to compensate for the potential loss of radioactivity during purification steps.

330

331   5.3.   Add Tz-PEG$_7$-DOTA in DMSO to the radioactive mixture in Step 5.2. The amount of Tz-
332   PEG$_7$-DOTA is dependent on the number of subjects being tested. More detail on this topic can
333   be found in Step 6.2.2.2.

334

335   5.4.   Allow the solution to incubate at 37 °C for 20 min.

336

337   5.5.   Verify the radiolabeling is complete using radio instant thin layer chromatography
338   (radio-iTLC) with 50 mM EDTA, pH 5.5 as the mobile phase. The labeled [^{177}Lu]Lu-DOTA-PEG$_7$-Tz
339   will remain at the baseline — $R_f = 0$ — while free [^{177}Lu]Lu^{3+} will be coordinated by the EDTA
340   and will travel with the solvent front, $R_f = 1.0$ (**Figure 3B**).

341

342   5.6.   If quantitative labeling is not achieved, add additional ligand to coordinate the free
343   radiometal. Alternatively, purify the labeled [^{177}Lu]Lu-DOTA-PEG$_7$-Tz using a C$_{18}$ cartridge.
344   Follow manufacturer instructions for use. A sample procedure is given below.

345

346   5.6.1.   Prime the cartridge by slowly passing through 5 mL of ethanol through the cartridge
347   with a large syringe. Then, pass through 5 mL of acetonitrile and then 5 mL of deionized (DI)
348   H$_2$O.

349

350   5.6.2. Draw up the radioligand solution from Step 5.3 in a smaller syringe and inject it slowly
351   onto the C_{18} cartridge. Then, wash the cartridge with 10 mL of DI H_2O to remove any unbound
352   [^{177}Lu]LuCl$_3$.

353

354   5.6.3. Elute with 500 µL of ethanol. Remove any ethanol from the final product by passing over
355   the vessel with a low flow rate of dry nitrogen or argon for 10-15 min. Subsequently, resuspend
356   the radioligand in saline in a volume determined in Step 6.2.2.2.

357

358   6. *In vivo* **Studies**

359

360   CAUTION: As in Section 5, this step of the protocol involves the handling and manipulation of
361   radioactivity. Before performing these steps, researchers should consult with their home
362   institution's Radiation Safety Department. Take all possible steps to minimize exposure to
363   ionizing radiation.

364

365   6.1.   **Preparation of animals**

366

367   6.1.1. In female athymic nude mice, subcutaneously implant 5 x 10^6 SW1222 colorectal cancer
368   cells suspended in 150 µL of a 1:1 mixture of cell media and matrix (*e.g.*, Matrigel) and allow
369   these to grow into a 100-150 mm^3 xenograft (14-18 days after inoculation).

370

371   6.1.2. Sorting of animals for a biodistribution experiment

372

373   6.1.2.1. Once the tumors are of sufficient size as determined by caliper measurement, sort the
374   animals to ensure each cohort has approximately the same average tumor volume. The animals
375   can be distinguished in each cage by markings on the tail with indelible ink (one band, two
376   bands, *etc.*).

377

378   6.1.3. Sorting of animals for a longitudinal therapy study

379

380   6.1.3.1. Once the tumors are of sufficient size as determined by caliper measurement, attach
381   ear tags to each of the animals to ensure correct tracking throughout the experiment.

382

383   NOTE: Numerical ear tags may fall off throughout the course of the experiment. As a result, it is
384   recommended to accompany these physical tags with ear notches in a systematic manner (*i.e.*,
385   right, left, bilateral, right x 2, left x 2).

386

387   6.1.3.2. Sort the animals so that the average tumor volume in each cohort is roughly equal. The
388   following method for animal sorting can be performed using a spreadsheet program.

389

390   6.1.3.2.1.   List the animal identification numbers, ear notch pattern, and tumor volume in
391   three separate columns.

392

393   6.1.3.2.2.   Sort the data from smallest to largest tumor volume.

394

395   6.1.3.2.3.   In a fourth column, assign each animal a cage number and cycle through the cages
396   in a snakelike pattern. For example, if there are 5 cages, this column would be filled "5, 4, 3, 2,
397   1, 1, 2, 3, 4, 5…" until all the animals are assigned a cage.

398

399   6.1.3.2.4.   Once the cages are assigned, sort the data by cage number. If ear notches are
400   being used, ensure that each animal in a given cage has a unique ear notch pattern. If there are
401   duplicates (two or more of the same pattern) in a given cage, swap one mouse with one from
402   another cage with the missing pattern until every cage has animals with unique ear notch
403   patterns.

404

405   **6.2.   Formulations and Injections**

406

407   NOTE: The sequence of injection for both biodistribution and therapy study proceeds as
408   follows: huA33-TCO is injected first, followed by [^{177}Lu]Lu-DOTA-PEG$_7$-Tz after a pre-determined
409   interval.

410

411   6.2.1.   Immunoconjugate

412

413   6.2.1.1. Dilute an aliquot of the huA33-TCO solution from Step 1.9 to a concentration of 0.8
414   mg/mL in 0.9% sterile saline.

415

416   6.2.1.2. Draw doses of 150 µL (100 µg) of huA33-TCO solution in syringes pretreated with 1%
417   bovine serum albumin (BSA) in PBS and store these syringes on ice.

418

419   NOTE: BSA treatment reduces the non-specific binding of the antibody to the walls of the
420   syringe.

421

422   6.2.1.3. Warm the animals under a heat lamp for 3 minutes to dilate the tail vein.

423

424   6.2.1.4. Inject the huA33-TCO solution into the tail vein of the xenograft-bearing mouse. Allow
425   24 h (or a different pre-determined time interval) for the huA33-TCO to accumulate in the
426   tumor of the mouse.

427

428   6.2.2. **Radioligand**

429

430   6.2.2.1. Radiolabel Tz-PEG$_7$-DOTA as outlined in Section 5.

431

432   6.2.2.2. Draw doses in 150 µL of 0.9% sterile saline containing 1.1 molar equivalents of Tz-
433   PEG$_7$-DOTA relative to the amount of huA33-TCO administered. As an example, if 100 µg (0.67
434   nmol) of huA33-TCO was injected into the animal and the [^{177}Lu]Lu-DOTA-PEG$_7$-Tz reaction
435   mixture was made with a specific activity of 12.4 GBq/µmol, then doses will be drawn

436 containing 9.14 MBq of activity each. This corresponds to 0.74 nmol of Tz (1.1 molar eq. relative
437 to huA33-TCO).

439 6.2.2.3. Warm the animals under a heat lamp for 3 min to dilate the tail vein.

441 6.2.2.4. Inject the dose of radioligand into the tail vein of the xenograft-bearing mouse. The
442 amount of activity to be injected is determined by the researcher. Published data have shown
443 dose-dependent therapeutic effects on tumor size within a range of 7.4 - 55.5 MBq.

445 6.3.   **Biodistribution**

447 6.3.1.   At the desired time point after the administration of the radioligand, euthanize each
448 cohort of mice using a 2% O_2 / 6% CO_2 gas mixture.

450 NOTE: Follow any institutional requirements regarding methods for secondary physical
451 euthanasia (*e.g.,* cervical dislocation).

453 6.3.2.   For each animal, remove all organs of interest, wash them in a water bath to remove
454 any excess blood, and dry them on a paper towel in open air for 5 min. Sample organ list in
455 suggested order: blood, tumor, heart, lungs, liver, spleen, stomach, small intestine, large
456 intestine, kidneys, muscle, bone, skin, tail.

458 6.3.3.   Once sufficiently dried, place the organs in pre-weighed disposable culture tubes. Weigh
459 the now-filled tubes once again to obtain the mass of each organ or tissue.

461 6.3.4.   Measure the activity in each of the tubes using a gamma counter. Calibrate the
462 measured activity in the gamma counter to the detector used for measuring the drawn dose.
463 Count radioactive standards of ^{177}Lu on each instrument and determine a calibration factor for
464 interconversion between activity and counts-per-minute (cpm).

466 6.3.5.   Plot the biodistribution data as a bar graph (see **Figure 5**) with the mean normalized
467 uptake for each organ displayed along with a bar denoting one standard deviation. Statistical
468 differences in uptake between sample groups may be assessed by an unpaired t-test, in which
469 significance is achieved when $p < 0.05$.

471 6.4.   **Therapy Monitoring**

473 6.4.1.   Using calipers, measure the longest side of the oblong tumor (length) as well as the
474 width, which is perpendicular to the length. Calculate volume using the formula for the volume
475 of an ellipsoid: $(4/3)\pi L \times W \times H$, which simplifies to $\frac{1}{2}L \times W^2$, assuming that the height is
476 approximately equal to the width.

478 NOTE: It is also possible to use a handheld tumor measuring device if one is available (see **Table
479 of Materials**).

480
481 <mark>6.4.2. Weigh each mouse on a balance to track weight gain or weight loss over time.</mark>
482
483 6.4.3. Importantly, monitor each animal's physical appearance for signs of distress, such as
484 hunched back or ruptured cutaneous blood vessels (which may indicate haematotoxicity).
485
486 NOTE: Tumor measurements should be collected every 1-2 days during the course of the
487 longitudinal therapy study.
488
489 6.4.4. Plot data from the longitudinal therapy study as average tumor volumes over time and
490 normalize to starting tumor volume if desired. Statistical differences in uptake on the same day
491 between sample groups may be assessed by an unpaired t-test, in which significance is
492 achieved when $P < 0.05$. More extensive statistical analyses can and should be carried out as
493 recommended by a trained biostatistician.
494
495 **REPRESENTATIVE RESULTS:**
496 The conjugation of TCO to huA33 is predicated on the coupling between the amine-reactive
497 TCO-NHS and the lysine residues on the surface of the immunoglobulin. This method is highly
498 robust and reproducible and reliably yields a degree-of-labeling of 2-4 TCO/mAb. In this case,
499 MALDI-ToF mass spectrometry was employed to confirm a degree of labeling of approximately
500 4.0 TCO/mAb; a similar value was obtained using a fluorophore-modified tetrazine as a
501 reporter[24]. The synthesis of the tetrazine ligand is performed in three steps: (1) the coupling of
502 Tz-NHS to a mono-Boc-protected PEG linker (2) the deprotection of this intermediate to yield
503 Tz-PEG$_7$-NH$_2$, and (3) the formation of a thiourea linkage between p-SCN-Bn-DOTA and Tz-PEG$_7$-
504 NH$_2$. This procedure is relatively facile and affords Tz-PEG$_7$-DOTA in an overall yield of ~75%.
505 Each of the intermediates has been characterized by HRMS and ^{1}H-NMR; this data is presented
506 in **Table 1**.
507
508 Moving on to the radiolabeling, ^{177}Lu^{3+} is typically obtained from commercial suppliers as a
509 chloride salt [^{177}Lu]LuCl$_3$ in 0.5 M HCl. The radiolabeling of Tz-PEG$_7$-DOTA with ^{177}Lu to yield the
510 radioligand [^{177}Lu]Lu-DOTA-PEG$_7$-Tz is very straightforward: in just 20 min, the reaction is
511 complete, producing the desired product in >99% radiochemical purity as determined by radio-
512 iTLC. Typically, no further purification is necessary prior to formulation. A survey of the
513 literature on Tz/TCO-based pretargeting suggests that a Tz:mAb molar ratio of ~1:1 produces
514 the best *in vivo* data[10]. As a result, it is not essential to obtain the radioligand in the highest
515 possible molar activity. For example, biodistribution experiments discussed here employ
516 [^{177}Lu]Lu-DOTA-PEG$_7$-Tz with a molar activity of ~12 GBq/μmol. For longitudinal therapy studies,
517 in contrast, [^{177}Lu]Lu-DOTA-PEG$_7$-Tz with higher molar activity was used in order to facilitate the
518 administration of larger doses of radioactivity without changing the number of injected moles
519 of tetrazine.
520
521 As will be addressed further in the discussion, biodistribution experiments are of paramount
522 importance to understanding and optimizing any approach to PRIT. In this case, biodistribution
523 experiments were conducted to determine the optimal interval time between the

524 administration of the immunoconjugate and the injection of the radioligand. To this end, we
525 employed athymic nude mice bearing subcutaneous A33 antigen-expressing SW1222
526 xenografts on their right shoulder. These animals received 100 µg (0.67 nmol) of huA33-TCO 24,
527 48, 72, or 120 h prior to the injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (9.14 MBq, 0.74 nmol). **Figure
528 5** shows that all injection intervals produce high activity concentrations in the tumor tissue as
529 well as low activity concentrations in healthy organs. The 24 h injection interval affords the
530 highest tumoral uptake at 120 h post-injection: 21.2 ± 2.9%ID/g. Each set of conditions also
531 produces impressive tumor-to-organ activity concentration ratios. Pretargeting with a 24 h
532 interval, for example, yields tumor-to-blood, tumor-to-liver, and tumor-to-muscle ratios of 20 ±
533 5, 37 ± 7, and 184 ± 30, respectively, 120 h after the administration of the radioligand. Based on
534 these findings, a 24 h interval was chosen for the subsequent longitudinal therapy study (see
535 below).
536
537 For the *in vivo* longitudinal therapy study, cohorts (n = 10) of athymic nude mice bearing
538 subcutaneous SW1222 xenografts on their right flank were administered huA33-TCO (100 ug,
539 0.67 nmol) 24 hours prior to injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz. Three different experimental
540 cohorts were employed, receiving 18.7, 37, or 55.5 MBq of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz
541 (corresponding to molar activities of 24, 45, and 70 GBq/µmol). In addition, two control cohorts
542 received one half of the PRIT regimen: either huA33-TCO (100 ug, 0.67 nmol) without [^{177}Lu]Lu-
543 DOTA-PEG$_7$-Tz *or* [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (55.5 MBq, 0.74 nmol) without huA33-TCO. These are
544 essential controls to ensure that the therapeutic response is not elicited by either the
545 immunoconjugate or radioligand alone. The volumes of the tumors were measured every 3
546 days for the first three weeks of the study and then once per week until the conclusion of the
547 experiment (70 days, 10 half-lives of ^{177}Lu). As seen in **Figure 6**, there is a stark difference in the
548 response of the experimental cohorts compared to the control groups. While the tumors in the
549 mice receiving only one component of the PRIT strategy continue to grow unchecked, the
550 tumors of the mice receiving the full PRIT regimen stop growing and ultimately shrink to
551 volumes well below those measured at the beginning of the study. Importantly, no toxic side
552 effects were observed, and all animals maintained a weight within 20% of their initial mass
553 (**Figure 7A**). A Kaplan-Meier plot of the data provides an even more striking visualization of the
554 study: while all mice in the control cohorts had to be euthanized within a few weeks, the mice
555 of the experimental cohorts had a perfect record of survival at the end of the investigation
556 (**Figure 7B**).
557
558 **FIGURE LEGENDS:**
559 **Figure 1. Cartoon schematic of pretargeted radioimmunotherapy based on the inverse
560 electron demand Diels-Alder reaction.** This figure has been modified from reference #28.
561 Reprinted (adapted) with permission from Membreno, R., Cook, B. E., Fung, K., Lewis, J. S., &
562 Zeglis, B. M. Click-Mediated Pretargeted Radioimmunotherapy of Colorectal Carcinoma.
563 *Molecular Pharmaceutics*. **15** (4), 1729-1734 (2018). Copyright 2018 American Chemical Society.
564
565 **Figure 2. Schematic of the construction of huA33-TCO.**
566
567 **Figure 3. Schematic of the synthesis of Tz-PEG$_7$-DOTA.**

568

569 **Figure 4. (A) Schematic of the radiolabeling of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz; (B) Representative**
570 **radio-iTLC chromatogram demonstrating the >98% radiochemical purity of [^{177}Lu]Lu-DOTA-**
571 **PEG$_7$-Tz.**

572

573 **Figure 5. Biodistribution in of *in vivo* pretargeting with huA33-TCO and [^{177}Lu]-DOTA-PEG$_7$-Tz**
574 **in athymic nude mice bearing subcutaneous SW1222 human colorectal cancer tumors using**
575 **pretargeting intervals of 24 (purple), 48 (green), 72 (orange), or 120 (blue) hours**. Data with
576 standard errors from cohorts of n = 4; Statistical analysis was performed by an unpaired
577 Student's t-test, **$p < 0.01$. This figure has been modified from reference #28. Reprinted
578 (adapted) with permission from Membreno, R., Cook, B. E., Fung, K., Lewis, J. S., & Zeglis, B. M.
579 Click-Mediated Pretargeted Radioimmunotherapy of Colorectal Carcinoma. *Molecular*
580 *Pharmaceutics*. **15** (4), 1729-1734 (2018). Copyright 2018 American Chemical Society.

581

582 **Figure 6. Longitudinal therapy study of 5 groups of mice (n = 10 each) bearing subcutaneous**
583 **SW1222 tumors depicted in average tumor volume as a function of time (A); and tumor**
584 **volume normalized to initial volume as a function of time (B).** The control groups received
585 either the immunoconjugate without the radioligand (blue) or the radioligand without the
586 immunoconjugate (red). The three treatment groups received huA33-TCO (100 μg, 0.6 nmol)
587 followed 24 h later by either 18.5 (green), 37.0 (purple), or 55.5 (orange) MBq (~0.8 nmol in
588 each case) of [^{177}Lu]-DOTA-PEG$_7$-Tz. By log-rank (Mantel–Cox) test, survival was significant (p <
589 0.0001) for all treatment groups. This figure has been modified from reference #28. Reprinted
590 (adapted) with permission from Membreno, R., Cook, B. E., Fung, K., Lewis, J. S., & Zeglis, B. M.
591 Click-Mediated Pretargeted Radioimmunotherapy of Colorectal Carcinoma. Molecular
592 Pharmaceutics. **15** (4), 1729-1734 (2018). Copyright 2018 American Chemical Society.

593

594 **Figure 7. Weight curves for animals during the longitudinal therapy study of 5 groups of mice**
595 **(n = 10 each) bearing subcutaneous SW1222 tumors (A); the corresponding Kaplan-Meier**
596 **survival curve (B).** The control groups received either the immunoconjugate without the
597 radioligand (blue) or the radioligand without the immunoconjugate (red). The three treatment
598 groups received huA33-TCO (100 μg, 0.6 nmol) followed 24 h later by either 18.5 (green), 37.0
599 (purple), or 55.5 (orange) MBq (~0.8 nmol in each case) of [^{177}Lu]-DOTA-PEG$_7$-Tz. This figure has
600 been modified from reference #28. Reprinted (adapted) with permission from Membreno, R.,
601 Cook, B. E., Fung, K., Lewis, J. S., & Zeglis, B. M. Click-Mediated Pretargeted
602 Radioimmunotherapy of Colorectal Carcinoma. *Molecular Pharmaceutics*. **15** (4), 1729-1734
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604

605 **Table 1.** Characterization data for the organic compounds described in this protocol.

606

607 **DISCUSSION:**
608 One of the strengths of this approach to *in vivo* pretargeting — especially in relation to
609 strategies predicated on bispecific antibodies and radiolabeled haptens — is its modularity:
610 *trans*-cyclooctene moieties can be appended to any antibody, and tetrazine radioligands can be
611 radiolabeled with an extraordinary variety of radionuclides without impairing their ability to

612 react with their click partners. Yet the adaptation of this approach to other antibody/antigen
613 system is not as simple as duplicating the protocol described here. Of course, any attempt to
614 create a new mAb-TCO immunoconjugate or a novel tetrazine-bearing radioligand should be
615 accompanied by the appropriate chemical and biological characterization assays, including tests
616 for stability and reactivity. But beyond this, there are two variables that are particularly
617 important to explore and optimize: (1) the mass of mAb-TCO immunoconjugate administered
618 and (2) the interval time between the injection of the mAb-TCO and the administration of the
619 radioligand. Both factors can dramatically influence the *in vivo* behavior of the pretargeting
620 system. For example, the use of overly high doses of immunoconjugate or interval periods that
621 are too short can result in undesirably high activity concentrations in the blood due to click
622 reactions between the radioligand and immunoconjugate remaining in circulation. Conversely,
623 employing masses of immunoconjugate that are too low or overly long interval periods can
624 needlessly reduce activity concentrations in the tumor due to a failure to saturate the antigen
625 or the inexorable (though slow) isomerization of the *trans*-cyclooctene to inactive *cis*-
626 cyclooctene. Along these lines, performing biodistribution experiments using a range of masses
627 of immunoconjugate and pretargeting intervals can be extraordinarily helpful. Of course, it is
628 also recommended that appropriate controls be run in tandem with any *in vivo* experiments.
629 These include — but are not limited to — experiments featuring antigen-negative cell lines,
630 blocking cohorts that receive a vast excess of unconjugated antibody, the administration of the
631 radioligand alone, the injection of the radioligand following a TCO-*lacking* immunoconjugate,
632 and *in vivo* pretargeting using a non-specific, isotype control TCO-bearing immunoconjugate.
633 Alternatively, imaging experiments can be used for optimization if the therapeutic radionuclide
634 emits positrons or 'imageable' photons *or* if an 'imageable' isotopologue of the therapeutic
635 radionuclide is available. Ultimately, the sets of variables that provide the best balance of high
636 tumoral activity concentrations and high tumor-to-background activity concentration ratios
637 should be selected for subsequent longitudinal therapy studies. In the case presented here, 100
638 µg of huA33-TCO was injected with an interval of 24 h. Dosimetry calculations — particularly
639 those that allow for the calculation of tumor doses and therapeutic ratios — can also be helpful
640 during the process of optimization.
641
642 It is important to note that even the promising [^{177}Lu]Lu-DOTA-PEG$_7$-Tz/huA33-TCO system that
643 has been developed could benefit from additional optimization. A comparison between the
644 dosimetry data from this approach to PRIT and traditional RIT with a ^{177}Lu-labeled variant of
645 huA33 reveals that the tumor dose of PRIT lies below that of traditional RIT. Furthermore, the
646 effective dose of the PRIT system (0.054 mSv/MBq) is only slightly lower than that of traditional
647 RIT (0.068 mSv/MBq).
648
649 Two remedies to these issues are currently being explored. First, a dendritic scaffold has been
650 developed capable of increasing the number of TCOs appended to each antibody[30]. In the
651 context of pretargeted PET imaging, this approach dramatically boosts tumoral activity
652 concentrations, and analogous experiments with [^{177}Lu]Lu-DOTA-PEG$_7$-Tz are underway.
653 Second, the use of tetrazine-bearing clearing agents may be useful in the context of PRIT. The
654 administration of clearing agents prior to the injection of the radioligand has been exploited in
655 a variety of pretargeting methodologies as a way to decrease the concentration of residual

656   immunoconjugate in the blood and thus reduce activity concentrations in healthy organs[23,31].
657   The use of clearing agents is not without its drawbacks, though; the most notable of which is
658   increasing the complexity of an already admittedly complicated therapeutic modality.
659   Nonetheless, researchers at Memorial Sloan Kettering Cancer Center recently published a
660   compelling report on the creation a Tz-labeled dextran clearing agent for pretargeted PET
661   imaging, and data on the use of this construct in conjunction with [^{177}Lu]Lu-DOTA-PEG$_7$-Tz and
662   huA33-TCO are forthcoming[32]. Another approach to maximizing the dosimetric benefits of PRIT
663   is the use of radionuclides with shorter physical half-lives. This has proven highly effective for
664   imaging; however, therapeutic radionuclides with short physical half-lives are few and far
665   between.
666
667   Finally, we would be remiss if we failed to properly address some of the intrinsic limitations of
668   pretargeting based on the inverse electron demand Diels-Alder reaction. The first of these
669   problems is inherent to *all* approaches to *in vivo* pretargeting: the antibody employed cannot
670   be internalized upon binding to the target tissue. This, of course, is essential, as the antibody
671   must remain accessible to the radioligand rather than sequestered in an intracellular
672   compartment. This limitation is admittedly hard to circumvent, though it has been shown
673   recently that antibodies with slow-to-moderate rates of internalizing *can* be used for *in vivo*
674   pretargeting[33,34]. Second, the slow *in vivo* isomerization of reactive *trans*-cyclooctene to
675   inactive *cis*-cyclooctene has the potential to limit the length of the interval between the
676   administration of the TCO-bearing immunoconjugate and the injection of the radioligand.
677   Critically, intervals of up to 120 h have still provided excellent results in the context of both
678   pretargeted PET imaging *and* PRIT. However, the use of these longer intervals is almost always
679   accompanied by slight reductions in tumoral activity concentrations, a result which may stem
680   from this isomerization. In order to address this issue, several laboratories have attempted to
681   create more stable *trans*-cyclooctenes without compromising reactivity, while others have tried
682   to develop entirely new dienophiles capable of reacting with tetrazine[35]. In the coming years, it
683   is our hope that these chemical developments will be leveraged for PRIT.
684
685   In the end, PRIT based on the inverse electron demand Diels-Alder ligation is undeniably an
686   emergent and somewhat immature technology. However, we are nonetheless encouraged by
687   the preclinical results we have obtained and excited for the clinical promise of this strategy. We
688   sincerely hope that this protocol encourages others to explore and optimize this approach and
689   thus fuel its journey from the laboratory to the clinic.
690

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792

Figure 1

Figure 2

Carbonate Buffer
RT, pH 8.8-9.0, 1 h

Figure 3

Figure 4

A.

B.

Figure 5   Click here to access/download;Figure;Fig5 BioDs.tif ⬇

A.

Mass (g)

Days Elapsed

● huA33-TCO only control
■ Radioligand-only control (55.5 MBq)
● 18.7 MBq
● 37.0 MBq
● 55.5 MBq

B.

Survival Percentage

Days Elapsed

Table 1
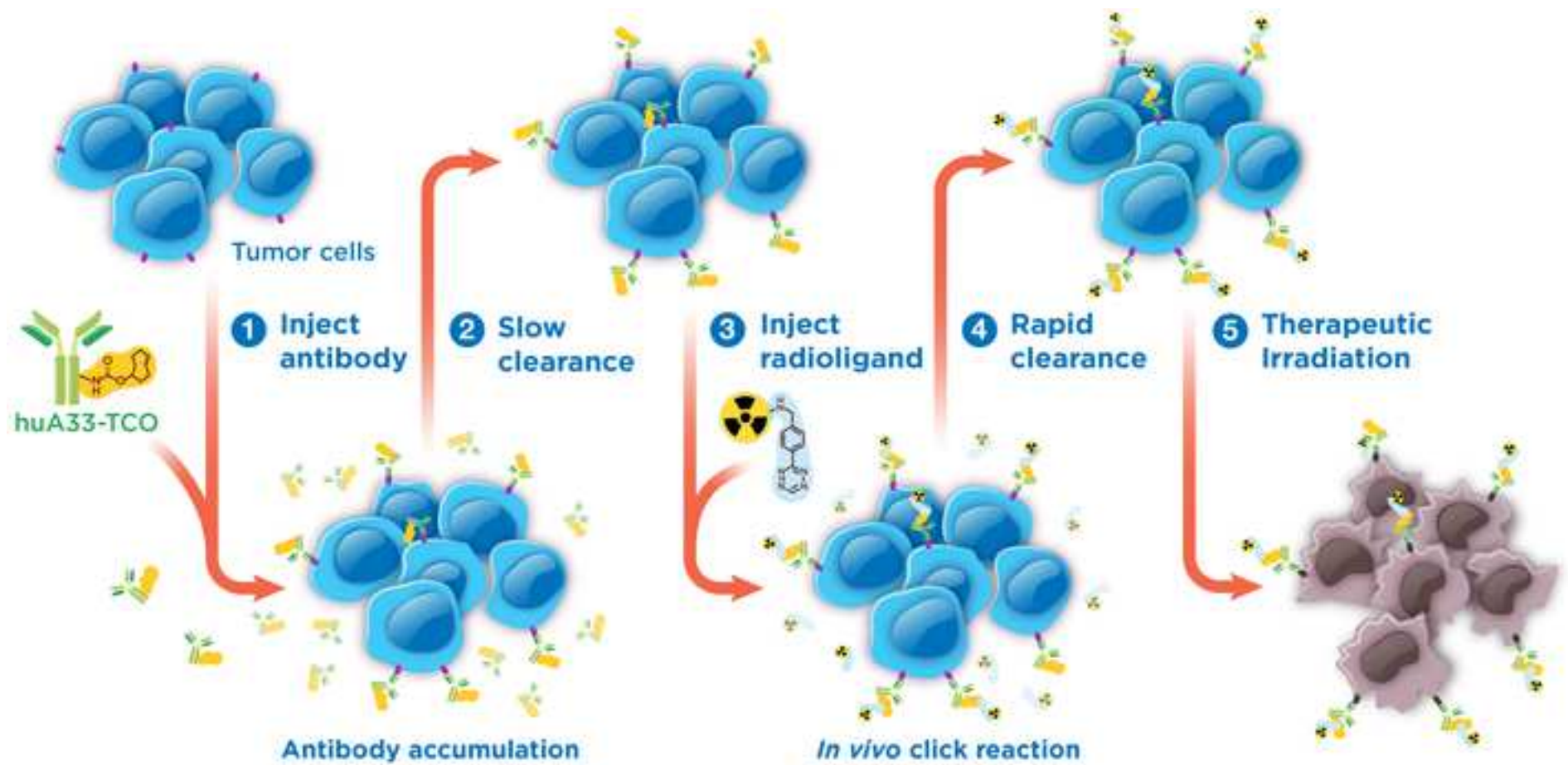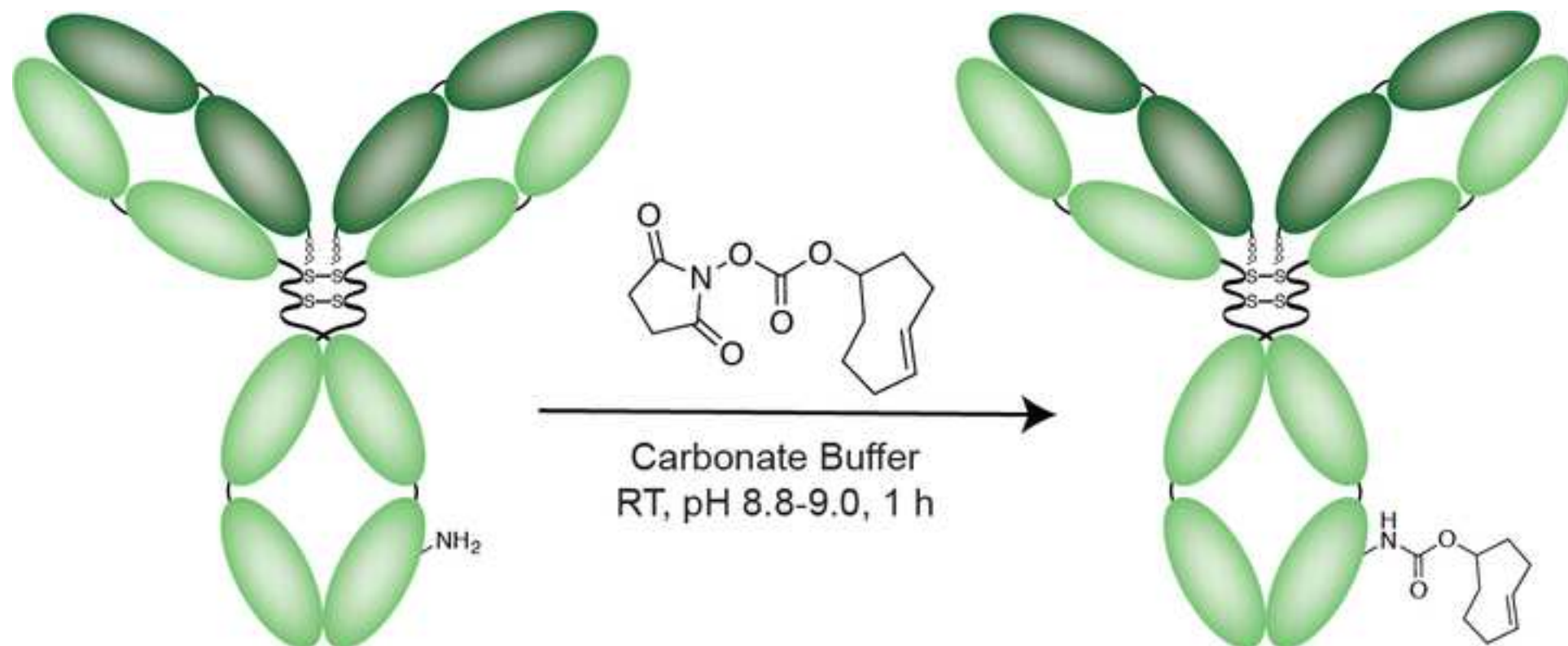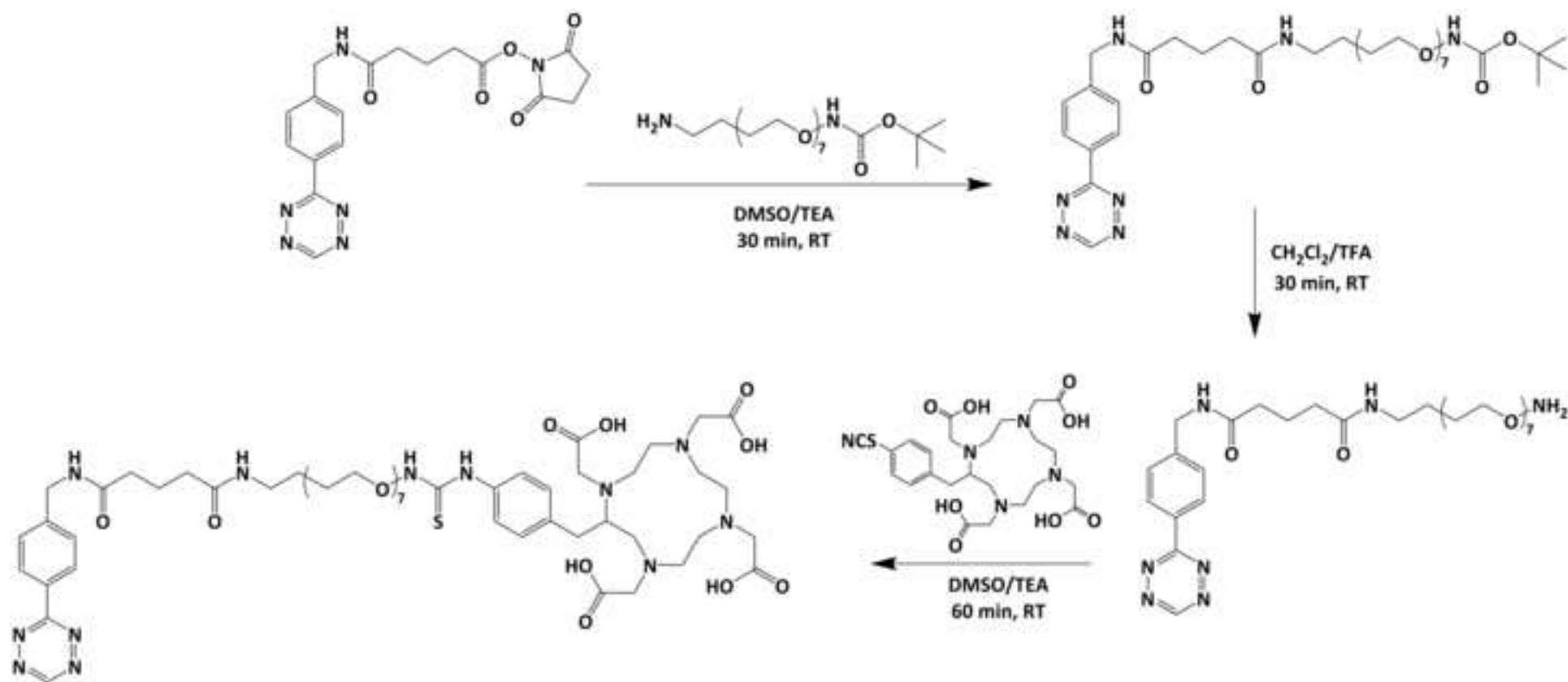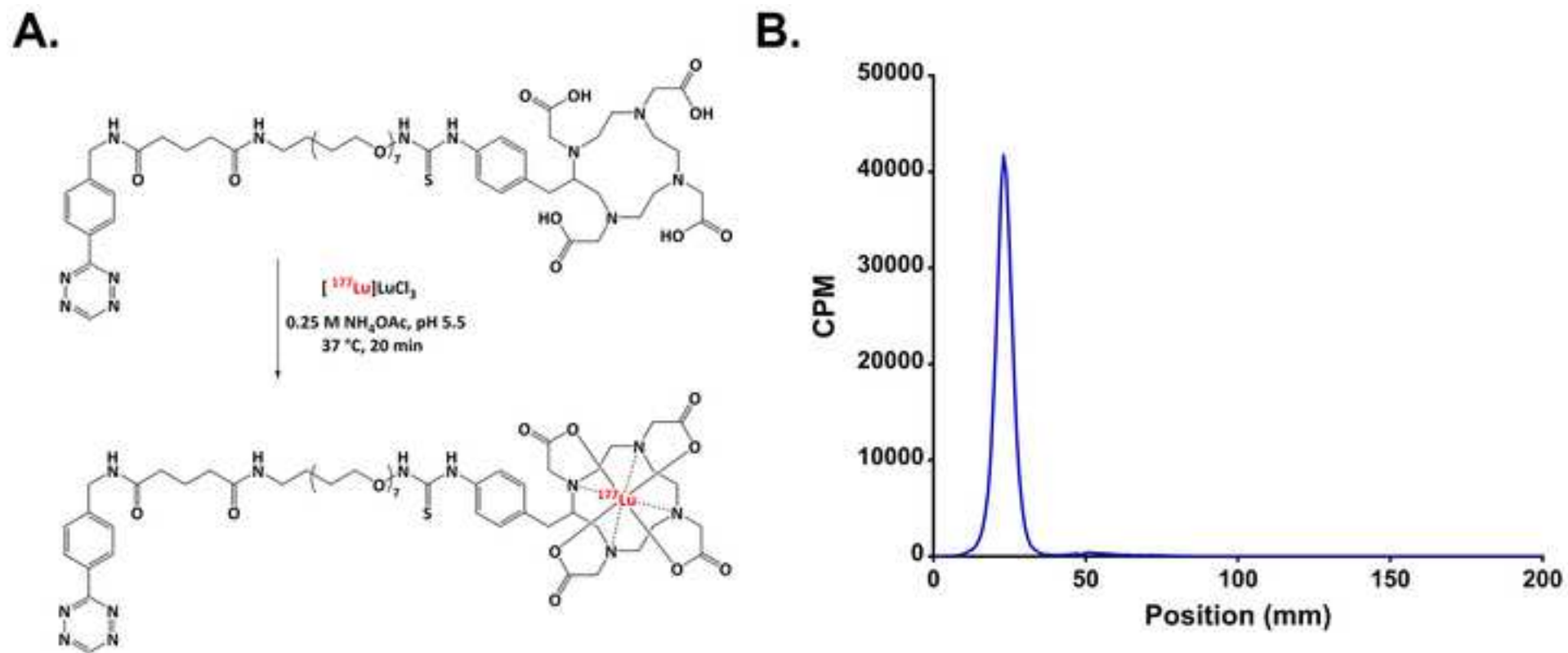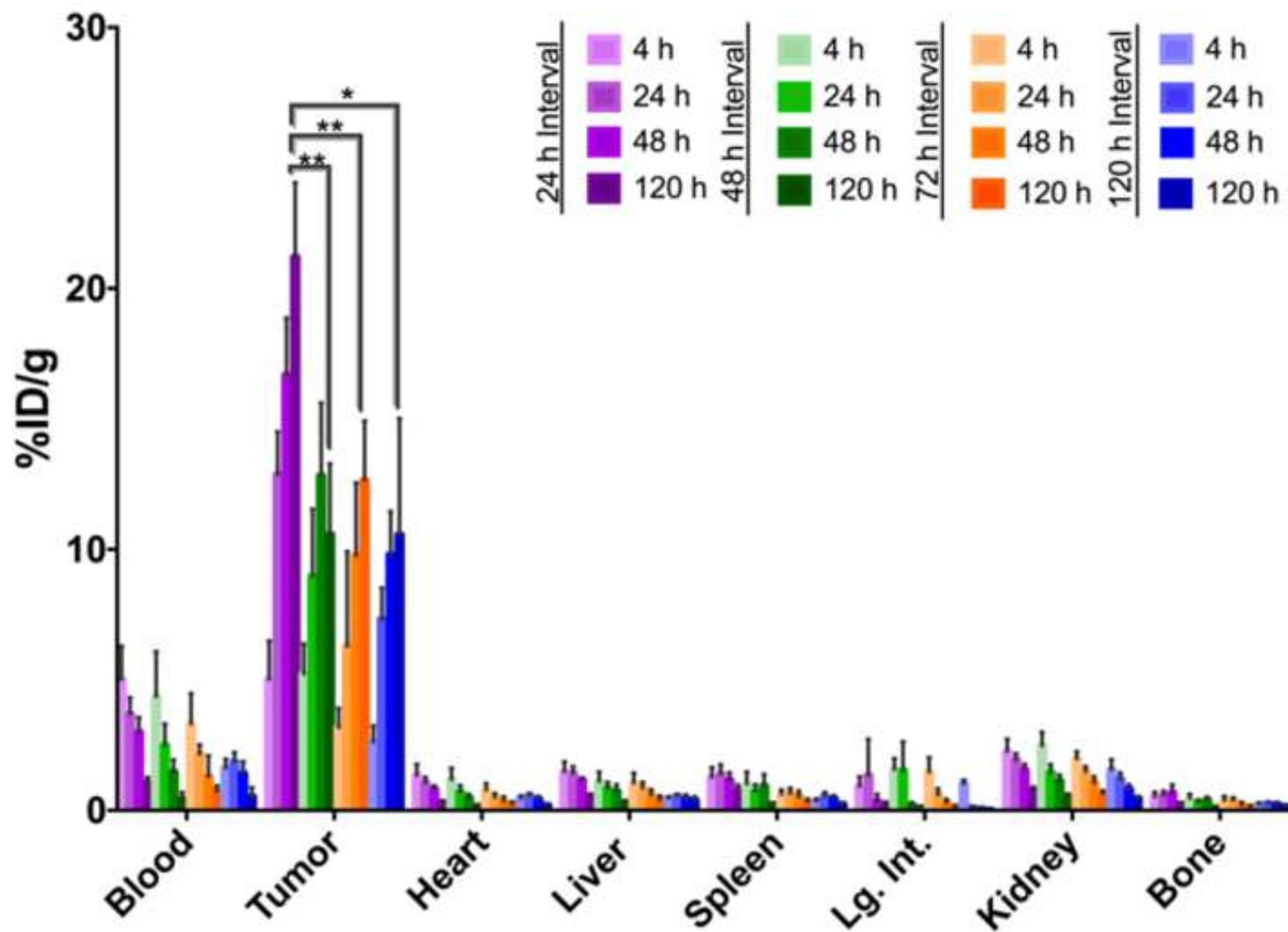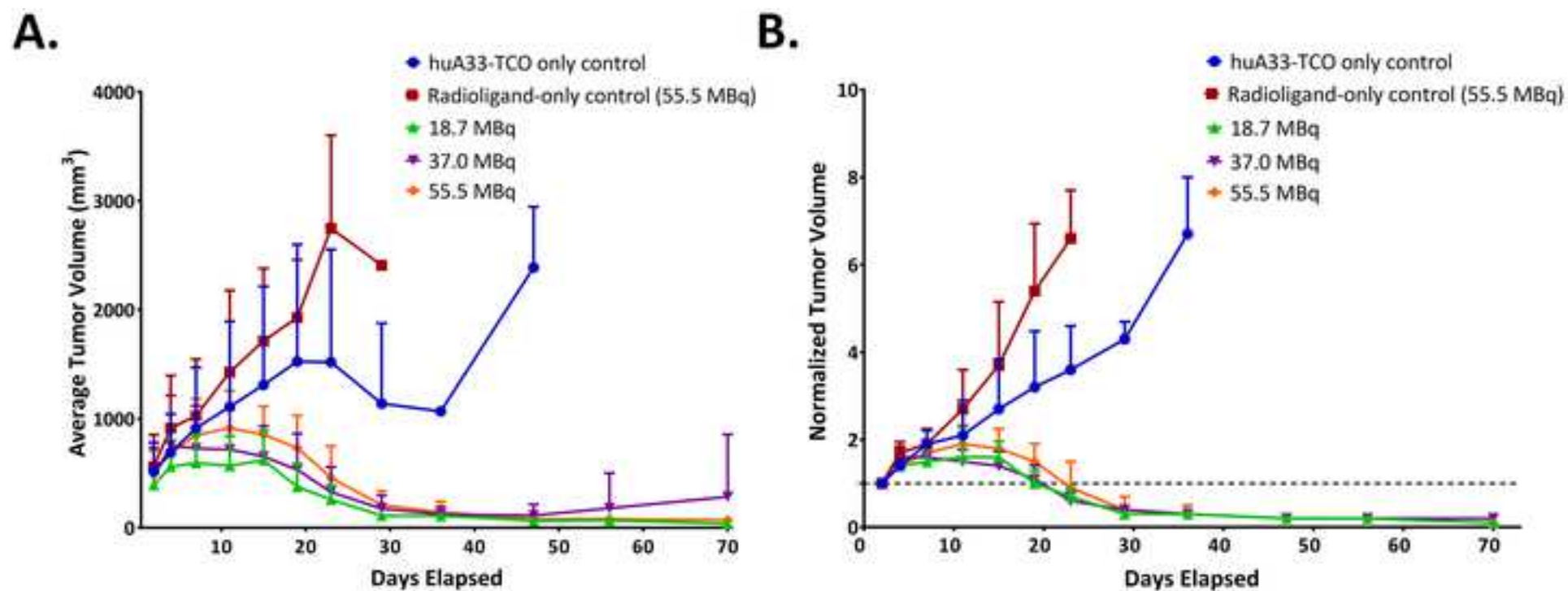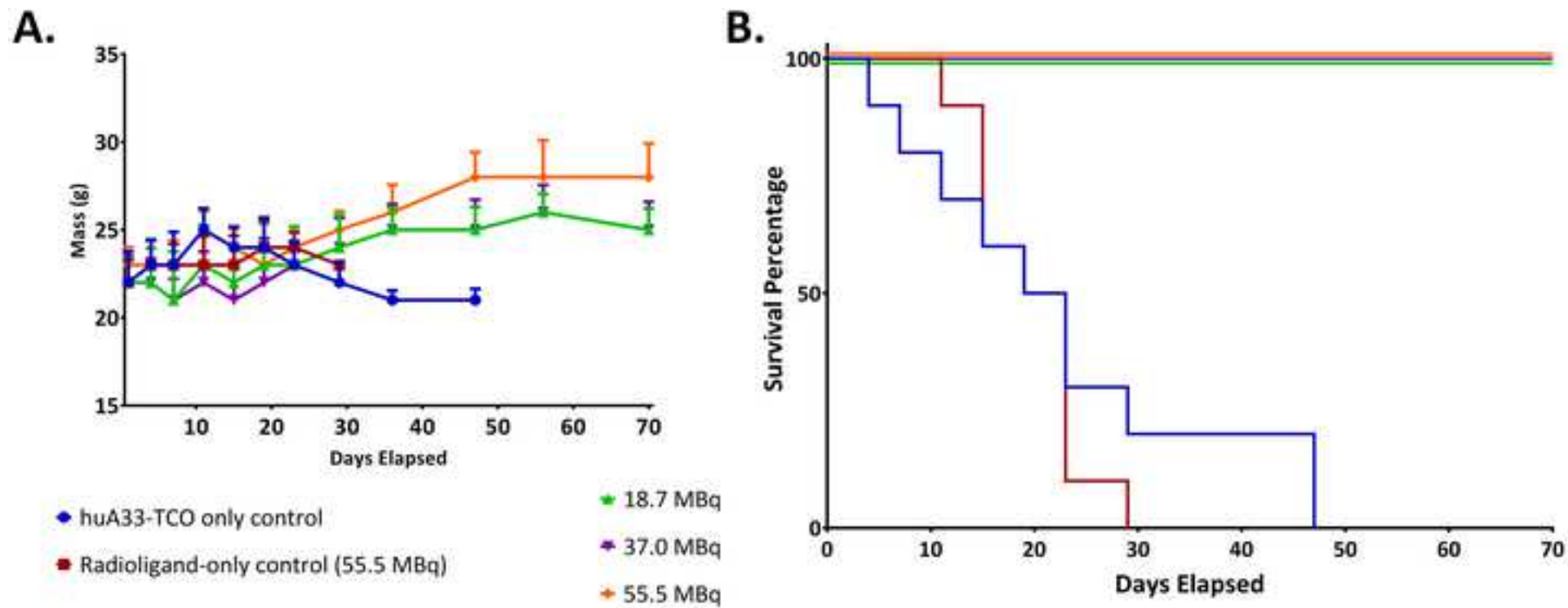
Compound	^{1}H-NMR Shifts 500 MHz, DMSO
Tz-PEG$_7$-NHBoc	10.52 (s, 1H), 8.50 (m, 3H), 7.82 (t, 1H), 7.46 (d, 2H), 6.69 (t, 1H), 4.33 (d, 2H), 3.42 (m, 22H), 3.33 (t, 2H), 3.31 (t, 2H), 3.12 (q, 2H), 2.99 (q, 2H), 2.12 (t, 2H), 2.03 (t, 2H), 2.12 (t, 2H), 1.70 (q, 2H), 1.29 (s, 9H)
Tz-PEG$_7$-NH$_2$	10.58 (s, 1H), 8.46 (m, 2H), 7.87 (t, 1H), 7.75 (d, 2H), 7.52 (d, 1H), 4.40 (d, 2H), 3.60-3.50 (m, 26H), 3.40 (t, 2H), 3.32 (bs, 2H), 3.20 (q, 2H), 2.99 (bs, 2H), 2.19 (t, 2H), 2.12 (t, 2H), 1.79 (q, 2H).
Tz-PEG$_7$-DOTA	10.57 (s, 1H), 9.63 (bs, 1H), 8.45 (m, 3H), 7.86 (m, 1H), 7.73 (bs, 1H), 7.54 (d, 2H), 7.41 (m, 2H), 7.19 (m, 2H), 6.54 (bs, 1H),

HRMS (ESI)

m/z calcd. for $C_{35}H_{57}N_7O_{11}Na$: 774.4005; found: 774.4014.

m/z calcd. for $C_{30}H_{50}N_7O_9$: 652.3670; found: 652.3676.

m/z calcd. for $C_{50}H_{76}N_{11}O_{15}S$: 1202.56; found: 1203.5741.

Table of Materials

Name	Company	Catalog Number
(E)-Cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS)	Sigma-Aldrich	764523
2,5-Dioxo-1-pyrrolidinyl 5-[4-(1,2,4,5-tetrazin-3-yl)benzylamino]-5-oxopentanoate (Tz-NHS)	Sigma-Aldrich	764701
Acetonitrile (MeCN)	Fisher Scientific	A998-4
Ammonium Acetate (NH4OAc)	Fisher Scientific	A639-500
Boc-PEG$_7$-amine (O-(2-Aminoethyl)-O'-[2-(Boc-amino)ethyl]hexaethylene glycol)	Sigma-Aldrich	70023
Dichloromethane (DCM)	Fisher Scientific	D143-1
Dimethyl sulfoxide (DMSO), anhydrous	Fisher Scientific	D12345
EMD Millipore Amicon Ultra-2 Centrifugal Filter Unit	Fisher Scientific	UFC205024
GE Healthcare Disposable PD-10 Desalting Columns	Fisher Scientific	45-000-148
N,N-Dimethylformamide (DMF), anhydrous	Fisher Scientific	AC610941000
Phosphate Buffered Saline (PBS)	Fisher Scientific	70-011-044
p-SCN-Bn-DOTA	Macrocyclics	B-205
Triethylamine (TEA)	Fisher Scientific	AC157911000
Trifluoroacetic Acid (TFA)	Fisher Scientific	A116-50
Tumor measuring device	Peira TM900	Peira TM900

Comments
Store at -80 °C
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Store at -20 °C
10x Concentrated
Store at -20 °C

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Author(s):	Rosemery Membreno and Brian Zeglis

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Signature: _B-3f_　Date: September 3, 2018

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October 16th, 2018

Bing Wu, Ph.D.
Review Editor, *JoVE*

Dear Dr. Wu,

Attached please find our revisions to the manuscript we have submitted to *JoVE* entitled "Pretargeted Radioimmunotherapy Based on the Inverse Electron Demand Diels-Alder Reaction" (JoVE59041). We would like to thank you and the reviewers for your consideration of our work and your careful review of the manuscript. We have made a number of critical modifications to the manuscript based on the critiques offered in the review. As requested, we have addressed these changes on a point-by-point basis. Below we have listed the comments of the Reviewers 1, 2, and 3 (in **bold**) along with our responses to said comments and the changes we have made (in normal text). For the sake of clarity, any addition or modification to the text in the manuscript are listed below in *purple italics*.

Editorial Comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have fully proofread the manuscript.

Please revise lines 50-52, 154-156, 192-194, 289-291, 315-318 to avoid previously published text.

These lines have now been revised.

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All of the figures in this manuscript are either original or have been modified from our own work that was recently published in *Molecular Pharmaceutics*. Therefore, according to the rules of the American Chemical Society, permissions are not required. However, we have added the appropriate citations for the original manuscript to each of the figure captions.

Please shorten the Summary to no more than 50 words.

The summary has been shortened, it now reads: *"This protocol describes the synthesis and characterization of a trans-cyclooctene (TCO)-modified antibody and a ^{177}Lu-labeled tetrazine (Tz) radioligand for pretargeted*

Please spell out each abbreviation the first time it is used.

We have corrected all instances of this error.

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All commercial language has been removed from the manuscript.

Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns where appropriate, but we have kept personal pronouns in places in which we think their use improves the pedagogical framework of the article. Please do not edit further.

Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have made the necessary revisions.

Please specify the volume of this solution to be prepared.

We have added the necessary information.

Does the antibody solution refer to the huA33 solution? Please specify.

We have added the necessary information.

What temperature is set during rotary evaporation?

We have added the necessary information.

Please specify the amount of [^{177}Lu]LuCl$_3$ added.

We have added the necessary information.

How to confirm that tumors are of sufficient size?

We have added the necessary information.

Please specify the specific step where the huA33-TCO solution is prepared, as Protocol Section 1 has many steps.

We have added the necessary information.

Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."

We have added the necessary information.

Please specify the dose of radioligand.

We have added the necessary information.

After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The appropriate sections have now been highlighted.

Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

These directions have been followed.

Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

These directions have been followed.

As Table 1 shows NMR and MS assignments, please indicate in the protocol that the organic compounds are characterized with NMR and MS.

This has been added.

Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

This has been done where appropriate.

Please reference Figure 3 and Figure 4 in the manuscript.

These references have been added

Figure 3: Please change "30 m" to "30 min" and "60 m" to "60 min" if the "m" is intended for time unit.

These changes have been made.

Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text. Please remove the embedded table(s) from the manuscript. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Please remove embedded Table of Materials from the manuscript. Please remove trademark (™) and registered (®) symbols.

All embedded tables and figures have been removed from the manuscript.

Reviewer #1:

The current manuscript by Membreno and Zeglis, builds on the extensive work published by their group and others on the in vivo pretargeting approach based on the inverse electron demand Diels-Alder reaction. This is a

high impact topic for the scientific community, with potential and imminent clinical applicability for the circumvention of the long residence times of radiolabeled antibodies. This is a very thorough methods article, which is well written, correctly organized and that describes the modification of the huA33 antibody with a TCO moiety, radiosynthesis of ^{177}Lu-labeled tetrazine and in vivo evaluation of this system. Only a minor revision is recommended.

We thank the reviewer for their thorough critique of our work.

Line 2: Although the title of this manuscript is very appealing, it seems generic also. There are several other pretargeted radioimmunotherapy approaches than the one described here by the authors. This is only one of them, using the IEDDA reaction. Please make the title more concise and informative.

This is an excellent point, we have added the phrase "*Based on the Inverse Electron Demand Diels-Alder Reaction*" to the title in order to be more specific and distinguish this approach from other pretargeting methodologies (i.e. streptavidin/biotin, oligonucleotides, bispecific antibodies, etc.).

Lines 78-80: To circumvent the problem of long residence times of the antibodies in the blood several strategies are presented. Recent findings using radiolabeled nanobodies emphasize their potential as therapeutic tools in cancer treatment. However, the authors fail to mention the nanobodies in their list of available alternative strategies.

This is a good point. We did not mean for list to be exhaustive. We have now added "*nanobodies*".

Line 102: The claim that "TCO is more stable in vivo than its Tz partner" should be rephrased. This is not always true. Physiologically stable radiolabeled tetrazines have been developed by different labs, including the one of Hannes Mikula in Vienna. Also, other authors have attempted the inverse strategy (Tz-modified antibody and a TCO-bearing radioligand) with success. Reformulate accordingly.

Great find! The wording of the section has now been modified to suggest that there are other methodologies to IEDDA pretargeting. We have elected not to include more discussion of these alternative tetrazine/TCO formulations, as it would be beyond scope of this protocol. That said, while there is certainly some interesting chemistry being evaluated, very little of it has been tested *in vivo*.

Line 150, 1.2: Were normal 1.7 mL microcentrifuge tubes used or low protein binding tubes? The latter could help minimize protein sample loss during sample manipulation.

For these experiments, we actually found that low protein binding tubes were not necessary for high-yield! The text has been modified accordingly.

Line 223, 2.6: For purification of the reaction, was the reaction diluted? Or was it injected directly on the HPLC?

Great catch! The reaction was diluted in water prior to purification. This change has been made to 2.5 and 4.5.

Line 230: I assume the mobile phase was used without any (acid) additive to prevent removal of the Boc protecting group. It would state the importance of this in the text, to be clear to the readers as well.

We had previously stated in Step 2.5 that the HPLC should be run without additive. However, we have updated the text to note that, "*Solvents without acid should be used to prevent the premature removal of the Boc protecting group*."

Line 234, 2.7: Could you provide an alternative to using liquid nitrogen to freeze the sample with before placing it in the lyophilizer? Some labs do not have access to it.

An excellent point. We have updated the protocol to include alternatives.

Lines 230-232 and 258-260: What was the HPLC flow rate used to get the retention times mentioned in the text? Please clarify.

We have clarified in both sections: a 1 mL/min flow rate is used.

Line 314, 5: For the radiolabeling of Tz-PEG$_7$-DOTA, is it important to work in metal-free conditions? For example, for the preparation of the ammonium acetate buffer.

Interestingly, across dozens of radiolabeling experiments, we have found that these procedures do not require metal-free buffers or solvents. At the beginning of Section 5, however, we have noted that this is a good practice!

Lines 330-332, 5.6: Please explain with more detail the procedure to alternatively purify the labeled [^{177}Lu]Lu-DOTA-PEG$_7$-Tz using a C$_{18}$ cartridge. The way it is written now is too much simplified.

Thank you for bringing this to our attention! We have now expanded this section to include more detail.

Line 343, 6.1.1: How do you identify the mice in the biodistribution experiment if they do not receive ear tags. You need to know which ones are euthanized at the different time points.

We have now updated this section to include our use of tail markings.

Lines 343-369: Sections 6.1.1 and 6.1.2 contain mostly the same information. The information could be mentioned in one section only, for both biodistribution and therapy studies.

We agree that the inclusion of tumor growth information was redundant, and this has been condensed accordingly. However, we have elected to keep sorting of animals separate for each, as the rigor needed for ensuring highly homogeneous tumor distributions for a therapy experiment is greater than that needed for a biodistribution experiment.

Lines 395, 396, <u>6.2.1.2</u>: Is this a critical step? Is it to prevent aggregation? Please clarify.

We have added the following clarification: "*NOTE: PBS treatment is necessary to prevent nonspecific binding of the antibody to the walls of the syringe.*"

Line 405, 6.2.2: This section is lacking the description of the use of controls. I can see in Figures 6 and 7 (huA33-TCO only and radioligand only controls) that you have used them. Therefore, these should also be mentioned in the text as well. Did you consider using a tumor which does not express A33 or only expresses it at low levels as negative control? It is not clear to me if the mAb is also injected in the biodistribution experiment. Please specify what is the injection sequence for the biodistribution experiment and the therapy study.

To address the reviewer's excellent points in order:

1. We have added the following passage following Line 768: "*It is also recommended that appropriate controls be run in tandem, including but not limited to the use of an antigen-negative cell lines, antigen blocking with an excess of unconjugated antibody, and the injection of isotype control immunoconjugates to determine non-specific accumulation at the tumor.*" We have also added emphasis to the use of key controls for the therapy study in Line 533.
2. Comparisons with an A33 negative cell line have been investigated in previously published work. Interestingly, comparing different cell lines in an experiment like this would be quite difficult, as they would likely have different growth rates, morphology, and responses to therapy. A blocking study could certainly be an option, but it would be much easier to run as a part of a biodistribution experiment.
3. The sequence of injections has now been addressed in Section 6.2 "*NOTE: The sequence of injections for both biodistribution experiments and therapy studies proceeds as follows: huA33-TCO is injected first, followed by [^{177}Lu]Lu-DOTA-PEG$_7$-Tz after a predetermined interval.*"

Line 431, 6.3.2: How and when is the blood collected? Is it using cardiac puncture? Is it before the mouse is euthanized? Please specify.

We have added "post-euthanasia" to remove any confusion. The exact method will be left to the reader based on institutional guidelines and group-specific methodologies.

A data analysis section is missing on the "PROTOCOL" section. Statistical analysis methods should also be specified.

While we have not added a data analysis section, we have included additional steps in the protocol (6.4.4 and 6.3.5) with suggestions on the presentation of data as well as basic statistical tests that can be utilized. In addition, the "Representative Results" should give readers an appropriate level of initial guidance in their analysis.

As ^{177}Lu can be used for SPECT imaging, together with biodistribution experiments, radiation dosimetry for this radiotracer could be determined. Why was this not performed?

Radiation dosimetry was calculated from biodistribution experiments and published in our *Molecular Pharmaceutics* manuscript! The data was not included in this protocol, as its calculation was deemed beyond the scope of this work.

Line 489: According to the latest nomenclature rules for radiotracers (cfr. Coenen et al., 2017. Nuclear Medicine and Biology. Consensus nomenclature rules for radiopharmaceutical chemistry — Setting the record straight.) instead of "specific activity", molar activity should be mentioned on the text since it is the measured radioactivity per mole of compound. Please reformulate this here and in the different parts of the text where "specific activity" is mentioned.

This is an excellent point. We have amended the manuscript accordingly.

Line 508: A small typo: "xenogarfts" should be xenografts instead.

Great catch! We have corrected this typo!

Table 1: The calculated m/z for Tz-PEG7-DOTA is missing two decimal digits.

Another great catch! We have corrected this typo, too!

Lines 795, 796: The use of the inverse strategy (Tz-modified antibody and a TCO-bearing radioligand) should also be discussed.

In this protocol, we have elected to only address the more established mAb-TCO/Tz-radioligand strategy. While the inverse strategy has shown some potential, the efficacy of PRIT based on this approach has yet to be demonstrated. Furthermore, we would never want to step on the toes of these authors if they were to want to publish a protocol in *JoVE*.

Reviewer #2:

This methods article describes in detail the concept for pretargeted radioimmunotherapy (PRIT) in a very elaborate way. They use the inverse electron demand Diels-Alder click reaction between a radiolabeled tetrazine (Tz) and a trans-cyclooctene (TCO) coupled to an antibody. The TCO-antibody is injected and allowed to accumulate at the target tissue (slow process) for 24 hours before the radiolabeled-Tz is administered. The Tz then clicks to the antibody (quick process) and excess radioactivity is cleared quickly. This approach minimizes radiation to healthy tissue and increase radioactivity concentration within the target region. The theoretical background is thoroughly described in the introduction that gives the reader a good overview followed by a

detailed protocol for how the technique should be executed and the results analyzed. I recommend to accept this article after minor revision.

We thank the reviewer for their kind words about our manuscript.

Line 147, page 5: The preparation of huA33-TCO: Please, specify which TCO is used or reference to "Table of materials".

Trans-cyclooctene NHS ester (TCO-NHS) is listed in the Table of Materials. We have added the commonly used abbreviation for clarification.

Some abbreviations should be added to the "Table of materials": For example, NHS, DMF, DMSO, MeCN or TEA. Please, check the whole manuscript.

This is a great point. We have added all these abbreviations to the Table of Materials!

Line 209, page 6 (2. The synthesis of Tz-PEG7-NHBoc): Please, specify what Tz is used.

The tetrazine used is specified in the Table of Materials. We have added the abbreviation for clarification.

Line 350, page 10 (6.1. Preparation of animals) "Once the tumors are of sufficient size". Please, indicate what this means. Are you referring to the size mentioned on 6.1.1.1 (100-150 mm2).

Good catch! We have updated this for the sake of clarity, referencing 6.1.1.1.

Lines 355-357 and 367-369, page 10 (6.1. Preparation of animals): These two paragraphs are repetitions of 6.1.1. Consider removing 6.1.1. or refer to it without repeating the text in these paragraphs.

We have consolidated these paragraphs to minimize repetitions.

Please, refer to GBq/mg when you use the term specific activity and GBq/μmol when molar activity is used. Recently, a Consensus nomenclature rules for radiopharmaceutical chemistry (2017) was published. Please, follow it.

We have updated the nomenclature to reflect "molar activity" rather than "specific activity".

Please, use in the whole manuscript GBq or MBq and not mCi (for example, line 321, page 9).

We have updated the manuscript accordingly.

Reviewer #3:

In this manuscript the authors described the construction of a TCO-modified immunoconjugate of the huA33 antibody, the synthesis of a ^{177}Lu-DOTA-labeled Tz radioligand, and the performance of in vivo biodistribution and therapy studies using this system in a murine model of colorectal carcinoma. The manuscript was comprehensive in the experiment procedures and provided adequate results. There are some minor revisions needed and are listed below:

We thank the reviewer for their careful consideration of our work.

In the step 4.3, since DOTA is not easily dissolved in organic solvent, should it be dissolved in DMSO first before adding to the reaction? Or is there a volume/concentration requirement for the reaction?

This is an interesting point! We have found that pre-dissolving the DOTA in DMSO prior to its addition to the reaction mixture results in the formation of additional byproducts that are not found when the solid is directly dissolved as stated in the protocol. While vortexing is required, the DOTA does fully enter solution, ultimately leading to a cleaner product and higher yield. We have added a note that this vortexing may take a couple of minutes.

In the step 5.3, how much of the Tz-PEG-DOTA is needed for each mCi of ^{177}Lu?

Our mistake! A reference has been added directing readers to Step 6.2.2.2, where more detail is given on the formulation of doses.

The breakthrough of functionalized TCO was made by Joseph Fox's group in 2008. The references should be cited.

Great catch! This reference slipped through the cracks. A proper citation has now been included: reference 26.

Thank you for your time, work, and consideration. Please let us know if you require any more information.

Respectfully,

Brian M. Zeglis, Ph.D.