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# Inverse electron demand Diels–Alder click chemistry for pretargeted PET imaging and radioimmunotherapy

Samantha M. Sarrett[1,2,8], Outi Keinänen[1,3,4,8], Eric J. Dayts[1], Guillaume Dewaele-Le Roi[1,3,5], Cindy Rodriguez[1,5], Kathryn E. Carnazza[6] and Brian M. Zeglis[1,2,3,5,7]✉

**Radiolabeled antibodies have shown promise as tools for both the nuclear imaging and endoradiotherapy of cancer, but the protracted circulation time of radioimmunoconjugates can lead to high radiation doses to healthy tissues. To circumvent this issue, we have developed an approach to positron emission tomography (PET) imaging and radioimmunotherapy (RIT) predicated on radiolabeling the antibody after it has reached its target within the body. This in vivo pretargeting strategy is based on the rapid and bio-orthogonal inverse electron demand Diels–Alder reaction between tetrazine (Tz) and *trans*-cyclooctene (TCO). Pretargeted PET imaging and RIT using TCO-modified antibodies in conjunction with Tz-bearing radioligands produce high activity concentrations in target tissues as well as reduced radiation doses to healthy organs compared to directly labeled radioimmunoconjugates. Herein, we describe how to prepare a TCO-modified antibody (humanized A33-TCO) as well as how to synthesize two Tz-bearing radioligands: one labeled with the positron-emitting radiometal copper-64 ([$^{64}$Cu]Cu-SarAr-Tz) and one labeled with the β-emitting radiolanthanide lutetium-177 ([$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz). We also provide a detailed description of pretargeted PET and pretargeted RIT experiments in a murine model of human colorectal carcinoma. Proper training in both radiation safety and the handling of laboratory mice is required for the successful execution of this protocol.**

## Introduction

### Background

Over the past two decades, radioimmunoconjugates have become increasingly important diagnostic and therapeutic tools in oncology. The ability of monoclonal antibodies (mAbs) to target tumor-associated antigens with high affinity and specificity has long made them effective vectors for the delivery of radionuclides to malignant tissue. The protracted circulation time of full-length immunoglobulins—i.e., they can take several days to reach their optimal biodistribution in the body—means that they must be labeled with radionuclides with multiday physical half-lives. Zirconium-89 ($^{89}$Zr; $t_{1/2}$: ~3.3 d) and iodine-124 ($^{124}$I; $t_{1/2}$: ~4.2 d) are typically used for positron emission tomography (PET), indium-111 ($^{111}$In; $t_{1/2}$: ~2.8 d) is the current gold standard for single-photon emission computed tomography and lutetium-177 ($^{177}$Lu; $t_{1/2}$: ~6.7 d), iodine-131 ($^{131}$I; $t_{1/2}$: ~8.0 d) and actinium-225 ($^{225}$Ac; $t_{1/2}$: ~10.0 d) are commonly used for radioimmunotherapy (RIT)[1–5]. Several antibody-based radiopharmaceuticals have emerged as clinical success stories, including [$^{131}$I]I-hu3F8 for the treatment of pediatric neuroblastoma and an ever-expanding array of [$^{89}$Zr]Zr-labeled antibodies for diagnostic and theranostic PET[6–8]. However, the unavoidable combination of long biological and physical half-lives can create high radiation dose rates to healthy tissues, a complication that has dampened enthusiasm for radioimmunoconjugates in the clinic.

Perhaps not surprisingly, several alternative approaches have been created in an attempt to circumvent this issue. For example, antibody fragments (e.g., F(ab)$_2$, Fab and single-domain antibodies) have attracted a great deal of attention as smaller format (~10–100 kDa) analogues of

[1]Department of Chemistry, Hunter College, City University of New York, New York, NY, USA. [2]PhD Program in Biochemistry, Graduate Center of the City University of New York, New York, NY, USA. [3]Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [4]Department of Chemistry, Radiochemistry, University of Helsinki, Helsinki, Finland. [5]Ph.D. Program in Chemistry, Graduate Center of the City University of New York, New York, NY, USA. [6]Brain and Mind Research Institute & Appel Institute for Alzheimer's Disease Research, Weill Cornell Medical College, New York, NY, USA. [7]Department of Radiology, Weill Cornell Medical College, New York, NY, USA. [8]These authors contributed equally: Samantha M. Sarrett, Outi Keinänen. ✉e-mail: bz102@hunter.cuny.edu

full-length immunoglobulins (~150 kDa) with more rapid pharmacokinetic profiles[9]. Although radiolabeled fragments have shown promise in both the laboratory and the clinic, their shorter biological half-lives come at a not-insignificant price: lower tumor accretion and higher retention in the kidneys[10].

This protocol is focused on yet another alternative methodology that seeks to leverage all of the advantages of full-length immunoglobulins while skirting their pharmacokinetic—and thus dosimetric—drawbacks: in vivo pretargeting.

In vivo pretargeting is predicated on injecting the immunoglobulin and radionuclide separately and relying on a bio-orthogonal ligation to join two components together within the body. Labeling the antibody with a fast-moving, small molecule radioligand after it has reached an optimal biodistribution in vivo—or at least has had a head start—limits the circulation time of the assembled radioimmunoconjugate in the blood and enables the use of radionuclides with shorter half-lives that are normally incompatible with full-length IgG. As a result, this approach can produce high activity concentrations in target tissues alongside reduced radiation doses to healthy organs compared to traditional, directly radiolabeled immunoconjugates.

The central feature of any pretargeting strategy is the mechanism of the in vivo combination of the vector and radionuclide. A handful of different approaches have been used, including the ligation of streptavidin and biotin[11], the hybridization of complementary oligonucleotides[12] and the ability of bispecific antibodies to bind both antigens and radiolabeled haptens[13]. Although these strategies have produced promising preclinical and, in some cases, clinical results, each possesses significant intrinsic limitations (see 'Comparison with other methods'). As a result, the development of novel approaches to in vivo pretargeting remains an unmet need.

## Development of the protocol

Our laboratory has worked for almost a decade to develop and optimize an approach to in vivo pretargeting based on the inverse electron demand Diels–Alder (IEDDA) reaction between 1,2,4,5-tetrazine (Tz) and *trans*-cyclooctene (TCO) (Fig. 1a). We have not been the only ones working in this field, of course. Indeed, several excellent teams have also been instrumental in the advancement of IEDDA-based pretargeting technology, most notably Jason S. Lewis and his group at Memorial Sloan Kettering Cancer Center, Raffaela Rossin and Mark Robillard and their coworkers at Tagworks Pharmaceuticals and Matthias Herth and his laboratory at the University of Copenhagen. Rondon et al.[14] have recently published an excellent review summarizing the field's progress as a whole.

The IEDDA ligation is a catalyst-free click chemistry transformation whose rapidity ($k_2$ >30,000 $M^{-1}s^{-1}$) and bio-orthogonality make it nearly ideal for in vivo applications[15]. This singular chemical technology underpins two of the most important advantages of this approach to pretargeting: its modularity and its reliance, unique among pretargeting strategies, on the formation of covalent bonds between the vector and the radioligand. IEDDA-based approaches for in vivo pretargeting generally rely on two components—a TCO-modified mAb and a Tz-bearing radioligand—and four sequential steps: (i) the intravenous administration of the TCO-based immunoconjugate, (ii) an interval period during which the mAb-TCO accumulates within target tissue and clears from the blood, (iii) the intravenous administration of the radiolabeled tetrazine and (iv) the click ligation of the two components in vivo followed by the rapid clearance of excess radioligand (Fig. 1b). Over the years, however, several variations on this theme have emerged, including the use of clearing agents and the sequential administration of two different radioligands[16–18]. Nonetheless, this original approach remains the simplest, most robust and most well optimized.

Our initial foray into pretargeted PET imaging, which is predated in the literature by the pretargeted single-photon emission computed tomography work of Rossin et al., used a [$^{64}$Cu]Cu-NOTA-Tz radioligand and a TCO-modified variant of the colorectal cancer-targeting antibody humanized A33 (huA33-TCO)[19,20]. Although this system produced promising imaging and biodistribution data in murine models of human colorectal carcinoma, the hepatobiliary clearance of the radioligand would prove an obstacle to clinical imaging. This result fueled the development of a second-generation $^{64}$Cu-labeled radioligand, [$^{64}$Cu]Cu-SarAr-Tz, that combined rapid renal clearance and excellent in vivo performance in pretargeted PET and biodistribution experiments[21]. Around the same time, exhaustive structure-activity studies were performed to optimize $^{18}$F- and $^{68}$Ga-labeled Tzs that facilitated pretargeted PET using radionuclides with even shorter half-lives[22–24]. Although these efforts produced several highly effective radioligands and provided important lessons in molecular design, none surpassed the in vivo performance of [$^{64}$Cu]Cu-SarAr-Tz[25].

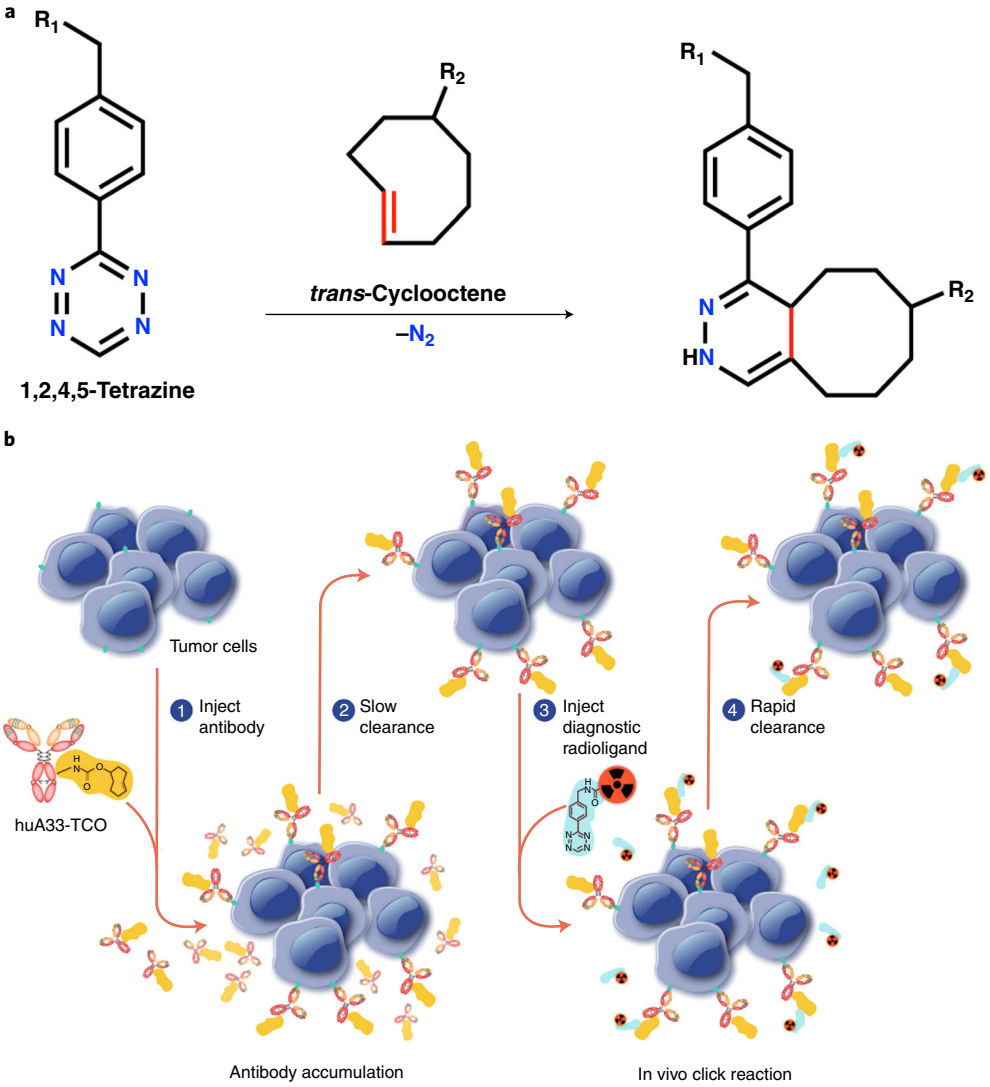


**Fig. 1 | In vivo pretargeting based on the IEDDA reaction. a**, The IEDDA ligation between Tz and TCO. **b**, Schematic of in vivo pretargeting based on the IEDDA reaction. huA33, humanized A33. Image reproduced with permission from ref. [26]. Copyright 2018 American Chemical Society.

Pretargeted RIT (PRIT) experiments soon followed after the development of the PET systems. To this end, a Tz radioligand labeled with the β-emitting radiometal $^{177}$Lu, [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz, was used in conjunction with a pair of TCO-bearing immunoconjugates in murine models of colorectal cancer (huA33-TCO) and pancreatic ductal adenocarcinoma (5B1-TCO)[24,26–28]. In both cases, biodistribution studies revealed high activity concentrations in tumor tissue along with low levels of uptake in healthy organs. Furthermore, longitudinal therapy studies subsequently revealed the efficacy of $^{177}$Lu-PRIT, illustrating that this strategy produces a dose-dependent therapeutic response in both murine models of disease. We have recently extended our exploration of IEDDA-based PRIT, with forays into radioligands bearing α-emitting radionuclides (i.e., [$^{225}$Ac]Ac-DOTA-PEG$_7$-Tz), TCO-modified dendrimers designed to amplify tumoral activity concentration levels and dual radionuclide systems that integrate PRIT with theranostic PET[16,17,27,29]. We are far from the only ones innovating in this space, however. Various laboratories—including, but not limited to, those mentioned previously—have produced ground-breaking work, including studies focused on alternative dienophile moieties, Tz-bearing masking agents, antibody fragment-based pretargeting, TCO-modified bone-seeking vectors and nanoparticulate radioligands[30–34].

We previously described our approach to in vivo pretargeting in a protocol in the *Journal of Visualized Experiments* that discussed PRIT with huA33-TCO and [$^{177}$Lu]Lu-PEG$_7$-DOTA[28].

**Box 1 | Preparation of Tz-PEG$_7$-AF680 and determination of TCO occupancy of huA33-TCO**

**Additional reagent**

- NHS-AF680 (Thermo Fisher Scientific, cat. no. A37567)

**Procedure**

**Synthesis of Tz-PEG$_7$-AF680** ● Timing ~1 d

1 In a 1.5-ml microcentrifuge tube, dissolve 1.0 mg of Tz-PEG$_7$-NH$_2$ (Procedure 1) (0.0015 mmol) in 400 µl of DMSO.
**! CAUTION** DMSO is a flammable liquid and may cause slight skin and eye irritation.
2 Add 2.0 mg of NHS-AF680 (0.0021 mmol, 1.4 eq.) to the solution and mix thoroughly.
3 Add 10.0 µl of TEA (7.3 mg, 0.072 mmol) to the solution and mix thoroughly.
**! CAUTION** TEA is a highly flammable liquid and has high oral, dermal and respiratory toxicity.
4 Place the solution on a dry block heating mixer and agitate at 300 r.p.m at 25 ºC for 30 min.
5 Take a small aliquot from the reaction mixture (~20 µl), perform a twofold dilution with acetonitrile and check the reaction via preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 7 ml/min ($t_{R,product}$ = ~17 min; potential impurities: $t_{R,Tz\text{-}PEG7\text{-}NH2}$ = ~14 min). If the reaction is deemed to be complete, filter the rest of the reaction mixture and continue on with HPLC purification. If the reaction is not complete, allow the reaction to incubate for longer or repeat steps 1–4. If the purification is unable to be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.
6 Purify the samples via preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 7 ml/min ($t_{R,product}$ = ~17 min; potential impurities: $t_{R,Tz\text{-}PEG7\text{-}NH2}$ = ~14 min). Collect the purified fractions (~3 ml per fraction, from 16.5 to 17.5 min, in a total of two to three fractions per run) in 3.5-ml glass collection tubes and combine them in a 50-ml conical centrifuge tube.
**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.
7 Lyophilize the HPLC eluant to yield Tz-PEG$_7$-AF680 as a deep blue powder (typical results: 2.0 mg; 0.0013 mmol; 79% yield).
**PAUSE POINT** The purified Tz-PEG$_7$-AF680 can be stored as a solid at −20 ºC for ~6 months. Make sure to protect the sample from light by wrapping the container in aluminum foil.

**Determination of the TCO occupancy of huA33-TCO** ● Timing ~1 d

Performing this assay once per prepared batch of huA33-TCO is sufficient.
8 Dilute an aliquot of the huA33-TCO solution (50.0 µg; 0.33 nmol) to 0.3 ml with 1× PBS (pH 7.4) in a 1.5-ml microcentrifuge tube (1.1 µM).
9 Add 10.0 µl of a 0.5 mM solution of Tz-PEG$_7$-AF680 in DMSO.
**! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.
10 Place the solution on a dry block heating mixer and agitate it at 500 r.p.m at 25 °C for 24 h.
11 Purify the reaction with a PD-10 desalting column and an Amicon Ultra-4 centrifugal filter.
(A) Equilibrate the PD-10 desalting column (see 'Equipment setup').
(B) Add the reaction mixture (0.3 ml) to the PD-10 desalting column and allow the solution to enter the packed bed completely.
(C) Discard the flow-through.
(D) Add 2.2 ml of 1× PBS (pH 7.4) to each column and allow the solution to enter the packed bed completely.
(E) Discard the flow-through.
(F) Elute the column with 2.0 ml of 1× PBS (pH 7.4) into an Amicon Ultra-4 centrifugal filter.
(G) Centrifuge the Amicon Ultra-4 centrifugal filters at 3,500*g* for 15 min and discard the flow-through.
(H) Transfer the remaining solution (~0.1 ml) from the Amicon Ultra-4 centrifugal filters into a microcentrifuge tube and vortex gently. Wash out the Amicon Ultra-4 centrifugal filter by using 0.2 ml of 1× PBS (pH 7.4) and combine this with the antibody solution in the microcentrifuge tube, bringing the total volume up to ~0.3 ml.
12 Measure the absorbance at 280 and 680 nm.
13 Calculate the degree of labeling by using the absorbances of the antibody at 280 and 680 nm and the equation below. Alternatively, the BioSpec Nano UV-visible spectrophotometer can automatically report the DOL.

$$A_{\mathrm{mAb}} = A_{280} - -A_{\max}(\mathrm{CF})$$
$$\mathrm{DOL} = [A_{\max} \times \mathrm{MW}_{\mathrm{mAb}}]/[[\mathrm{mAb}] \times \varepsilon_{\mathrm{AF680}}]$$

where the correction factor (CF) for AF680 was given as 0.05 by the supplier, $\mathrm{MW}_{\mathrm{huA33}}$ = 150,000 Da, $\varepsilon_{\mathrm{AF680}}$ = 82,030 $\mathrm{cm}^{-1}\mathrm{M}^{-1}$ and $\varepsilon_{280,\mathrm{huA33}}$ = 210,000 $\mathrm{cm}^{-1}\mathrm{M}^{-1}$.

This protocol expands on this earlier work by describing the synthesis of radioligands for both pretargeted PET ([$^{64}$Cu]Cu-SarArTz) and RIT ([$^{177}$Lu]Lu-PEG$_7$-DOTA) and by providing detailed descriptions of in vivo imaging, biodistribution and longitudinal therapy studies. In addition, we also provide a method for determining the TCO occupancy of immunoconjugates by using Tz-PEG$_7$-AF680 (Box 1) as well as procedures for cell culture and the implantation of xenografts (Supplementary Methods).

## Comparison with other methods

The IEDDA reaction is not the first ligation to be harnessed for in vivo pretargeting. Several different 'molecular couples' have been used over the years, each with its own distinct set of advantages and

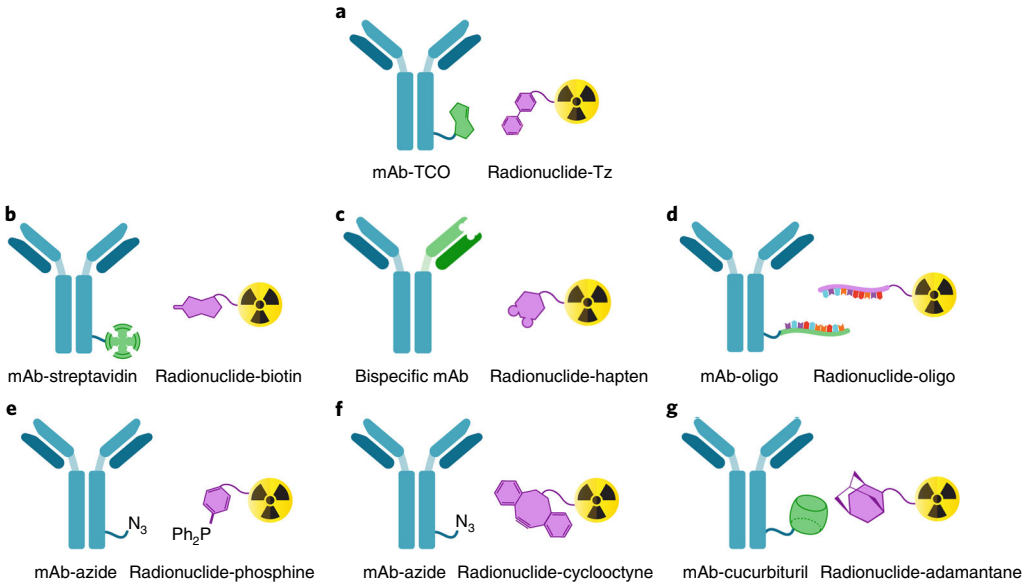


**Fig. 2 | Several ligation mechanisms have been leveraged to facilitate in vivo pretargeting. a**, The IEDDA reaction. **b**, The interaction between streptavidin and biotin. **c**, The ability of bispecific antibodies to bind both a cancer antigen and a hapten. **d**, The hybridization of complementary oligonucleotides. **e**, The Staudinger ligation. **f**, The strain-promoted azide-alkyne click reaction. **g**, The host–guest relationship between adamantane and cucurbituril.

disadvantages (Fig. 2). The earliest approaches to pretargeting relied on the extraordinarily strong binding interaction between streptavidin (a protein) and biotin (a small molecule). Indeed, strategies were developed that used biotin-bearing immunoconjugates and streptavidin-based radioligands as well as streptavidin-bearing immunoconjugates and biotin-based radioligands[35]. These methodologies produced excellent preclinical results and promising data in early trials. However, clinical trials revealed that many patients experienced mild to severe immune responses related to the streptavidin moieties[36,37]. This immunogenicity ultimately proved a fatal flaw for the approach that has not been overcome in nearly two decades.

Another, arguably more elegant strategy for in vivo pretargeting is predicated on the use of bispecific antibodies that can bind to both tumor antigens and radiolabeled haptens. Methods using both chelator- and peptide-based haptens have been explored, with both producing excellent preclinical results[38–40]. Even more importantly, in vivo pretargeting using bispecific antibodies and radiolabeled variants of IMP288, an oligopeptide hapten, has produced extremely promising clinical results in recent years[41]. IMP288 is a histamine-succinyl-glycine peptide with two important advantages: modularity—it can be modified with different chelators and radionuclides without dramatically affecting its binding to the bispecific antibody—and a pharmacokinetic profile that boasts rapid clearance from the blood and low levels of uptake in the kidneys[42,43]. Yet despite this success, the construction of bispecific antibodies is complex and expensive, factors that dramatically reduce the modularity of this approach and can represent significant barriers to laboratories interested in exploring pretargeting.

The third major approach to pretargeting relies on the hybridization of complementary oligonucleotide chains for the in vivo ligation of the two components. This strategy has used both antibodies and affibody molecules as targeting vectors and has typically relied on phosphorodiamidate morpholino oligomers and peptide nucleic acids rather than natural nucleic acids[12,44]. Unlike natural DNA and RNA, phosphorodiamidate morpholino oligomers and peptide nucleic acids are not prone to degradation via nucleases, giving them significantly increased in vivo stability[45]. Like IEDDA-based methods, oligonucleotide-based pretargeting has not yet reached the clinic, but it has produced promising preclinical results in murine models of skin and ovarian cancer. The clinical translation of this approach is anticipated in the near future, yet the inherent instability of oligonucleotides and the non-covalent nature of hybridization remain concerns going forward. Creating a covalent link between the antibody and radioligand will always be the ideal, because this

removes the possibility of the dissociation of the radiolabeled hapten and its subsequent washout from the tumor.

Finally, a handful of publications have emerged in which other in vivo ligation mechanisms have been interrogated over the years, including the Staudinger reaction, the strain-promoted azide-alkyne cycloaddition and the host–guest relationship between cucurbituril and adamantane[46–49]. These methods, however, have either proven ineffective in vivo or have not been the subject of a large enough body of data to merit further discussion here.

## Limitations of the IEDDA approach

It is important to carefully examine the drawbacks associated with IEDDA-based pretargeting as well. To begin, IEDDA-based pretargeting inevitably shares the issues common to all approaches to in vivo pretargeting. For example, in vivo pretargeting is most effective when the TCO-modified vector, usually a monoclonal antibody, is not internalized upon binding its molecular target. In this protocol, we use huA33, a humanized mAb that targets the A33 antigen, a transmembrane glycoprotein that is expressed on >95% of colorectal carcinomas, and has been shown to remain surface persistent after binding its molecular target[50]. Although various non-internalizing antibodies have been discovered, many are internalized upon binding their targets, a process that (understandably) can make pretargeting more difficult. That said, our laboratory and others have demonstrated that in vivo pretargeting can be successful by using antibodies that are internalized at slow or moderate rates (e.g., the CA19.9-targeting mAb 5B1, ~40% of which has been shown to internalize in BxPC3 cells in 24 h)[51,52].

In addition, the intrinsic complexity of in vivo pretargeting is often held up as a potential impediment to its clinical viability. Typically, these worries are not centered around the efficacy of the methodology in humans but rather the increased logistical burden associated with translating multicomponent systems (i.e., performing toxicology studies on and garnering regulatory approval for not one but two parts of a system). To some degree, these concerns are valid and inevitable; it is our hope, however, that these issues will become less daunting and provoke fewer knee-jerk reactions as more clinical trials emerge in the future.

The principal drawback unique to IEDDA-based pretargeting is the in vivo isomerization of TCO to *cis*-cyclooctene (CCO). This transformation of reactive TCO to inert CCO reduces the number of reaction partners for the Tz radioligand, thereby decreasing the frequency of in vivo ligations and limiting the accumulation of radioactivity in target tissues. Pioneering work by Rossin et al. determined that the in vivo stability half-life of TCO can range from ~4 to ~10 d depending on the structure of the moiety[53]. Although the exact mechanism of this interconversion remains unknown, it is hypothesized that it stems from the interaction between TCO and circulating transition metals or, more likely, circulating transition metal–containing proteins[54]. Various attempts have been made to address this issue. One study, for example, has suggested that reducing the length of the linker between the vector and the TCO can increase the half-life of the latter[55]. Others have attempted to circumvent this problem by moving the Tz to the vector and using TCO-based radioligands, although this approach is somewhat suspect because Tz is even less stable in vivo than TCO[56–58]. In the absence of a clear mitigation strategy—and considering that some instability may be inevitable as part of the trade-off for rapid reactivity—the best 'insurance' against isomerization may simply be to use immunoconjugates with degrees of labeling of TCO as high as possible without compromising the biochemical integrity of the vector. In our hands, a range of two to five TCOs per antibody have been shown to facilitate successful in vivo experiments without impairing the immunroeactivity of the antibody. To be sure, as IEDDA-based pretargeting enters the clinic, it will be important to interrogate the degree to which TCO isomerization occurs in human patients.

## Experimental design

Herein, we first describe the synthesis and characterization of three components of our IEDDA-based approach to pretargeting: a pair of radioligands, [$^{64}$Cu]Cu-SarAr-Tz (Fig. 3) and [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz (Fig. 4), and a single immunoconjugate, huA33-TCO (Fig. 5). We then provide a detailed description of how to perform pretargeted PET imaging and PRIT experiments in athymic nude mice bearing subcutaneous SW1222 colorectal carcinoma xenografts. We selected these radioligands, this immunoconjugate and this tumor model for the protocol at hand because they represent the best characterized and most effective systems that we have developed to date. Yet, one of the greatest strengths of IEDDA-based pretargeting is the ease with which it can be adapted to new applications.

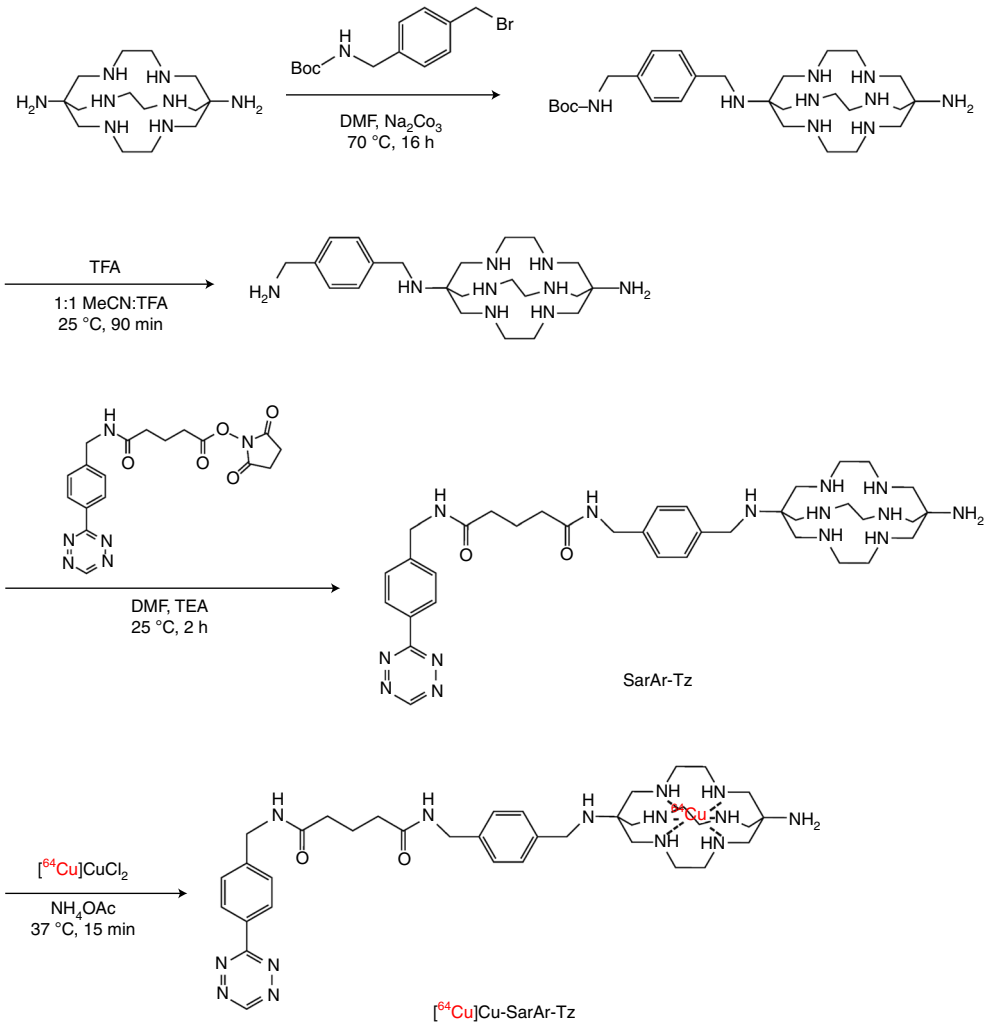


**Fig. 3 |** The synthesis of [$^{64}$Cu]Cu-SarAr-Tz. Boc, *tert*-butoxycarbonyl; DMF, *N,N*-dimethylformamide; MeCN, acetonitrile; TEA, triethylamine; TFA, trifluoroacetic acid.

Tz-bearing radioligands have been synthesized by using a range of nuclides, including $^{11}$C, $^{68}$Ga, $^{18}$F, $^{64}$Cu, $^{111}$In, $^{44}$Sc, $^{99m}$Tc and $^{225}$Ac[27,59–65]. Similarly, various vectors, including antibodies, antibody fragments, nanoparticles, peptides and small molecules, have been modified with TCO[34,61,66,67]. Of course, the mass of the TCO-bearing construct injected as well as the interval time between the administrations must be optimized for each vector (*vide infra*). Readers interested in attempting pretargeted PET or PRIT with a TCO-modified IgG are encouraged to use the optimized bioconjugation methods and $^{64}$Cu- and $^{177}$Lu-labeled radioligands described in this protocol. Those seeking to leverage other types of targeting vectors or use different radionuclides are welcome to combine the framework of this protocol with experimental procedures found in the references we cite throughout.

## Validating immunoreactivity

Before embarking on a new set of in vivo experiments, it is important to confirm the immunoreactivity of the immunoconjugate. Historically, we have used two types of immunoreactivity assays, both of which require radiolabeling and purifying the TCO-modified immunoconjugate. The first is a cell-based assay in which the radioimmunoconjugate is incubated with the antigen-expressing cells that will be used in the in vivo experiments[68]. The second is a bead-based assay in which the radioimmunoconjugate is incubated with nickel nitroacetic acid (Ni-NTA)-tagged beads coated with His-tagged antigen according to the manufacturer's instructions[69]. Each method has a distinct set of

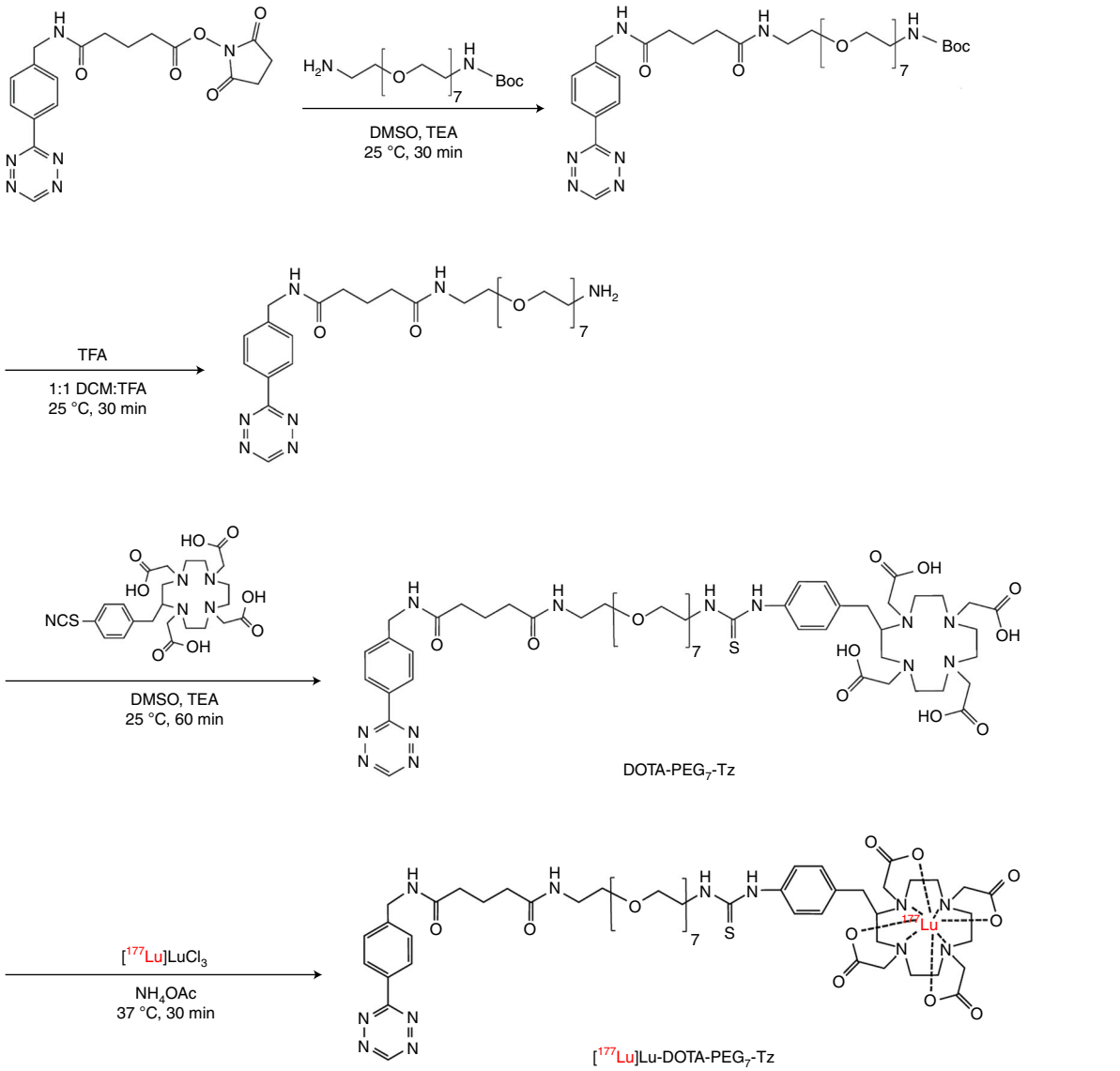


**Fig. 4 |** The synthesis of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz. DCM, dichloromethane; DMSO, dimethyl sulfoxide.

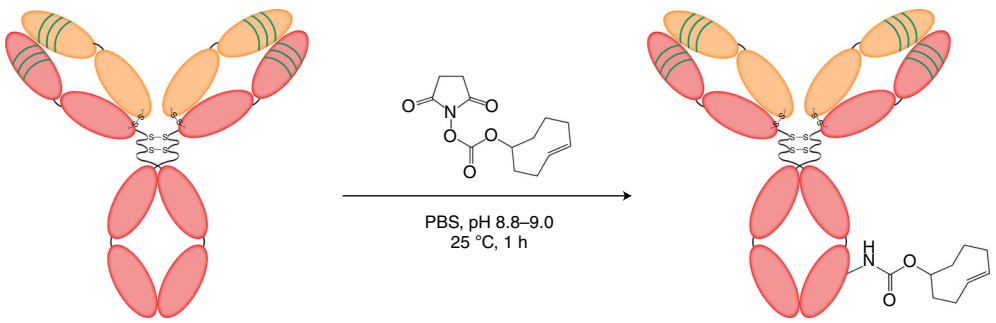


**Fig. 5 |** The synthesis of huA33-TCO.

advantages. The bead-based assay removes all of the variables associated with using live cells as well as the need for cell culture. The cell-based assay, on the other hand, provides information on the interaction between the radioimmunoconjugate and the cells that will subsequently be used in vivo.

If the binding of the immunoconjugate to the cells that will be used in vivo has been well established previously, we recommend the bead-based assay. If not, the cell-based assay is probably more useful.

### Optimizing the interval between injections

One of the key variables to optimize in the context of in vivo pretargeting is the length of the interval time between the injections of the antibody and the radioligand. This has a significant influence on the success of the experiment. If the interval is too short, this can result in high activity concentrations in the blood because of the ligation of the radioligand with still-circulating immunoconjugate. On the other hand, if the interval is too long, the number of click ligations at the tumor could be reduced because of the internalization of the immunoconjugate or the isomerization of TCO to CCO.

In the past, we have used imaging experiments using a directly radiolabeled antibody to determine the point at which the immunoconjugate reaches an optimal biodistribution in vivo and thereby identify the appropriate injection interval. For example, imaging experiments with [$^{89}$Zr]Zr-DFO-huA33 in murine models of colorectal cancer illustrated that the radioimmunoconjugate reaches an optimal biodistribution between 1 and 4 d after injection, data that has led us to use injection intervals of 24–96 h when performing pretargeting experiments with huA33-TCO. In the absence of biodistribution or imaging data, the biological half-life of the vector could be used as a general guidepost for choosing an appropriate injection interval, although there is no replacement for experimentation. In the end, we recommend that those developing a new pretargeting system explore several different intervals to identify the timing that produces the best in vivo results.

#### Control experiments

In pretargeted PET imaging experiments, we have used cohorts of animals treated with three sets of control conditions: (i) the radioligand alone, (ii) the radioligand in conjunction with an unmodified (i.e., TCO-less) vector (e.g., wild-type huA33) and (iii) the TCO-bearing vector in conjunction with the radioligand in very low specific activity. Perhaps not surprisingly, each of these conditions produced images with very low tumor-to-background contrast as well as minimal activity concentrations (i.e., a percentage of injected dose per gram of tissue (%ID/g) of <0.5) in tumor tissue.

Armed with the PET data, we have typically used only two control conditions in longitudinal PRIT studies: the radioligand alone (at the highest dose used for PRIT) and the TCO-modified immunoconjugate alone. In these experiments, the cohorts subjected to both control conditions experience dramatically more rapid tumor growth than those treated with even the lowest doses of PRIT (Fig. 6). Finally, in both PET and PRIT studies, a control cohort in which the radioligand alongside a non-targeted yet TCO-bearing vector (e.g., non-specific $IgG_1$-TCO) is advisable if the specificity of the vector has not previously been demonstrated. These control experiments should be performed during the in vivo evaluation of any new pretargeting system.

### Necessary expertise

#### Radiation safety training

Only personnel who have received proper radiation safety training should perform the parts of this protocol that involve radioactivity. Furthermore, these experiments must only be performed in authorized and regulated laboratory spaces that have been approved for work with radioactivity. Finally, survey meters, dosimeters, adequate shielding and proper personal protective equipment (PPE) must be used at all times.

#### Laboratory animal training

Only personnel who have received thorough training in the handling and use of mice for preclinical in vivo experimentation should perform the parts of the protocol that involve mice. Regulated and well-staffed animal facilities are required for all animal experiments, and all in vivo procedures must be performed according to institutional, state and federal guidelines. Again, proper PPE must be used at all times during in vivo experimentation.

## Materials

### Biological materials

- Athymic nude mice (The Jackson Laboratory, cat. no. 007850) bearing SW1222 human colorectal cancer xenografts (see Supplementary Methods for a protocol for xenograft implantation; RRID_3886)

**! CAUTION** All animal experiments should be performed according to institutional, state and federal

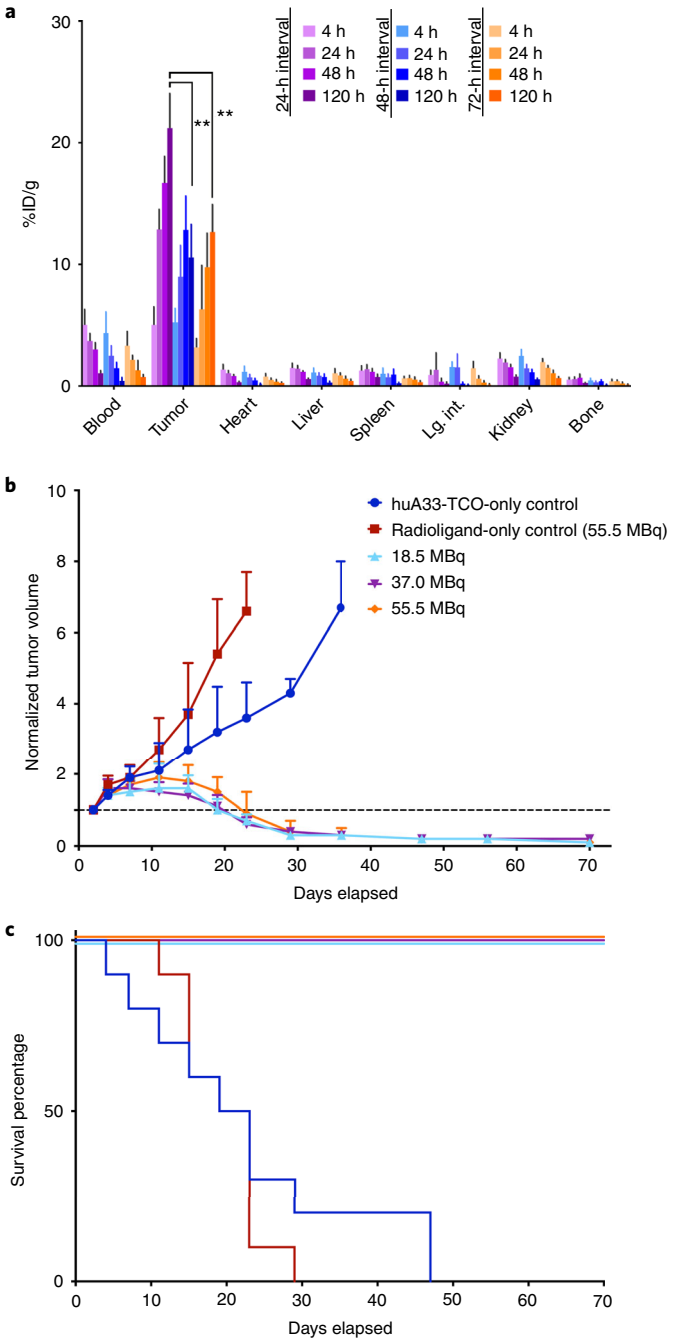


**Fig. 6 | In vivo pretargeted radioimmunotherapy data. a**, huA33-TCO and [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz were used in athymic nude mice (four per cohort) bearing subcutaneous SW1222 human colorectal cancer xenografts by using pretargeting intervals of 24 (purple), 48 (light blue) or 72 (orange) h. For each pretargeting interval, the mice were sacrificed at 4, 24, 48 and 120 h after the administration of the radioligand. The data are presented as the uptake value in %ID/g ± s.d. Longitudinal therapy study of five groups of mice (10 each) bearing subcutaneous SW1222 tumors depicted in a graph of normalized tumor volume as a function of time (**b**) and the corresponding Kaplan-Meier survival curve (**c**). The control groups received either the immunoconjugate without the radioligand (blue) or the radioligand without the immunoconjugate (red). The three treatment groups received huA33-TCO (100 μg, 0.7 nmol) followed 24 h later by 18.5 (light blue), 37.0 (purple) or 55.5 (orange) MBq (0.5, 1.0 or 1.5 mCi, respectively) (~0.7 nmol in each case) of [$^{177}$Lu]Lu-DOTA-PEG7-Tz. By log-rank (Mantel–Cox) test, survival was significant ($P < 0.0001$) for all treatment groups. Lg. Int., large intestine. $^{**}P < 0.01$. Image reproduced with permission from ref. [26]. Copyright 2018 American Chemical Society.

guidelines. All animal experiments in this protocol were performed in accordance with protocols approved by the institutional animal care and use committees of Memorial Sloan Kettering Cancer Center, Weill Cornell Medical College and Hunter College.

## Reagents

- [$^{64}$Cu]$CuCl_2$ in 0.05 M HCl (MIR Cyclotron Facility, Washington University School of Medicine) **! CAUTION** Copper-64 represents a radiological hazard and must be used according to institutional, state and federal regulations as well as ALARA (as low as reasonably achievable) principles. Proper shielding during the handling of all radioactive reagents, mixtures, glassware and instruments is essential. Furthermore, it is critical that appropriate PPE, dosimeters and survey meters always be used when handling radioactive material.
- [$^{177}$Lu]$LuCl_3$ in 0.05 M HCl (PerkinElmer Life and Analytical Sciences) **! CAUTION** Lutetium-177 represents a radiological hazard and must be used according to institutional, state and federal regulations as well as ALARA principles. Proper shielding during the handling of all radioactive reagents, mixtures, glassware and instruments is essential. Furthermore, it is critical that appropriate PPE, dosimeters and survey meters always be used when handling radioactive material.
- huA33 antibody (Olivia Newton-John Cancer Research Institute) **▲ CRITICAL** Store antibodies according to the manufacturer's instructions.
- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A9418)
- Chelex 100 resin (100–200 mesh, sodium form; Bio-Rad Laboratories, cat. no. 143-2832)
- Dimethyl sulfoxide (DMSO; ThermoFisher Scientific, cat. no. MT-25950CQC) **! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.
- Ethylenediaminetetraacetic acid (EDTA; ThermoFisher Scientific, cat. no. AC327211000) **! CAUTION** EDTA can cause serious eye damage and is hazardous to aquatic life.
- Hydrochloric acid (HCl 6 M; Thermo Fisher Scientific, cat. no. 60-047-420) **! CAUTION** HCl can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.
- Ultrapure water (18.2 MΩ/cm, Milli-Q Advantage A10 water purification system;MilliporeSigma, cat. no. Z00Q0V0WW; or equivalent)
- Phosphate-buffered saline (PBS; 10× solution, BP3991; Thermo Fisher Scientific)
- $Na_2CO_3$(; Sigma-Aldrich, cat. no. S7795) **! CAUTION** $Na_2CO_3$ can cause serious eye irritation.
- (*E*)-Cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS; Sigma-Aldrich, cat. no. 764523)
- Acetic acid ($C_2H_4O_2$, 99.8%; Thermo Fisher Scientific, cat. no. AC222140010) **! CAUTION** Acetic acid can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.
- Ammonium acetate ($NH_4OAc$; Sigma-Aldrich, cat. no. 372331)
- Acetonitrile (Thermo Fisher Scientific, cat. no. A998SK-4) **! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity.
- *N*-Boc-4-(bromomethyl)-benzylamine (Boc, *tert*-butoxycarbonyl; AstaTech, cat. no. 54463) **! CAUTION** *N*-Boc-4-(bromomethyl)-benzylamine has acute oral toxicity and is potentially irritating to the skin.
- Benzylamino tetrazine *N*-hydroxysuccinimidyl ester (Tz-NHS; Sigma-Aldrich, cat. no. 764701) **! CAUTION** Tz-NHS has acute oral and dermal toxicity.
- DiAmSar (SarAr-Bn-$NH_2$; Macrocyclics, cat. no. M-190)
- *N,N*-Dimethylformamide (DMF, anhydrous, 99.8%; Thermo Fisher Scientific, cat. no. AC448381000) **! CAUTION** DMF is a highly flammable liquid and may cause serious eye and respiratory irritation.
- Triethylamine (TEA; Acros Organics, cat. no. 219510500) **! CAUTION** TEA is a highly flammable liquid and has high oral, dermal and respiratory toxicity.
- Trifluoroacetic acid (TFA, 99+%; Thermo Fisher Scientific, cat. no. AAA1361414) **! CAUTION** TFA is highly toxic and can cause severe skin burns, eye damage and lung irritation.
- Boc-$PEG_7$-amine (*O*-(2-aminoethyl)-*O*′-[2-(Boc-amino)ethyl]hexaethylene glycol; Sigma-Aldrich, cat. no. 70023)
- Dichloromethane ($CH_2Cl_2$, DCM; Thermo Fisher Scientific, cat. no. AC610300010) **! CAUTION** DCM can cause skin irritation, eye irritation, drowsiness and dizziness. DCM is also a suspected carcinogen.
- *p*-SCN-Bn-DOTA (Macrocyclics, cat. no. B-205)

## Equipment

- Centrifugal filters (Amicon Ultra-4 centrifugal filters with a 50-kDa cutoff; Sigma-Aldrich, cat. no. UFC8050)
- UV-visible spectrophotometer (Biospec Nano, Shimadzu; or equivalent)
- Analytical balance (XPE105, Mettler Toledo; or equivalent)
- Dose calibrator (Capintec CRC-15R dose calibrator, Capintec; or equivalent)

- 1.5-ml microcentrifuge tubes (Eppendorf LoBind microcentrifuge tubes: protein; Thermo Fisher Scientific, cat. no. 13-698-794; or equivalent)
- 50-ml conical centrifuge tubes (Corning CentriStar Cap, 50-mL centrifuge tubes, sterile; Thermo Fisher Scientific, cat. no. 05-538-60; or equivalent)
- Gamma counter (Automatic Wizard[2] gamma counter, PerkinElmer; or equivalent)
- Heat lamp (Infrared heating and drying lamp; VWR, cat. no. 36547-009; or equivalent)
- Insulin syringes (Thermo Fisher Scientific, cat. no. 14-826-79; or equivalent)
- Instant thin-layer chromatography (iTLC) plates, glass microfiber chromatography paper impregnated with silica gel (Thermo Fisher Scientific, NC0283947; or equivalent)
- Ultrapure water system (Milli-Q Advantage A10 water purification system; MilliporeSigma, cat. no. Z00Q0V0WW; or equivalent)
- Mouse restrainer (TV-RED 150-STD, Braintree Scientific; or equivalent)
- Ethanol wipes (Thermo Fisher Scientific, cat. no. 22-363-750) **! CAUTION** Ethanol is highly flammable.
- Gauze (Dynarex sterile gauze pads; Dynarex, cat. no. 3353; or equivalent)
- Mouse surgery kit (Kent Scientific mouse surgical kit; Thermo Fisher Scientific, cat. no. 13-005-204; or equivalent)
- Disposable culture tubes (DWK Life Sciences Kimble plain disposable plastic tubes, 12 × 75 mm polystyrene; Thermo Fisher Scientific, cat. no. 13-674-60A; or equivalent)
- Disposable culture tube caps (Simport Scientific flange plug caps; Thermo Fisher Scientific, cat. no. 22-045-563; or equivalent)
- PET scanner (microPET Focus 120 dedicated small-animal scanner, Siemens Medical Solutions or equivalent)
- pH paper (Sigma-Aldrich, cat. no. 1.09543.0001; or equivalent)
- Pipette tips (1–1,000 µl, Fisherbrand SureOne aerosol barrier pipette tips, micropoint; Thermo Fisher Scientific, cat. nos. 02-707-439 (0.1–10 µl), 02-707-432 (2–20 µl), 02-707-430 (20–200 µl) and 02-707-404 (100–1,000 µl); or equivalent)
- Pipettes (1–1,000 µl, Brand Transferpette S pipette, adjustable single channel; Sigma-Aldrich, cat. nos. Z646512 (0.5–10 µl), Z646520 (2–20 µl), Z646547 (20–200 µl) and Z646555 (100–1,000 µl); or equivalent)
- Disposable PD-10 desalting columns (Sephadex G-25 medium; Thermo Fisher Scientific, cat. no. 45-000-148)
- Dry block heating and cooling shaker (Eppendorf Thermomixer F1.5; Thermo Fisher Scientific, cat. no. 05-412-500; or equivalent)
- Thin-layer chromatography (TLC) plate reader (Bioscan AR-2000 Radio-TLC plate reader + Winscan Radio-TLC software, Bioscan; or equivalent)
- Tumor-measuring device (Peira TM900 or equivalent)
- Vortex mixer (VWR analog vortex mixer, 300–3,200 r.p.m; Avantor, VWR, cat. no. 10153-838; or equivalent)
- Filter units (Thermo Scientific Nalgene rapid-flow sterile disposable filter units with 75-mm polyethersulfone membrane, 0.2-µm pore size; Thermo Fisher Scientific, cat. no. 09-741-02; or equivalent)
- Centrifuge for 1.5-ml microcentrifuge tubes (Eppendorf Centrifuge 5430R; Sigma-Aldrich, cat. no. EP022620603; or equivalent)
- Tweezers (Fisherbrand high precision straight broad strong point tweezers/forceps; Thermo Fisher Scientific, cat. no. 12-000-128; or equivalent)
- Permanent marker (Thermo Scientific Nalgene black ink lab markers; Thermo Fisher Scientific, cat. no. 13-382-51; or equivalent)
- 5-mm thin-wall precision NMR sample tube, 7 inches long, 600 MHz (Wilmad-LabGlass, cat. no. 535-PP-7; or equivalent)
- Lyophilizer (FreeZone 2.5-liter −84 °C benchtop freeze dryer; Labconco, cat. no. 7670520; or equivalent)
- Glass collection tubes, 3.5 ml (Shimadzu, cat. no. 228-25315-91; or equivalent)
- Glass Pasteur pipettes (Fisherbrand disposable soda-lime glass Pasteur pipettes; Thermo Fisher Scientific, cat. no. 13-678-6B; or equivalent)
- Pasteur pipette bulbs (Fisherbrand dropper bulbs, natural, 1 ml; Thermo Fisher Scientific, cat. no. 14-127-515; or equivalent)
- High field NMR spectrometer capable of performing $^{1}H$ and $^{13}C$ NMR analysis (Bruker Avance III 600 MHz, Bruker Avance DRX 500 MHz and/or Bruker Avance III 400 MHz, Bruker Scientific; or equivalent)

- Reverse-phase HPLC system with analytical, semi-preparative and preparative columns (degassing unit: DGU-20A 3R; pump system: LC-20AP; communications bus module: CMB-20A; UV-visible absorbance detector: SPD-M20A; fraction collector: FRC-10A; software: LabSolutions LC/GC; Shimadzu; or equivalent)
- Analytical HPLC column (Jupiter Proteo HPLC column, 250 × 2 mm, 5 µm, 300 Å; Phenomenex, cat. no. 00G-4053-B0; or equivalent)
- Semi-preparative HPLC column (Jupiter Proteo HPLC column, 250 × 4.6 mm, 5 µm, 300 Å; Phenomenex, cat. no. 00G-4053-E0; or equivalent)
- Preparative HPLC column (Jupiter Proteo HPLC column, 250 × 10 mm, 5 µm, 300 Å; Phenomenex, cat. no. 00G-4053-N0; or equivalent)
- Magnetic stir bar (Sigma-Aldrich, cat. no. Z328650-10EA)
- Magnetic stirrer hot plate (Sigma-Aldrich, cat. no. Z742542)
- Mass spectrometer capable of performing ESI analysis (Agilent 6340 ion trap with electron transfer dissociation liquid chromatography/mass spectrometry (MS) system, Agilent; or equivalent)
- Single-neck 10-ml round-bottom flask (Sigma-Aldrich, cat. no. Z100633)
- Single-neck 25-ml round-bottom flask (Sigma-Aldrich, cat. no. Z278262)
- Needles (hypodermic needle precision glide without safety 26 gauge 3/8 inch length; Thermo Fisher Scientific, cat. no. 14-826-10)
- Vacuum evaporator (Biotage V-10 Touch, V10-2XX, Biotage)
- 20-ml scintillation vials (FisherBrand 20-ml borosilicate glass scintillation vials with white urea caps; Thermo Fisher Scientific, cat. no. 03-337-5)

## Reagent setup

**Chelex ultrapure water (1.0 liter)**

Add 5.0 g of Chelex resin to 1.0 liter of ultrapure water and stir the mixture overnight at room temperature (20–25 °C). Remove the resin by filtration using filter units. This water can be stored at room temperature for ~1 year. ▲ **CRITICAL** We recommend treating all ultrapure water with Chelex resin before performing radiometallations of chelator-bearing conjugates (e.g., SarAr-Tz and DOTA-$PEG_7$-Tz). Chelex resin is a styrene divinylbenzene copolymer that acts as a chelating agent for polyvalent metal ions. Therefore, treating ultrapure water with the Chelex resin will remove any metal ions that could interfere with the subsequent coordination of radiometals.

**1× PBS, pH 7.4 buffer (1.0 liter)**

Combine 100.0 ml of 10× PBS with 900.0 ml of ultrapure water and mix thoroughly. This buffer can be stored at room temperature for ~1 year. If 1× PBS is used for diluting doses for mice injections, filter the solution by using a sterile filter unit on the day of use.

**0.25 M ammonium acetate buffer, pH 5.5 (100.0 ml)**

Dissolve 1.927 g of ammonium acetate in 95.0 ml of Chelex ultrapure water. Adjust the pH to 5.5 with acetic acid (~0.65 ml) and pH papers. Bring the volume to 100.0 ml with Chelex ultrapure water. This buffer can be stored at room temperature for ~1 year. ! **CAUTION** Acetic acid can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.

**0.1 M $Na_2CO_3$ (50.0 ml)**

Dissolve 0.53 g of $Na_2CO_3$ in 50.0 ml of ultrapure water. This solution can be stored at room temperature for ~1 year. ! **CAUTION** Sodium carbonate can cause serious eye irritation.

**25.0 mg/ml TCO-NHS in DMSO (1.0 ml)**

Dissolve 25.0 mg of TCO-NHS in 1.0 ml of DMSO. Mix the solution thoroughly by using a vortex mixer, divide it into aliquots and store the aliquots at −80 °C for ~3 years. ! **CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.

**50 mM EDTA, pH 5.0 (250.0 ml)**

Add 5.65 g of EDTA to 200.0 ml of ultrapure water. Adjust the pH to 5.0 with 6.0 M HCl and pH papers. Bring the volume to 250.0 ml with ultrapure water. This solution can be stored at room temperature for ~1 year. ! **CAUTION** EDTA can cause serious eye damage and is hazardous to aquatic life. Hydrochloric acid can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.

**1% BSA in 1× PBS (mass/vol, 100.0 ml)**
Dissolve 1.0 g of BSA in 100.0 ml of 1× PBS. Mix the solution thoroughly, divide it into aliquots and store the aliquots at −20 °C for ~1 year.

**HPLC eluent A (0.1% TFA in ultrapure water, 1.0 liter)**
Add 1.0 ml of TFA to 999.0 ml of ultrapure water. This solvent can be stored at room temperature for ~6 months. **! CAUTION** TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation. TFA should be avoided in the HPLC eluent when purifying Boc-protected compounds, because it can hydrolyze the protecting group. For Boc-protected compounds, use 1.0 l of pure $H_2O$ as eluent A.

**HPLC eluent B (0.1% TFA in acetonitrile, 1.0 liter)**
Add 1.0 ml of TFA to 999.0 ml of acetonitrile. This solvent can be stored at room temperature for ~6 months. **! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation. TFA should be avoided in the HPLC eluent when purifying Boc-protected compounds, because it can hydrolyze the protecting group. For Boc-protected compounds, use 1.0 liter of pure acetonitrile as eluent B.

## Equipment setup

**UV-visible spectrophotometer**
Turn on the instrument and replace the wiper cloth if necessary (one cloth can be used for 100 measurements). Choose the 'protein quantification' parameters, with the settings set for mAb measurements: molecular weight = 150,000 g/mol; extinction coefficient = 210,000 $M^{-1}cm^{-1}$; concentration output = μM. Next, add 1–3 μl of 1× PBS (pH 7.4) to the sensor by using a 0.1–10-μl pipette to obtain a blank measurement (repeat three times). Acquire a spectrum by using 1.0 μl of the antibody sample with five total replicates. The instrument will provide the absorbance value and automatically calculate the concentration of the antibody. Calculate the average of the five measurements to determine the total antibody concentration.

**Microcentrifuge**
Turn on the microcentrifuge and adjust the settings (temperature, speed and time) as needed. Use 1.5-ml microcentrifuge tubes with the lids closed tightly as well as counterbalances if necessary.

**TLC chamber and plate**
Add 2–3 ml of 50 mM EDTA (pH 5.5) to a 50-ml conical centrifuge tube. Make a dot on the bottom of the iTLC plate by using a pencil or permanent marker. Apply a drop (~1 μl) of the radioligand solution onto the mark and insert the iTLC plate into the centrifuge tube by using tweezers. Wait for the solvent front to ascend the iTLC plate. When the solvent front is 1 cm from the top of the plate, use tweezers to remove the plate from the chamber. Dry the radio-iTLC plate in air. **! CAUTION** EDTA can cause serious eye damage and is hazardous to aquatic life.

**RadioTLC plate reader**
Wrap the dried radio-iTLC plates in cellophane wrap to prevent the contamination of the instrument. Tape the iTLC plates to the plate reader and adjust the instrument markers so that they line up with the iTLC plates. Turn on the P10 gas and, using the software provided, run the plate reader. Adjust the ROIs on the resulting chromatogram to determine the radiochemical purity of the solution.

**Dry block heating and cooling mixer**
Turn on the mixer and adjust the settings (i.e., r.p.m and temperature) as necessary. Use 1.5-ml microcentrifuge tubes with the lids closed tightly.

**Disposable PD-10 desalting columns**
Set up the PD-10 columns according to the manufacturer's instructions. Remove the cap and pour out the storage solution. Cut the sealed end of the column at the notch. Equilibrate the column with 25 ml of 1× PBS (pH 7.4) and discard the flow-through. The maximum sample volume for the column is 2.5 ml. For sample volumes <2.5 ml, add the sample volume and then add 1× PBS (pH 7.4) buffer to the column after the sample has entered the packed bed completely to adjust the total volume up to 2.5 ml. After allowing the sample and buffer to enter the packed bed completely, discard

the flow-through. Subsequently, place a collection tube under the column, elute the column with 2.0 ml of 1× PBS buffer and collect the eluate. ▲ CRITICAL It is critical to equilibrate the column, because UV-absorbing stabilizers are used during the packing of the column. Do not use >2.5 ml of total solution when loading the sample.

**Preparation of the HPLC system**

Before using the HPLC system, de-gas and filter eluent A (0.1% TFA in water (vol/vol)) and eluent B (0.1% TFA in acetonitrile (vol/vol)). Wash the column with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 1 h with a relatively low flow rate (0.5 ml/min) before the injection of the sample. Finally, perform a purge (parameters set by the manufacturer) on the HPLC lines with eluent A and eluent B before every run to ensure that no air bubbles are in the system. ! CAUTION Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

**Lyophilization**

To prepare samples for lyophilization, weigh an empty container (typically a 50.0-ml conical centrifuge tube) with the cap on. Add the liquid samples to the container and cover again with the cap. Place the samples in a −80 °C freezer upright for a minimum of 12 h or expose them to liquid nitrogen for ~30 s. Once the samples are completely frozen, replace the original cap with a new cap that has been punctured several times with a needle. Be sure to keep the original cap for weighing the sample again after lyophilization. Wrap each container with aluminum foil if the protocol states that the samples are sensitive to light. Place them in the lyophilizer. Lyophilize the samples until all of the solvent has been removed. Replace the cap with holes with the original cap and re-weigh the container to determine the mass of the product. ! CAUTION Take great care if handling liquid nitrogen; always use proper PPE (i.e., cryo gloves).

**Vacuum evaporator**

To remove the solvent from a solution by using a vacuum evaporator, transfer the solution to a 20.0-ml scintillation vial (the solution needs to occupy less than 1/4 of the vial; if the volume of the solution is too great, transfer some to a separate vial or vials). Place the vial in the vacuum evaporator and adjust the parameters of the instrument according to the solvent being evaporated (refer to the manufacturer's instructions). Start the evaporation. After 30 min, stop the evaporation and check the progress. If the vial is completely dry, reconstitute the powder in the solvent of choice (as per the protocol). If the vial is not completely dry, repeat the evaporation for another 30 min. Continue this process until the vial is completely dry and then reconstitute the powder in the solvent of choice (per the protocol).

## Procedure 1: synthesis of Tz-bearing precursors

### Synthesis of $N^1$-(4-(((pivaloyloxy)amino)methyl)-benzyl)-3,6,10,13,16,19-hexaazabicyclo [6.6.6]icosane-1,8-diamine (SarAr-Bn-NHBoc) ● Timing ~3 d

1 Combine 30.0 mg (0.094 mmol) of DiAmSar and 4.0 ml of anhydrous DMF in a 25.0-ml round-bottom flask and stir the solution vigorously with a magnetic stir bar and a magnetic stir plate.
! CAUTION DMF is a highly flammable liquid and may cause serious eye and respiratory irritation.

2 Slowly add 37.0 mg (0.12 mmol) of *N*-Boc-4-(bromomethyl)-benzylamine to the stirring slurry (add in four batches; 9.25 mg, 9.25 mg, 9.25 mg and 9.25 mg). Between each addition, allow the solution to mix thoroughly. Continue stirring for ~1–2 min until the white solid has completely dissolved.
! CAUTION *N*-Boc-4-(bromomethyl)-benzylamine has acute oral toxicity and is potentially irritating to the skin.
? TROUBLESHOOTING

3 Add 34.0 mg (0.32 mmol) of $Na_2CO_3$ to the mixture and continue to stir vigorously.
! CAUTION $Na_2CO_3$ can cause serious eye irritation.

4 Heat the slurry to 70 °C and continue stirring for 16 h at 70 °C. Over the course of the heating, the solution will slowly change from colorless to pale yellow.

5 After 16 h, take a small aliquot from the reaction mixture, perform a twofold dilution with acetonitrile and check the reaction via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 20:80 (water/acetonitrile) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~16 min; potential impurities: $t_{R,BocNH\text{-}Bn\text{-}SarAr\text{-}Bn\text{-}NHBoc}$ = ~17 min). If the reaction is not complete, allow the solution to incubate longer. $t_R$ = retention time.

▲ CRITICAL STEP Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines. We recommend using solvents without 0.1% TFA when purifying Boc-protected compounds.

6 Once the reaction is deemed complete, stop heating the solution. Dilute the reaction solution up to 6.0 ml with ultrapure water and distribute 1,000-µL aliquots of the reaction mixture into six 1.5-ml microcentrifuge tubes. If HPLC purification cannot be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

■ PAUSE POINT The crude SarAr-Bn-NHBoc can be stored at −80 °C for ~1 month.

7 Purify the samples via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 20:80 (water/acetonitrile) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~16 min; potential impurities: $t_{R,BocNH\text{-}Bn\text{-}SarAr\text{-}Bn\text{-}NHBoc}$ = ~17 min). Collect the fractions (~3 ml per fraction, from 15.5 to 16.5 min, in a total of one to two fractions per run) in 3.5-ml glass collection tubes and combine them in a 50-ml conical centrifuge tube.

! CAUTION Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity.

▲ CRITICAL STEP We recommend using solvents without the 0.1% TFA additive when purifying the Boc-protected compound.

? TROUBLESHOOTING

8 Lyophilize the HPLC eluant to yield SarAr-Bn-NHBoc as a white powder (typical results: 35 mg; 0.065 mmol; 65–75% yield).

■ PAUSE POINT The purified SarAr-Bn-NHBoc can be stored as a solid at 4 °C for ~1 year.

? TROUBLESHOOTING

9 Confirm the identity of the product with ESI-MS and $^1$H-NMR.

### Synthesis of $N^1$-(4-(aminomethyl)benzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine (SarAr-Bn-$NH_2$) ● Timing ~2 d

10 Combine 31.0 mg (0.058 mmol) of SarAr-Bn-NHBoc and 2.0 ml of acetonitrile in a 10.0-ml round-bottom flask and stir vigorously with a magnetic stir bar and a magnetic stir plate.

! CAUTION Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity.

11 Place the setup in a water bath. Using a glass pipette, add 2.0 ml of TFA dropwise to the vigorously stirring slurry.

! CAUTION TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

▲ CRITICAL STEP The reaction is exothermic and therefore must be performed in a water bath.

12 Continue stirring for 90 min at room temperature (20–25 °C).

13 Take a small aliquot from the reaction mixture (~20 µl) and check the reaction via HPLC with a mobile-phase gradient of 95:5 to 20:80 (eluent A/eluent B) over 20 min with a flow rate of 6 ml/min ($t_{R,product}$ = ~7 min; no major impurities observed). If the reaction is deemed complete, continue with HPLC purification. If the purification cannot be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

▲ CRITICAL STEP Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product.

■ PAUSE POINT The crude SarAr-Bn-$NH_2$ can be stored at −80 °C for ~1 month.

14 Remove the solvent completely via vacuum evaporation (as described in 'Equipment setup') to provide crude SarAr-Bn-$NH_2$ as a yellow/brown oil.

15 Dissolve the product in 1.0 ml of DMSO and 1.0 ml of acetonitrile.

! CAUTION DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.

▲ CRITICAL STEP Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product.

16 Purify the samples via preparative HPLC with a mobile-phase gradient of 95:5 to 20:80 (eluent A/eluent B) over 20 min with a flow rate of 6 ml/min ($t_{R,product}$ = ~7 min; no major impurities observed). Collect the purified fractions (~3 ml per fraction, from 6.5 to 7.5 min, in a total of two to three fractions per run) in 3.5-ml glass collection tubes and combine them in a 50-ml conical centrifuge tube.

**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

**? TROUBLESHOOTING**

17 Lyophilize the HPLC eluant to yield SarAr-Bn-$NH_2$ as a white powder (typical results: 26 mg; 0.059 mmol; quantitative yield).

**■ PAUSE POINT** The purified SarAr-Bn-$NH_2$ can be stored as a solid at 4 °C for ~1 year.

**? TROUBLESHOOTING**

18 Confirm the identity of the product with ESI-MS and $^1$H-NMR.

### Synthesis of $N^1$-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-$N^5$-(4-(((8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosan-1-yl)amino)methyl)benzyl)glutaramide (SarAr-Tz)

● **Timing ~2 d**

19 Combine 5.0 mg (0.013 mmol) of Tz-NHS and 400 µl of anhydrous DMF in a 1.5-ml microcentrifuge tube and mix the solution thoroughly.

**! CAUTION** Tz-NHS has acute oral and dermal toxicity. DMF is a highly flammable liquid and may cause serious eye and respiratory irritation.

**▲ CRITICAL STEP** Tz-NHS is light sensitive; protect the reaction mixture from light. Our laboratory uses aluminum foil to cover the reaction vessel.

20 In a separate 1.5-ml microcentrifuge tube, combine 5.4 mg (0.013 mmol) of SarAr-Bn-$NH_2$ and 200 µl of anhydrous DMF and agitate the solution at 500 r.p.m on a dry block heating mixer at room temperature for 1–2 min.

**! CAUTION** DMF is a highly flammable liquid and may cause serious eye and respiratory irritation.

21 Transfer the Tz-NHS solution into the stirring slurry of SarAr-Bn-$NH_2$.

22 Add 2.79 µl (0.020 mmol) of TEA to the stirring slurry. Continue stirring the solution for 2 h at room temperature.

**! CAUTION** TEA is a highly flammable liquid and has high oral, dermal and respiratory toxicity.

**? TROUBLESHOOTING**

23 Take a small aliquot from the reaction mixture (~20 µl), perform a twofold dilution of the sample mixture with acetonitrile and check the reaction via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 20:80 (eluent A/eluent B) over 15 min with a flow rate of 2 ml/min ($t_{R,product}$ = 9.5 min; no major impurities observed). If the reaction is not complete, allow the solution to incubate longer.

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines.

24 Once the reaction is complete, perform a twofold dilution of the reaction mixture with acetonitrile and continue with the HPLC purification. If the purification cannot be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running HPLC, to prevent precipitation in the lines.

**■ PAUSE POINT** Crude SarAr-Tz can be stored at −80 °C for ~1 month.

25 Purify the samples via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 20:80 (eluent A/eluent B) over 15 min with a flow rate of 2 ml/min ($t_{R,product}$ = 9.5 min; no major impurities observed). Collect the purified fractions (~3 ml per fraction, from 9.0 to 10.0 min, in a total of one to two fractions per run) in 3.5-ml glass collection tubes and then combine them in a 50-ml conical centrifuge tube.

**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

**? TROUBLESHOOTING**

26 Cover the 50.0-ml conical centrifuge tube with aluminum foil to protect samples from light.

27 Lyophilize the HPLC eluant to yield SarAr-Tz as a pink powder (typical results: 3.9 mg; 0.0054 mmol; 40–45% yield).

**■ PAUSE POINT** The purified SarAr-Tz can be stored as a solid at −80 °C for ~1 year.

**? TROUBLESHOOTING**

28 Confirm the identity of the product with ESI-MS and $^1$H-NMR.

### Synthesis of Tz-PEG$_7$-NHBoc ● Timing ~1 d

29 Dissolve 10.0 mg (0.025 mmol) of Tz-NHS in 400 µl of DMSO in a 1.5-ml microcentrifuge tube. Mix the solution thoroughly.

**! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation. Tz-NHS has acute oral and dermal toxicity.

**▲ CRITICAL STEP** Tz-NHS is light sensitive; protect the reaction mixture from light. Our laboratory uses aluminum foil to cover the reaction vessel.

30 Add 15.0 mg of Boc-PEG$_7$-NH$_2$ (0.032 mmol; 1.3 equivalents (eq.)) to the solution and mix well.

31 Add 10.0 µl of TEA (7.2 mg; 0.072 mmol) to the solution and place mixture on a dry block heating mixer at 300 r.p.m at 25 °C for 30 min.

**! CAUTION** TEA is a highly flammable liquid and has high oral, dermal and respiratory toxicity.

**? TROUBLESHOOTING**

32 Take a small aliquot from the reaction mixture (~20 µl), perform a twofold dilution of the sample mixture with acetonitrile and check the reaction progress via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (water/acetonitrile) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~22 min; potential impurities: $t_{R,Tz\text{-}NHS}$ = ~19 min, $t_{R,Boc\text{-}PEG7\text{-}NH2}$ = ~21 min).

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines. We recommend using solvents without 0.1% TFA when purifying Boc-protected compounds.

33 Once the reaction is complete, perform a twofold dilution of the reaction mixture with acetonitrile and continue with HPLC purification. If the purification is unable to be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines. We recommend using solvents without 0.1% TFA when purifying Boc-protected compounds.

**■ PAUSE POINT** Crude Tz-PEG$_7$-NHBoc can be stored at −80 °C for ~1 week.

34 Purify the samples via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (water/acetonitrile) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~22 min; potential impurities: $t_{R,Tz\text{-}NHS}$ = ~19 min, $t_{R,Boc\text{-}PEG7\text{-}NH2}$ = ~21 min). Collect the purified fractions (~3 ml per fraction, from 20.5 to 21.5 min, in a total of one to two fractions per run) in 3.5-ml glass collection tubes and then combine them in a 50-ml conical centrifuge tube.

**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity.

**▲ CRITICAL STEP** We recommend using solvents without the 0.1% TFA additive when purifying the Boc-protected compound.

**? TROUBLESHOOTING**

35 Lyophilize the HPLC eluant to yield Tz-PEG$_7$-NHBoc as a bright pink powder (typical results: 16 mg; 0.021 mmol; 80–90% yield).

**■ PAUSE POINT** The purified Tz-PEG$_7$-NHBoc can be stored as a solid at −20 °C for ~6 months.

**? TROUBLESHOOTING**

36 Confirm the identity of the product with ESI-MS and $^1$H-NMR.

### Synthesis of Tz-PEG$_7$-NH$_2$ ● Timing ~1 d

37 In a 1.5-ml microcentrifuge tube, dissolve 10.0 mg (0.014 mmol) of Tz-PEG$_7$-NHBoc in 400.0 µl of 1:1 DCM/TFA and mix the solution thoroughly.

**! CAUTION** TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation. DCM can cause skin irritation, eye irritation, drowsiness and dizziness. DCM is also a suspected carcinogen.

38 Place the solution on a dry block heating mixer and agitate at 300 r.p.m at 25 °C for 30 min.

39 Remove the solvent completely via vacuum evaporation (as described in 'Equipment setup') and reconstitute the residue in 0.5 ml of ultrapure $H_2O$.

40 Take a small aliquot from the reaction mixture (~20 µl) and check the reaction progress via analytical HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 1 ml/min ($t_{R,product}$ = ~13 min; no major impurities observed). If the reaction is

deemed to be complete, filter the rest of the reaction mixture and continue on with HPLC purification. If the reaction is not complete, repeat Steps 37–39. If the purification is unable to be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

**PAUSE POINT** The crude Tz-PEG$_7$-NH$_2$ can be stored at −80 °C for ~1 month.

41 Purify the samples via preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 8 ml/min ($t_{R,product}$ = ~15 min; no major impurities observed). Collect the purified fractions (~3 ml per fraction, from 14.5 to 15.5 min, in a total of three to four fractions per run) in 3.5-ml glass collection tubes and combine them in a 50-ml conical centrifuge tube.

**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

**? TROUBLESHOOTING**

42 Lyophilize the HPLC eluant to yield Tz-PEG$_7$-NH$_2$ as a bright pink powder (typical results: 9 mg; 0.013 mmol; 90–95% yield).

**PAUSE POINT** The purified Tz-PEG$_7$-NH$_2$ can be stored as a solid at −20 °C for ~6 months.

**? TROUBLESHOOTING**

43 Confirm the identity of the product with ESI-MS and $^1$H-NMR analysis.

### Synthesis of DOTA-PEG$_7$-Tz ● Timing ~1 d

44 In a 1.5-ml microcentrifuge tube, dissolve 11.5 mg (0.0176 mmol) of Tz-PEG$_7$-NH$_2$ in 400 µl of DMSO.

**! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.

45 Add 14.8 mg of *p*-SCN-Bn-DOTA (0.0022 mmol; 1.2 eq.) to the solution and mix well.

46 Add 20.0 µl of TEA (14.8 mg; 0.15 mmol) to the solution and mix well.

**! CAUTION** TEA is a highly flammable liquid and has high oral, dermal and respiratory toxicity.

47 Place the solution on a dry block heating mixer and agitate at 300 r.p.m at 25 °C for 60 min.

**? TROUBLESHOOTING**

48 Take a small aliquot from the reaction mixture (~20 µl), perform a twofold dilution of the sample mixture with acetonitrile and check the reaction progress via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~18 min; potential impurities: $t_{R,p\text{-}SCN\text{-}Bn\text{-}DOTA}$ = ~21 min, $t_{R,Tz\text{-}PEG7\text{-}NH2}$ = ~15 min). If the reaction is not complete, allow the solution to incubate longer.

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines.

49 Once the reaction is complete, perform a twofold dilution of the sample mixture with acetonitrile and continue with the HPLC purification. If the purification is unable to be performed immediately after the reaction, we recommend separating the sample into 200-µl aliquots in 1.5-ml microcentrifuge tubes and storing the crude product at −80 °C until purification.

**▲ CRITICAL STEP** Keep the reaction mixture away from heat, moisture and light to prevent the degradation of the product. We recommend first diluting the reaction solution with water or acetonitrile before running the HPLC, to prevent precipitation in the lines.

**PAUSE POINT** Crude DOTA-PEG$_7$-Tz can be stored at −80 °C for ~1 month.

50 Purify the samples via semi-preparative HPLC with a mobile-phase gradient of 95:5 to 5:95 (eluent A/eluent B) over 30 min with a flow rate of 2 ml/min ($t_{R,product}$ = ~18 min; potential impurities: $t_{R,p\text{-}SCN\text{-}Bn\text{-}DOTA}$ = ~21 min, $t_{R,Tz\text{-}PEG7\text{-}NH2}$ = ~15 min). Collect the purified fractions (~3 ml per fraction, from 15.5 to 16.5 min, in a total of one to two fractions per run) in 3.5-ml glass collection tubes and combine them in a 50-ml conical centrifuge tube.

**! CAUTION** Acetonitrile is a highly flammable liquid and can cause acute oral, dermal and inhalation toxicity. TFA is highly toxic and may cause severe skin burns, eye damage and lung irritation.

**? TROUBLESHOOTING**

51 Lyophilize the HPLC eluant to yield DOTA-PEG$_7$-Tz as a bright pink powder (typical results: 15.4 mg; 0.0128 mmol; 70–75% yield).

**PAUSE POINT** The purified DOTA-PEG$_7$-Tz can be stored as a solid at −20 °C for ~6 months.

**? TROUBLESHOOTING**

52 Confirm the identity of the product with ESI-MS and $^1$H-NMR.

## Procedure 2: preparation of huA33-TCO, radiochemistry and in vivo pretargeting experiments

**! CAUTION** All hazardous chemicals should be used by trained personnel using protocols approved by an environmental health and safety officer. Only personnel who have received proper radiation safety training should perform the parts of this protocol that involve radioactivity. Furthermore, these experiments must be performed only in authorized and regulated laboratory spaces that that have been approved for work with radioactivity. Finally, survey meters, dosimeters, adequate shielding and proper PPE must be used at all times.

**! CAUTION** Only personnel who have received thorough training in the use of mice for preclinical in vivo experimentation should perform the parts of the protocol that involve mice. Regulated and well-staffed animal facilities are required for all animal experiments, and all in vivo procedures must be performed according to institutional, state and federal guidelines. Again, proper PPE must be used at all times during in vivo experimentation.

### Preparation and purification of huA33-TCO ● Timing ~3 h

1 Take the vial containing the manufacturer-supplied solution of huA33 antibody (1.0 ml) from the freezer and let it thaw slowly to room temperature (20–25 °C).

2 Measure the concentration of this solution with a UV-visible spectrophotometer (for the sake of this protocol, the concentration of our 1.0 ml of antibody solution was 9.8 mg/ml).

#### Preparation of the antibody, huA33

**▲ CRITICAL** Purify and concentrate the huA33 by using a PD-10 desalting column and an Amicon Ultra-4 centrifugal filter.

3 Equilibrate three PD-10 desalting columns (see 'Equipment setup').

4 Add one third of the 1.0-ml sample (0.33 ml) to each PD-10 desalting column and allow the solution to enter the packed bed completely.

5 Discard the flow-through.

6 Add 2.17 ml of 1× PBS (pH 7.4) to each column and allow the solution to enter the packed bed completely.

7 Discard the flow-through.

8 Elute each column by using 2.0 ml of 1× PBS (pH 7.4) into an Amicon Ultra-4 centrifugal filter (three centrifugal filters in total).

9 Centrifuge the Amicon Ultra-4 centrifugal filters at 3,500*g* for 15 min and discard the flow-through.

10 Combine the solutions from the three Amicon Ultra-4 centrifugal filters (3 × ~0.2 ml after centrifugation) into a microcentrifuge tube. Wash out each Amicon Ultra-4 centrifugal filter by using the same 0.2 ml of 1× PBS (pH 7.4) and combine this with the antibody solution in the microcentrifuge tube, bringing the total volume up to ~0.8 ml.
**? TROUBLESHOOTING**

11 Mix this solution gently by using a vortex mixer on a low setting.

12 Measure the concentration of the purified huA33 solution by using a UV-visible spectrophotometer (in this case, the 0.8 ml of solution had a concentration of 11 mg/ml).
**■ PAUSE POINT** You can prepare aliquots of purified huA33 and store them at −80 °C for ~3 years.
**? TROUBLESHOOTING**

#### Reaction with TCO-NHS

13 Adjust the pH of the huA33 solution (8.8 mg, 58.7 nmol, in 0.8 ml of 1× PBS (pH 7.4)) to 8.8–9.0 by using 0.1 M $Na_2CO_3$ (~0.04 ml) and pH paper.
**! CAUTION** $Na_2CO_3$ can cause serious eye irritation.

14 Slowly add 25.0 µl of TCO-NHS in DMSO (25 mg/ml, 40 molar eq.) to the huA33 solution.
**! CAUTION** DMSO is a flammable liquid and may cause slight skin and eye irritation.
**▲ CRITICAL STEP** We recommend using a reaction stoichiometry of 40:1 (TCO-NHS/huA33). To avoid precipitation, add the TCO-NHS slowly by gently swirling the pipette tip in the mixture while continuously pushing more TCO-NHS into the mixture at a rate of ~2 µl/s.

15 Incubate the reaction mixture on an agitating dry block heating mixer for 1 h at 25 °C at 500 r.p.m.

**huA33-TCO purification**

▲ **CRITICAL** Purify and concentrate the huA33-TCO by using a PD-10 desalting column as well as an Amicon Ultra-4 centrifugal filter.

16 Equilibrate two PD-10 desalting columns (see 'Equipment setup').

17 Add half of the sample (~0.6 ml) to each PD-10 desalting column and allow the solution to enter the packed bed completely.

18 Discard the flow-through.

19 Add 1.9 ml of 1× PBS (pH 7.4) to each column and allow the solution to enter the packed bed completely.

20 Discard the flow-through.

21 Elute each column by using 2.0 ml of 1× PBS (pH 7.4) into an Amicon Ultra-4 centrifugal filter (two centrifugal filters in total).

22 Centrifuge the Amicon Ultra-4 centrifugal filters at 3,500*g* for 15 min and discard the flow-through.

23 Combine the solutions from the two Amicon Ultra-4 centrifugal filters (2 × ~0.2 ml after centrifugation) into a 1.5-ml microcentrifuge tube. Wash out each Amicon Ultra-4 centrifugal filter by using the same 0.2 ml of 1× PBS (pH 7.4) and combine this with the antibody solution in the microcentrifuge tube, bringing the total volume up to ~0.6 ml.

**? TROUBLESHOOTING**

24 Mix this solution gently by using a vortex mixer on a low setting.

25 Measure the concentration of the purified huA33-TCO solution by using a UV-visible spectrophotometer (in this case, the 0.6 ml of solution had a concentration of 13.3 mg/ml).

■ **PAUSE POINT** Aliquots of purified huA33-TCO can be stored at −80 °C for ~2 years.

**? TROUBLESHOOTING**

26 Determine the TCO occupancy of huA33-TCO by using Tz-PEG$_7$-AF680 (Box 1).

**? TROUBLESHOOTING**

27 Alternatively, determine the TCO occupancy of huA33-TCO by performing MALDI-ToF MS on both the native antibody and the TCO-modified immunoconjugate. Use the following equation to determine the degree of labeling (DOL).

$$\frac{(mass\ of\ modified\ antibody - mass\ of\ native\ antibody)}{mass\ of\ modification} = number\ of\ TCO\ per\ antibody\ on\ average$$

**? TROUBLESHOOTING**

## Radiolabeling of Tz-bearing radioligands

▲ **CRITICAL** If you want to perform pretargeted PET imaging, continue with Step 28. If you want to perform PRIT, skip to Step 39.

## Radiolabeling of SarAr-Tz with [$^{64}$Cu]CuCl$_2$ ● Timing ~1 h

**! CAUTION** Copper-64 represents a radiological hazard and must be used according to institutional, state and federal regulations as well as ALARA principles. Proper shielding during the handling of all radioactive reagents, mixtures, glassware and instruments is essential. Furthermore, it is critical that appropriate PPE, dosimeters and survey meters always be used when handling radioactive material.

▲ **CRITICAL** If the β-emitting therapeutic isotope $^{67}$Cu is available, these procedures can also be used to synthesize a therapeutic radioligand, [$^{67}$Cu]Cu-SarAr-Tz, that can be used in lieu of [$^{177}$Lu]Lu-PEG$_7$-DOTA-Tz and thus enable the creation of an identical theranostic pair of radioligands: [$^{64}$Cu]Cu-SarAr-Tz for pretargeted PET and [$^{67}$Cu]Cu-SarAr-Tz for PRIT.

28 Dilute 36.0 µl of the SarAr-Tz stock solution (12.9 µg; 18 nmol; 0.36 mg/ml in DMSO; see Procedure 1) with 200.0 µl of $NH_4OAc$ buffer (0.25 M, pH 5.5).

**! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.

29 Add 380 MBq (10.3 mCi) of [$^{64}$Cu]CuCl$_2$ in 0.05 M HCl to the reaction solution.

**! CAUTION** Hydrochloric acid can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.

▲ **CRITICAL STEP** For the in vivo pretargeting experiments, we recommend administering a 1:1 ratio of [$^{64}$Cu]Cu-SarAr-Tz/huA33-TCO. For example, using the amounts listed in Steps 37–38, a 14.8-MBq (0.4-mCi) dose of [$^{64}$Cu]Cu-SarAr-Tz will give each mouse 0.7 nmol (equivalent to the

0.7-nmol dose of huA33-TCO each mouse will receive). If a higher radioactivity dose per mouse is desired (while maintaining a 1:1 molar ratio of the two components), we recommend increasing the activity of [$^{64}$Cu]$CuCl_2$ used in Step 29 and keeping the amount of Tz precursor the same.

30 Incubate the reaction mixture on an agitating dry block heating mixer at 500 r.p.m at 37 °C for 15 min.

**Radio-iTLC to determine the progress of [$^{64}$Cu]Cu-SarAr-Tz radiolabeling**

31 Apply a small droplet (1 µl) of the reaction mixture at the baseline of the iTLC plate.

32 Insert the iTLC plate into a TLC chamber containing 50 mM EDTA (pH 5.5).
**! CAUTION** EDTA can cause serious eye damage and is hazardous to aquatic life.

33 Allow the solvent front to reach ~1 cm from the top of the iTLC plate and then remove the plate from the chamber.

34 Dry the iTLC plate in air.

35 Wrap the iTLC plate in cellophane wrap to prevent the contamination of the instrument.

36 Analyze the iTLC plate on a radio-iTLC reader to determine the progress of the reaction. Free $^{64}Cu^{2+}$ will be chelated by EDTA and will travel with the solvent front, whereas [$^{64}$Cu]Cu-SarAr-Tz will remain at the baseline. The radiochemical purity should be >99%.
**? TROUBLESHOOTING**

**Prepare for injection into mice**

37 Dilute the reaction mixture with sterile 1× PBS (pH 7.4) to a concentration of 148 MBq (4 mCi)/ml. Each mouse will receive a 0.1-ml dose containing 0.7 nmol of [$^{64}$Cu]Cu-SarAr-Tz [14.8 MBq (0.4 mCi)].

38 Draw 0.1 ml of the solution of [$^{64}$Cu]Cu-SarAr-Tz into each insulin syringe. Determine the final weight of each syringe containing the solution of radiotracer. Measure the activity of each syringe by placing it into a lead-shielded dose calibrator and recording the activity along with the date and time. Each syringe should contain 14.8 MBq (0.4 mCi).
**▲ CRITICAL STEP** Ensure that there are no air bubbles in the syringes. Refer to your institution's guidelines to determine the maximum volume that may be injected into each mouse.

### Radiolabeling of DOTA-PEG$_7$-Tz with [$^{177}$Lu]$LuCl_3$ ● Timing ~1.5 h

**! CAUTION** Lutetium-177 represents a radiological hazard and must be used according to institutional, state and federal regulations as well as ALARA principles. Proper shielding during the handling of all radioactive reagents, mixtures, glassware and instruments is essential. Furthermore, it is critical that appropriate PPE, dosimeters and survey meters always be used when handling radioactive material.

39 Dilute 13.0 µl of the DOTA-PEG$_7$-Tz stock solution (29.9 µg; 24.8 nmol; 2.3 mg/ml in DMSO; see Procedure 1) with 200.0 µl of $NH_4OAc$ buffer (0.25 M, pH 5.5).
**! CAUTION** DMSO is a flammable liquid. DMSO may cause slight skin and eye irritation.

40 Add 1.26 GBq (34.1 mCi) of [$^{177}$Lu]$LuCl_3$ in 0.05 M HCl to the reaction solution.
**! CAUTION** HCl can cause severe skin burns and eye damage and may cause respiratory irritation if inhaled.
**▲ CRITICAL STEP** For in vivo PRIT experiments, we recommend administering a 1:1 ratio of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz/huA33-TCO. For example, using the amounts listed in Steps 49 and 50, a 37-MBq (1-mCi) dose of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz will give each mouse 0.7 nmol (equivalent to the 0.7-nmol dose of huA33-TCO each mouse will receive). If a higher radioactivity dose per mouse is desired (while maintaining a 1:1 molar ratio of the two components), we recommend increasing the activity of [$^{177}$Lu]$LuCl_3$ used in Step 40 and keeping the amount of Tz precursor the same.

41 Incubate the reaction mixture on an agitating dry block heating mixer at 700 r.p.m at 37 °C for 30 min.

### iTLC to monitor [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz radiolabeling

42 After incubation, determine the progress of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz radiolabeling with radio-iTLC.

43 Apply a small droplet (1 µl) of the reaction mixture at the baseline of the iTLC plate.

44 Insert the iTLC plate into a TLC chamber containing 50 mM EDTA (pH 5.5).
**! CAUTION** EDTA can cause serious eye damage and is hazardous to aquatic life.

45 Allow the solvent front to reach ~1 cm from the top of the iTLC plate and then remove the plate from the chamber.

46 Dry the iTLC plate in air.
47 Wrap the iTLC plate in cellophane wrap to prevent the contamination of the instrument.
48 Analyze the iTLC plate on a radio-iTLC reader to determine the progress of the reaction. Free $^{177}Lu^{3+}$ will be chelated by EDTA and will travel with the solvent front, whereas [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz will remain at the baseline. The radiochemical purity should be >99%.
**? TROUBLESHOOTING**

**Prepare for injection into mice**

49 Dilute the reaction mixture with sterile 1× PBS (pH 7.4) to a concentration of 370 MBq (10 mCi)/ml. Each mouse will receive a 0.1-ml dose containing 0.7 nmol of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz (37.0 MBq (1 mCi)).
50 Draw 0.1 ml of the [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz solution into each insulin syringe. Determine the final weight of each syringe with the radiotracer. Measure the activity of each syringe by placing it into a lead-shielded dose calibrator and recording the activity along with the date and time. Each syringe should contain 37.0 MBq (1 mCi).
**▲ CRITICAL STEP** Ensure that there are no air bubbles in the syringes. Refer to your institution's guidelines to determine the maximum volume that may be injected into each mouse.

## In vivo pretargeting experiments ● Timing ~2 h for injections

**! CAUTION** Only personnel who have received thorough training in the use of mice for preclinical in vivo experimentation should perform the parts of the protocol that involve mice. Regulated and well-staffed animal facilities are required for all animal experiments, and all in vivo procedures must be performed according to institutional, state and federal guidelines. Again, proper PPE must be used at all times during in vivo experimentation.

**▲ CRITICAL** The nature of protocol writing means that some of the steps that we have already described (i.e., the synthesis of the radioligands) should actually take place after some of the steps described below (i.e., injection of huA33-TCO). To be clear, the radioligand precursors, SarAr-Tz and DOTA-PEG$_7$-Tz, should be prepared well in advance of any in vivo experiments. In addition, the radioligands themselves, either [$^{64}$Cu]Cu-SarAr-Tz or [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz, should always be prepared immediately before administration to the animals and thus during the interval period between injections.

51 Warm the mice bearing SW1222 tumors under a heat lamp to promote venodilation (~25 cm away from the lamp for 3–4 min; see Supplementary Methods for the procedure used for the implantation of subcutaneous xenografts).
52 Place one mouse in the restraining device.
53 Clean the mouse's tail thoroughly with a sterile ethanol wipe.
54 Locate the lateral tail vein and insert the needle of the syringe containing huA33-TCO (0.1 mg, 0.7 nmol, in 0.1 ml of 1× PBS), bevel up, into the vein toward the head of the mouse.
55 Inject the huA33-TCO solution into the vein slowly (0.1 ml injected over a period of ~3 s) and then carefully withdraw the needle.
56 Gently hold a piece of gauze over the injection site and apply pressure to prevent the formation of a hematoma (~30 s).
57 Return the mouse to its cage.
58 Repeat Steps 51–57 with the rest of the mice.
59 Wait for the desired interval time before the radiosynthesis and administration of the radioligand. We have successfully used interval times ranging from 1 to 5 d for this huA33-TCO/SW1222 system.
60 Warm the mice under a heat lamp to cause venodilation (~25 cm away from the lamp for 3–4 min).
61 Take one mouse at a time and place it in the restraining device to prepare for PET imaging or RIT studies.
62 Perform the PET imaging experiment as described in option A or the RIT experiment as described in option B.

(A) **Pretargeted PET imaging ● Timing ~2 h per time point, ~2 d overall**

(i) Clean the mouse's tail thoroughly with a sterile ethanol wipe.
(ii) Locate the lateral tail vein and insert the needle of the syringe containing [$^{64}$Cu]Cu-SarAr-Tz ((14.8 MBq (0.4 mCi), 0.7 nmol, prepared in Step 38), bevel up, into the vein toward the head of the mouse.
(iii) Inject the [$^{64}$Cu]Cu-SarAr-Tz solution into the vein slowly (0.1 ml injected over a period of ~3 s) and carefully withdraw the needle.

(iv) Gently hold a piece of gauze over the injection site and apply pressure to prevent the formation of a hematoma (~30 s).
(v) Return the mouse to its cage.
(vi) Repeat Step 62A(i–v) with the rest of the mice.
(vii) Measure the activity and weight of each syringe after administration to determine the dose administered.
(viii) Perform the PET imaging at the desired time points after the administration of the radioligand. With this [$^{64}$Cu]Cu-SarAr-Tz/huA33-TCO/SW1222 system, we have found that 4-, 12-, 24- and 48-h imaging time points work best.
**? TROUBLESHOOTING**

(B) **PRIT ● Timing ~2 h for tumor measurements, ~100 d overall**
(i) Clean the mouse's tail thoroughly with a sterile ethanol wipe.
(ii) Locate the lateral tail vein and insert the needle of the syringe containing [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz (37 MBq (1 mCi), 0.7 nmol, prepared in Step 50), bevel up, into the vein toward the head of the mouse.
(iii) Inject the [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz solution into the vein slowly (0.1 ml injected over a period of ~3 s) and carefully withdraw the needle.
(iv) Gently hold a piece of gauze over the injection site and apply pressure to prevent hematoma formation (~30 s).
(v) Return the mouse to its cage.
(vi) Repeat Step 62B(i–v) with the other mice.
(vii) Measure the activity and weight of each syringe after administration to determine the dose administered.
(viii) Follow the tumor growth and well-being of the mice at least twice a week for the duration of the experiment by measuring the size of the xenografts by using either calipers or a tumor measurement device (e.g., Peira TM900), weighing the mice and observing their behavior for any signs of distress.

### Ex vivo biodistribution ● Timing ~2 h for injections, ~2 h per time point

**▲CRITICAL** Biodistribution experiments can be useful companion procedures for both pretargeted PET imaging and PRIT. Biodistribution data can provide a quantitative measure of the activity concentrations produced in tumor tissue as well as healthy organs, can help validate PET imaging results and can facilitate dosimetry calculations for PET imaging and, even more critically, RIT.

**▲CRITICAL** Ex vivo biodistribution experiments require smaller doses of radioactivity compared to PRIT experiments. When preparing radioligands for ex vivo biodistribution studies (Steps 28–50), modify the radiolabeling procedure so that the final injection activity of [$^{64}$Cu]Cu-SarAr-Tz and [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz is 11.1 MBq (0.30 mCi, 0.7 nmol). When altering the radiolabeling procedure for ex vivo biodistribution studies, it is critical that the mass of the radioligand does not change, only the specific activity; the molar ratios of the radioligand and the antibody need to remain equal.

63 Administer the radioligand of choice as described in Step 62A ([$^{64}$Cu]Cu-SarAr-Tz) or 62B ([$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz). With [$^{64}$Cu]Cu-SarAr-Tz, we have found that 4-, 12- and 24-h time points are the most informative in a biodistribution study. With [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz, we have found that 4-, 24-, 72- and 120-h time points work best.

64 At the appropriate time point, euthanize the mice in the selected cohort by using $CO_2$ asphyxiation (or whichever euthanasia methods are listed in the laboratory's animal protocol).

65 Collect the blood via cardiac heart puncture and use a surgery kit to dissect each mouse and collect the desired tissues. For a typical biodistribution study, we collect 14 tissues: blood, tumor, heart, lungs, liver, spleen, stomach, small intestine, large intestine, kidneys, muscle, bone, skin and tail. Place each organ into pre-weighed and labeled biodistribution tubes.

66 To prepare radioactivity standards, pipette 5–10 µl of the radioligand used into four pre-weighed tubes.

67 Weigh the standards as well as the tubes containing the organs.

68 Measure all the tubes with a gamma counter that has been calibrated for $^{64}$Cu or $^{177}$Lu.
**▲CRITICAL STEP** Gamma counters typically decay-correct radioactivity values to the same time point. We usually set the gamma counter to decay-correct all of the measurements to the start of the measurement cycle. In this way, the values given by the gamma counter can be directly compared to one another. The standards should always be measured alongside the tissue-containing tubes.
**? TROUBLESHOOTING**

69 Calculate the average activity concentration of the standards with the following equation:

$$Ac_{std} = \frac{\frac{A_{std1}}{m_{std1}} + \frac{A_{std2}}{m_{std2}} + \frac{A_{std3}}{m_{std3}} + \frac{A_{std4}}{m_{std4}}}{4}$$

where $Ac_{std}$ = the average activity concentration of the standards (counts per minute (cpm)/g), $A_{stdi}$ = the activity of standard $i$ (cpm) and $m_{stdi}$ = the mass of standard $i$ (g).

70 Calculate total injected activity for each animal with the following equation:

$$A_i = Ac_{std} \times m_i$$

where $A_i$ = the total injected activity for mouse $i$ (cpm), $Ac_{std}$ = the average activity concentration of the standards (cpm/g) and $m_i$ = the mass of radioligand injected in mouse $i$ (g).

71 Calculate the %ID/g with the following equation:

$$\%ID/g_{organ\,i} = 100\% \times \frac{\frac{A_{organ\,i}}{A_i}}{m_{organ\,i}}$$

where $A_{organ\ i}$ = the activity of the organ from mouse $i$ (cpm), $A_i$ = the total injected activity for mouse $i$ (cpm) and $m_{organ\ i}$ = the mass of organ $i$ (g).

## Troubleshooting

Troubleshooting advice can be found in Tables 1 and 2.

**Table 1 | Troubleshooting for Procedure 1**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 2 | The *N*-Boc-4-(bromomethyl)-benzylamine does not dissolve completely | The reaction mixture is too concentrated | Add anhydrous DMF to the reaction mixture until the solution becomes clear |
| 7, 16, 25, 34, 41 and 50 | The product is impure according to HPLC | The product was exposed to light, moisture, and/or heat | Repeat the HPLC purification or perform the reaction again |
| | The product is impure according to HPLC | The reaction mixture was contaminated | Ensure that proper laboratory techniques are followed while performing these reactions. Make sure that all solvents and starting reagents are fresh and pure before beginning |
| 7, 34 | The yield of the Boc-protected product is low | The intermediates were deprotected during purification | Ensure that the HPLC solvents do not contain TFA |
| 8 | The yield of the isolated product is low | Water in the reaction mixture may have affected the formation of the product | Ensure that the DMF is anhydrous and that the reaction is performed in a moisture-free environment |
| 8, 17, 27, 35, 42 and 51 | The yield of the isolated product is low | The incubation time for the reaction was too short | Increase the incubation time |
| | The yield of the isolated product is low | The reaction either failed or was incomplete | Repeat the reaction; ensure that all solvents and starting reagents are fresh and pure before beginning |
| 22, 31 and 47 | The reaction solution turns orange or pale yellow | The Tz moiety has degraded | Reduce the amount of TEA in the reaction mixture |
| | The reaction solution turns orange or pale yellow | The Tz moiety has degraded | Ensure that the reaction vessel is protected from light |
| | The reaction solution turns orange or pale yellow | The Tz moiety has degraded | Ensure that the DMSO and TEA are anhydrous |
| 51 | The yield of the isolated product is low | The *p*-SCN-Bn-DOTA was not fully dissolved or precipitated during the reaction | Add the *p*-SCN-Bn-DOTA to the reaction mixture slowly and vortex the reaction mixture thoroughly to ensure dissolution |

**Table 2 | Troubleshooting for Procedure 2**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 10 and 23 | The residual volume in the Amicon Ultra-4 centrifugal filter is too large (>0.5 ml) | The filter is clogged because of too much mAb | Reduce the amount of mAb used per Amicon Ultra-4 centrifugal filter unit |
| | The residual volume in the Amicon Ultra-4 centrifugal filter is too large (>0.5 ml) | More time is needed for filtration | Centrifuge the unit for another 10 min and repeat until a desired volume is reached |
| 12 and 25 | A low absorbance signal is obtained at 280 nm during UV-visible measurements | The concentration of antibody is too low for the instrument to detect | Concentrate the sample further with an Amicon Ultra-4 centrifugal filter |
| | A low absorbance signal is obtained at 280 nm during UV-visible measurements | The sample was lost during purification | Double-check the calculations for the volume of antibody solution and buffer added to the PD-10 column. Ensure that it was not >2.5 ml. Review the instructions for preparing PD-10 desalting columns |
| 26 | The degree of labeling of TCO on the antibody is too low (as determined via the Tz-$PEG_7$-AF680 method) | Not enough Tz-$PEG_7$-AF680 was added to the solution to react with all of the TCO moieties on the antibody | Increase the ratio of Tz-$PEG_7$-AF680 to TCO-mAb |
| | The degree of labeling of TCO on the antibody is too low (as determined via the Tz-$PEG_7$-AF680 method) | The reaction of Tz-$PEG_7$-AF680 and TCO-huA33 was incomplete or unsuccessful | Incubate the mixture longer |
| 26 and 27 | The degree of labeling of TCO on the antibody is zero (as determined via either method) | The pH of the TCO-NHS/mAb conjugation reaction was incorrect | Make sure that the pH of the reaction is 8.8–9.0 before adding the TCO-NHS |
| | The degree of labeling of TCO on the antibody is zero (as determined via either method) | The TCO-NHS was added too quickly, leading to the formation of a precipitate | Make sure to add the TCO-NHS slowly. While adding it, gently swirl the pipette tip around in the solution to mix |
| | The degree of labeling of TCO on the antibody is zero (as determined via either method) | The mAb solution contained compounds that interfered with the conjugation reaction | Purify the mAb solution carefully before conjugation |
| | The degree of labeling of TCO on the antibody is too low (as determined via either method) | The molar ratio of TCO-NHS to mAb in the bioconjugation reaction was too low | Increase the amount of TCO-NHS added to the reaction vial while keeping the amount of the antibody the same |
| | The degree of labeling of TCO on the antibody is too low (as determined via either method) | The TCO-NHS conjugation reaction was not allowed to incubate for enough time | Increase the incubation time of the reaction |
| 27 | The degree of labeling of TCO on the antibody is negative (as determined via MALDI) | A calculation error was made | Double-check the equation used to calculate the DOL by using MALDI |
| 36 | The radiolabeling of [$^{64}$Cu]Cu-SarAr-Tz is not quantitative and/or exhibits low radiochemical purity | The radiolabeling reaction was incomplete | Increase the incubation time of the reaction |
| | The radiolabeling of [$^{64}$Cu]Cu-SarAr-Tz is not quantitative and/or exhibits low radiochemical purity | The radiolabeling was performed at the wrong pH | Prepare new buffers and double-check the pH of said buffers during the radiolabeling process by using pH papers |
| | The radiolabeling of [$^{64}$Cu]Cu-SarAr-Tz is not quantitative and/or exhibits low radiochemical purity | The radionuclide in the stock solution has low specific activity | Check with the producers of the radionuclide to ensure that the specific activity of the radionuclide is sufficient |
| | The radiolabeling of [$^{64}$Cu]Cu-SarAr-Tz is not quantitative and/or exhibits low radiochemical purity | Not enough precursor was added to the radiolabeling reaction | Make sure that the correct amount of precursor was used |
| 36 and 48 | The peaks in the radio-iTLC chromatogram are smeared | The iTLC plate was smeared from wrapping the plate with plastic wrap | Make sure that the plate is completely air-dried before wrapping it in plastic wrap |
| 48 | The radiolabeling of [$^{177}$Lu]Lu-DOTA-$PEG_7$-Tz is not quantitative and/or exhibits low radiochemical purity | The click reaction was incomplete | Increase the incubation time of the reaction |
| | The radiolabeling of [$^{177}$Lu]Lu-DOTA-$PEG_7$-Tz is not quantitative and/or exhibits low radiochemical purity | The radiolabeling was performed at the wrong pH | Prepare new buffers and double-check the pH of said buffers during the radiolabeling process by using pH papers |

Table continued

**Table 2 (continued)**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| | The radiolabeling of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz is not quantitative and/or exhibits low radiochemical purity | The radionuclide in the stock solution has low specific activity | Check with the producers of the radionuclide to ensure that the specific activity of the radionuclide is sufficient |
| | The radiolabeling of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz is not quantitative and/or exhibits low radiochemical purity | Not enough precursor was added to the radiolabeling reaction | Make sure that the correct amount of precursor was used |
| 62A (viii) and 68 | High concentrations of $^{64}$Cu are observed in the liver | There is free [$^{64}$Cu]Cu$^{2+}$ in the injectate | Check the radiochemical purity of the radioligand |
| | Low activity concentrations are observed in the tumor | The wrong molar ratio of mAb-TCO and Tz radioligand was used | Check the molar ratio of the two components administered to ensure that it is ~1:1 |
| | Low activity concentrations are observed in the tumor | The mAb-TCO has been rapidly internalized in the tumor | If the mAb is internalized rapidly, in vivo pretargeting is not a viable method |
| | Low activity concentrations are observed in the tumor | The interval time between the injection of the two components is too long | Shorten the interval time between the administration of the two components |
| | Low activity concentrations are observed in the tumor | The radioligand was not prepared correctly | Validate the synthesis of the radioligand in question via HPLC. See the rows above that address troubleshooting the preparation of the radioligands |
| | Low activity concentrations are observed in the tumor | The mAb-TCO conjugate was not prepared correctly | See the rows above that address troubleshooting the preparation of the immunoconjugate |
| | Low activity concentrations are observed in the tumor | The mAb has lost immunoreactivity because of improper handling | Ensure that the antibody and immunoconjugate are handled and stored in an appropriate manner |
| | Low activity concentrations are observed in the tumor | The mAb has lost immunoreactivity because of the conjugation of TCO moieties in the antigen-binding domains | Perform the bioconjugation reaction again and be sure to perform immunoreactivity assays to ensure that the conjugation did not affect the reactivity of the antibody. If needed, decrease the DOL of the immunoconjugate to reduce the likelihood that TCOs are affecting the antigen-binding domains |
| | Low activity concentrations are observed in the tumor | The TCO has isomerized to CCO and is thus no longer reactive | Prepare a new batch of mAb-TCO and store it appropriately |
| | High activity concentrations are observed in the blood | Free antibody is still circulating at the time of the injection of the radioligand | Increase the interval time between the administration of the mAb-TCO and the radioligand |
| | High activity concentrations are observed in healthy tissues | The reactivity of the immunoconjugate has been compromised so that it is not binding its target | See the sections above that address ensuring the biological integrity of the immunoconjugate |
| | High activity concentrations are observed in healthy tissues | The target of the immunoconjugate is present in healthy tissues | This immunoconjugate may not be suitable for this approach |

## Timing

**Procedure 1**

Steps 1–9, synthesis of SarAr-Bn-NHBoc: ~3 d

Steps 10–18, synthesis of SarAr-Bn-NH$_2$: ~2 d

Steps 19–28, synthesis of SarAr-Tz: ~2 d

Steps 29–36, synthesis of Tz-PEG$_7$-NHBoc: ~1 d

Steps 37–43, synthesis of Tz-PEG$_7$-NH$_2$: ~1 d

Steps 44–52, synthesis of DOTA-PEG$_7$-Tz: ~1 d

**Procedure 2**

Steps 1–27, preparation and purification of huA33-TCO: ~3 h

Steps 28–38, radiolabeling of SarAr-Tz with [$^{64}$Cu]CuCl$_2$: ~1 h

Steps 39–50, radiolabeling of DOTA-PEG$_7$-Tz with [$^{177}$Lu]LuCl$_3$: 1.5 h
Steps 51–61, injection of huA33-TCO vector (highly variable depending on the size and number of the cohorts used): 2 h
Step 62A(i–vii), injection of PET radiotracer (highly variable depending on the size and number of the cohorts used): 2 h
Step 62A(viii), pretargeted PET imaging (for each PET time point, highly variable depending on the size and number of the cohorts used): 2 h
Step 62B(i–vii), injection of PRIT radiotracer (highly variable depending on the size and number of the cohorts used): 2 h
Step 62B(viii), checking on well-being of mice and measuring tumor sizes for PRIT (for each time point, highly variable depending on the size and number of the cohorts used): 2 h
Steps 63–71, ex vivo biodistribution (for each time point, highly variable depending on the size and number of the cohorts used): 2 h

## Anticipated results

### SarAr-Bn-NHBoc

Analytical data for SarAr-Bn-NHBoc were as follows: white powder (typical results: 35 mg; 0.065 mmol; 65–75% yield); $^1$H NMR (600 MHz, DMSO-d$_6$) p.p.m.: δ = 7.38–7.50 (m, 4H), 4.18 (m, 2H), 2.31–3.98 (m, 42H), 1.35 (s, 9H); ESI-MS(+): mass-to-charge ratio (*m/z*) = 534.5 [M+H]$^+$; high-resolution mass spectrometry (HRMS) (ESI)—*m/z* calculated for $C_{27}H_{52}N_9O_2$: 534.4244; found: 534.4250.

### SarAr-Bn-NH$_2$

Analytical data for SarAr-Bn-NH$_2$ were as follows: white powder (typical results: 26 mg; 0.059 mmol; quantitative yield); $^1$H NMR (600 MHz, DMSO-d$_6$) p.p.m.: δ = 7.55 (d, 2H), 7.51 (d, 2H), 4.23 (m, 2H), 2.56–4.05 (m, 27H), 1.35 (s, 9H); ESI-MS(+): *m/z* = 434.4 [M+H]$^+$; HRMS (ESI)—*m/z* calculated for $C_{22}H_{44}N_9$: 434.3720; found: 434.3715.

### SarAr-Tz

Analytical data for SarAr-Tz were as follows: pink powder (typical results: 3.9 mg; 0.0054 mmol; 40–45% yield); $^1$H NMR (600 MHz, D$_2$O) p.p.m.: δ = 10.25 (s, 1H), 8.31 (d, 2H), 7.46 (d, 2H), 7.22–7.27 (m, 4H), 4.39 (m, 2H), 4.24 (m, 3H), 2.46–3.95 (m, 24H), 2.41–2.44 (m, 4H), 1.83 (m, 2H); $^{13}$C NMR (125 MHz, D$_2$O): δ = 21.7, 34.7, 42.5, 42.7, 47.4, 52.5, 55.9, 57.2, 58.8, 60.1, 116.2 (TFA), 127.7, 128.0, 128.5, 130.3, 135.4, 138.5, 143.5, 157.3, 162.9 (TFA), 166.3, 175.7, 175.8; ESI-MS(+): *m/z* = 717.6 [M+H]$^+$; HRMS (ESI)—*m/z* calculated for $C_{36}H_{57}N_{14}O_2$: 717.4789; found: 717.4788.

### Tz-PEG$_7$-NHBoc

Analytical data for Tz-PEG$_7$-NHBoc were as follows: bright pink powder (typical results: 16 mg; 0.021 mmol; 80–90% yield); $^1$H NMR (500 MHz, DMSO) p.p.m.: δ = 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); ESI-MS (+): *m/z* (%) = 753.1 [M+H]$^+$; HRMS (ESI)—*m/z* calculated for $C_{35}H_{57}N_7O_{11}Na$: 774.4005; found: 774.4014; UV-visible: $\varepsilon_{525}$ = 530 M$^{-1}$cm$^{-1}$.

### Tz-PEG$_7$-NH$_2$

Analytical data for Tz-PEG$_7$-NH2 were as follows: bright pink powder (typical results: 9 mg; 0.013 mmol; 90–95% yield); $^1$H NMR (500 MHz, DMSO) p.p.m.: δ = 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); ESI-MS(+): *m/z* (%) = 652.9 [M+H]$^+$; HRMS (ESI)—*m/z* calculated for $C_{30}H_{50}N_7O_9$: 652.3670; found: 652.3676; UV-visible: $\varepsilon_{525}$ = 535 M$^{-1}$cm$^{-1}$.

### DOTA-PEG$_7$-Tz

Analytical data for DOTA-PEG$_7$-Tz were as follows: bright pink powder (typical results: 15.4 mg; 0.0128 mmol; 70–75% yield); $^1$H NMR (600 MHz, DMSO) p.p.m.: δ = 12.2 (bs, 4H), 11.74 (s, 1H) 10.59 (s, 1H), 8.55 (t, 1H, J = 5.9 Hz), 8.51 (t, 1H, J = 5.9 Hz), 8.46 (d, 2H, J = 8.2 Hz), 7.79 (d, 2H, J

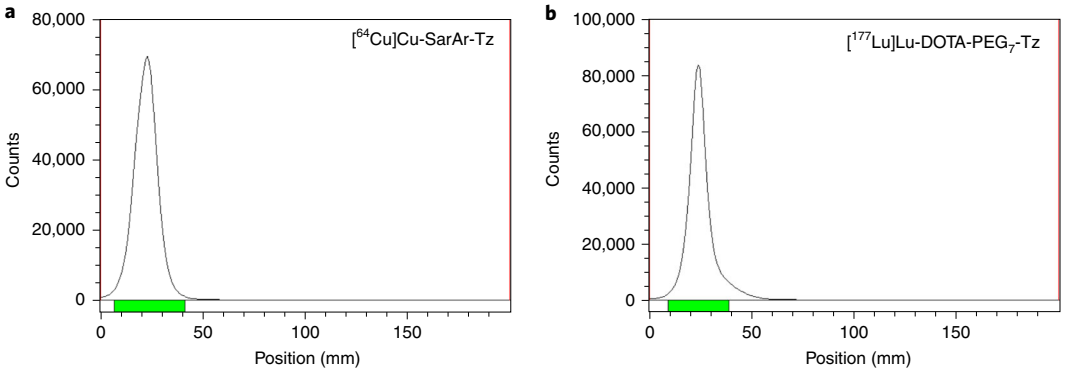


**Fig. 7 | Representative radio-iTLC chromatograms for radiosynthesis. a**, [$^{64}$Cu]Cu-SarAr-Tz. **b**, [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz. The reaction times were 15 and 30 min, respectively.

= 8.2 Hz), 7.55 (d, 2H, J = 8.3 Hz), 7.44 (d, 2H, J = 8.2 Hz), 7.42 (t, 1H, J = 7.7 Hz), 4.42 (d, 2H, J = 5.9 Hz), 3.80–3.40 (m, 38H), 2.51 (s, 1H), 2.69 (t, 1H, J = 6.0 Hz), 2.50–2.30 (m, 16H), 2.09 (t, 4H, J = 7.0 Hz); $^{13}$C NMR (600 MHz, DMSO) p.p.m.: δ = 173.88, 173.16, 170.96, 169.99, 169.31, 165.89, 158.61, 146.09, 145.41, 132.69, 130.77, 128.80, 128.47, 128.22, 70.36, 70.13, 70.11, 70.07, 70.06, 67.35, 66.68, 42.25, 42.18, 40.48, 36.65, 36.59, 35.17, 32.64, 31.19, 28.94, 27.28; ESI-MS(−): *m/z* (%) = 1203.0 $[M-H]^-$; 601.8 $[M-2H]^{-2}$; HRMS (ESI)—*m/z* calculated for $C_{50}H_{76}N_{11}O_{15}S$: 1202.5642; found: 1203.5741; UV-visible: $\varepsilon_{525} = 500\ M^{-1}cm^{-1}$.

### Preparation of huA33-TCO

The preparation of huA33-TCO is a straightforward task. The huA33-TCO immunoconjugate prepared as described in this protocol has between two and five TCO groups per antibody (this occupancy is determined as described in Box 1) and has an immunoreactive fraction of >0.90 (when labeled with [$^{64}$Cu]Cu-SarAr-Tz).

### Radiosynthesis of [$^{64}$Cu]Cu-SarAr-Tz

The radiolabeling of SarAr-Tz is likewise relatively simple. The reaction is typically complete after 15 min, affording [$^{64}$Cu]Cu-SarAr-Tz in >99% radiochemical yield, >99% radiochemical purity and a molar activity of 15.3–16.4 GBq (413.4–443.3 mCi)/μmol ($n$ = 3) without any need for further purification (Fig. 7).

### Radiosynthesis of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz

The radiolabeling of DOTA-PEG$_7$-Tz is also a facile endeavor. The reaction is usually complete after 30 min, affording [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz in >99% radiochemical yield, >99% radiochemical purity and a molar activity of 46.8–71.1 GBq (1.264–1.921 Ci)/μmol ($n$ = 3) without any need for further purification (Fig. 7).

### Pretargeted PET imaging

The pretargeted PET imaging experiments described in this protocol were performed in athymic nude mice bearing A33 antigen-expressing SW1222 human colorectal carcinoma xenografts. For the pretargeted PET imaging, the mice were first injected with huA33-TCO (100 μg; 0.7 nmol) via tail vein injection and then administered [$^{64}$Cu]Cu-SarAr-Tz (14.8 MBq (0.4 mCi); 0.7 nmol, also via the tail vein) 24, 48 or 120 h later. For the pretargeted biodistribution study, huA33-TCO (100 μg; 0.7 nmol) was administered to athymic nude mice bearing SW1222 xenografts 24, 48 or 120 h before the injection of [$^{64}$Cu]Cu-SarAr (11.1 MBq (0.30 mCi); 0.7 nmol). Critically, both the mass of the huA33-TCO immunoconjugate and the 1:1 molar ratio of the two components were selected on the basis of values from the literature; indeed, the excellent work of Duijnhoven et al.[30] was particularly important with regard to the chosen molar ratio. In light of the 12.7-h half-life of $^{64}$Cu and the rapid pharmacokinetic profile of [$^{64}$Cu]Cu-SarAr-Tz, we have found that time points of 4, 12 and 24 h after the administration of the radioligand are best suited for the collection of PET images and tissue samples.

Both the PET images and biodistribution data reveal the accumulation of [$^{64}$Cu]Cu-SarAr-Tz in tumor tissue as early as 4 h after injection, with the activity concentration in the xenograft increasing

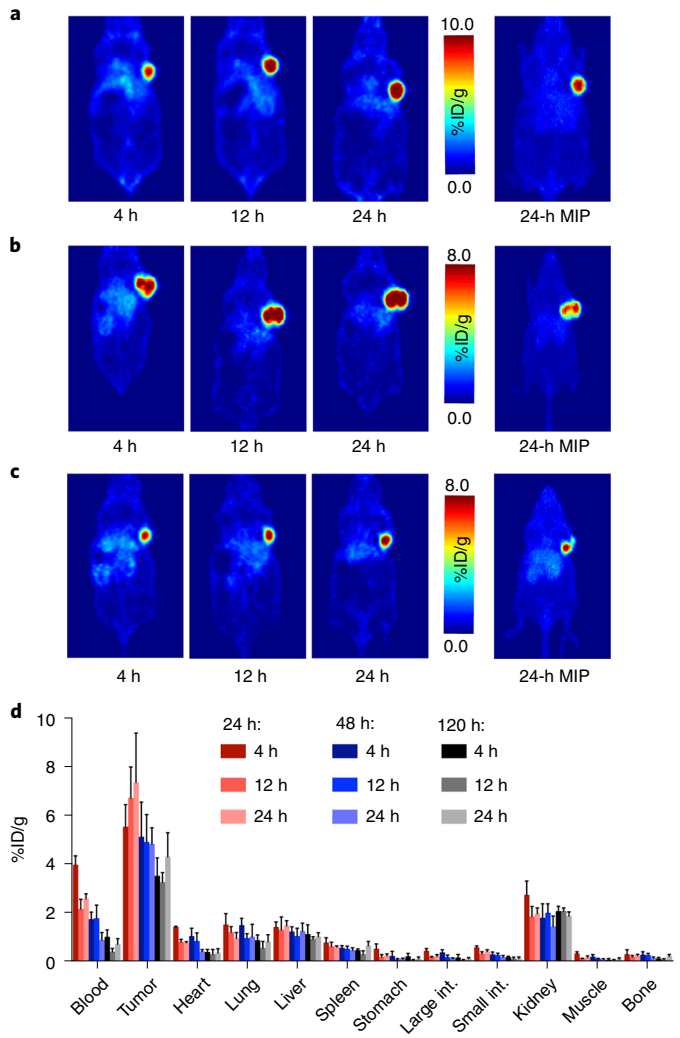


**Fig. 8 | Representative pretargeted PET images.** [$^{64}$Cu]Cu-SarAr-Tz and huA33-TCO were used with 24- (**a**), 48- (**b**) and 120-h (**c**) interval times between the two injections. **d**, Biodistribution data for in vivo pretargeting with huA33-TCO and [$^{64}$Cu]Cu-SarAr-Tz in athymic nude mice bearing subcutaneous SW1222 human colorectal cancer xenografts ($n = 4$) by using pretargeting intervals of 24 (red), 48 (blue) or 120 (gray) h. At each interval, mice were sacrificed at 4, 12 and 24 h after the administration of the radioligand. int., intestine; MIP, maximum intensity projection. Image reproduced with permission from ref. [21]. Copyright 2015 American Chemical Society.

over the course of the experiment to maximum values of 8–10 %ID/g by 24 h after injection (Fig. 8). Indeed, even as early as 4 h after injection, the tumor is easily the most readily visualized tissue. The activity concentrations in healthy organs generally remain low (<1 %ID/g) throughout the experiment, with the highest level of background uptake in the kidneys (typically ~2 %ID/g at 24 h after injection). The activity concentration in the blood decreases relatively slowly over the course of the study, probably because of ligations between [$^{64}$Cu]Cu-SarAr-Tz and circulating huA33-TCO that create $^{64}$Cu-labeled huA33 in the blood that subsequently accumulates in the tumor. The frequency of these click reactions—and thus the activity concentration in the blood—can be reduced by increasing the interval time between the administration of the two components. For example, the activity concentration in the blood at 24 h after injection drops from 2.6 ± 0.2 to 0.7 ± 0.2 %ID/g upon increasing the interval time from 24 to 120 h. It is important to note, however, that extending the interval time can also decrease tumoral accretion—albeit to a lesser degree—probably because of the increased isomerization of TCO to CCO over longer lag times. To wit, the tumoral activity concentration at 24 h after injection decreases from 7.4 ± 2.0 to 4.3 ± 1.0 %ID/g (mean value ± standard deviation) when the lag time is increased from 24 to 120 h. Critically, control experiments using [$^{64}$Cu]Cu-SarAr-Tz alone, [$^{64}$Cu]Cu-SarAr-Tz in conjunction with unmodified huA33 and huA33-TCO in conjunction with low–specific activity [$^{64}$Cu]Cu-SarAr-Tz produce only minimal activity concentrations in the tumor (<0.5 %ID/g) (see ‘Experimental design’).

### PRIT

The most fundamental difference between the pretargeted PET and PRIT experiments is the use of [$^{64}$Cu]Cu-SarAr-Tz in the former and [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz in the latter. The biodistribution data for the PRIT system mirror that produced by huA33-TCO and [$^{64}$Cu]Cu-SarAr-Tz from the PET studies (Fig. 6a). In the biodistribution experiments, huA33-TCO (100 µg, 0.7 nmol) was administered to athymic nude mice bearing SW1222 xenografts 24, 48 or 72 h before the injection of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz (11.1 MBq (0.30 mCi), 0.7 nmol). Activity concentrations in the tumor are substantial at early time points (~5 %ID/g at 4 h after injection) and increase with time, ultimately reaching 15–20 %ID/g by 120 h after injection. Generally speaking, uptake in healthy tissues remains low. However, the gradual decrease in the activity concentrations in the blood suggests that [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz—like [$^{64}$Cu]Cu-SarAr-Tz—combines with circulating huA33-TCO to create $^{177}$Lu-labeled huA33 that remains in the blood before accumulating in the tumor. As in the PET experiments, longer injection intervals produce decreased activity concentrations in the blood but also reduced tumoral uptake values.

Longitudinal therapy studies clearly demonstrate the efficacy of this approach. Significant reductions in tumor growth are observed in the SW1222 xenograft-bearing mice receiving huA33-TCO (100 µg, 0.7 nmol) followed 24 h later by 18.5, 37.0 or 55.5 MBq (0.5, 1.0 or 1.5 mCi) of [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz (0.7 nmol in each case) (Fig. 6b). After a brief initial period of growth, the xenografts in the mice of each experimental cohort decrease in size dramatically, whereas those in the animals of the two control groups receiving only one half of the PRIT system (i.e., either huA33-TCO alone or 55.5 MBq (1.5 mCi) [$^{177}$Lu]Lu-DOTA-PEG$_7$-Tz alone) continue to grow unabated. A Kaplan-Meier plot underscores the potency of $^{177}$Lu-PRIT: although the mice of the two control cohorts exhibited median survival times of 20–25 d after the injection of the radiotracer, all of the mice in the three experimental cohorts survived for the duration of the 10 half-life (70-d) investigation (Fig. 6c).

### Data availability

The data described in 'Anticipated results' were derived from refs. [21] and [26], which are available in the public domain from the National Library of Medicine Database at https://pubmed.ncbi.nlm.nih.gov. Source data are provided with this paper.

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## Author contributions

All authors contributed extensively to the work presented and wrote the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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**Correspondence and requests for materials** should be addressed to B.M.Z.

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