INTRODUCTION

The use of radiolabeled monoclonal antibodies (mAbs) directed against tumor-associated antigens to visualize, characterize, and/or treat tumor lesions has been widely studied in preclinical and clinical studies (1–3). After intravenous injection, the accumulation of the radiolabeled mAbs in the tumor is slow due to the slow extravasation and tissue penetration of intact antibodies. Various physiological barriers between the circulation and the tumor cell surface, such as the vascular endothelium, the relatively large transport distances, and the high interstitial fluid pressure in tumors, prevent the rapid accretion of the antibodies in the tumor (4). As a result, only a small fraction of the injected activity will localize in the tumor. Despite this inefficient targeting, in various clinical settings this approach can be effective: especially radiosensitive tumors, like Non-Hodgkin’s lymphomas, can respond to treatment with radiolabeled anti-CD20 antibodies (5). Effective radioimmunotherapy (RIT) requires high tumor uptake and rapid clearance of radioactivity from normal tissues. Various approaches have been investigated to improve the tumor targeting such as reducing the circulatory half-life of the antibodies (4). Molecular engineering techniques made it possible to produce antibody formats that clear faster from the blood. However, this is the central dilemma in antibody targeting of tumors: on one hand, the high level of mAb in the circulation is the driving force for the accumulation of the mAb in the tumor; on the other hand, the clearance from normal tissues should be rapid. Therefore, innovative approaches are needed to enhance tumor accumulation while limiting retention in normal tissues. Pretargeting is an approach to solve this problem. Several pretargeting systems have been developed including the avidin/streptavidin–biotin pretargeting system (4, 6) exploiting the extremely high binding affinity between biotin and (strept)avidin ($K_D = 4 \times 10^{-14} \text{ M}$) (7), the DNA-complementary DNA binding pretargeting system, which relies on the high affinity interaction between complementary oligomers (8–11), pretargeting systems based on the use of bispecific antibodies (bsAb), and recently a novel pretargeting approach has been reported based on highly selective bioorthogonal chemical reactions (12, 13). All pretargeting systems are promising; however, each of these approaches has its advantages and limitations. For the avidin/streptavidin, the most significant limitation is the immunogenicity of the pretargeting agents: (strept)avidin being a protein that cannot be humanized. Additionally, the presence of endogenous biotin that could interfere with the system and the need to use a clearing agent to remove the residual antibody from the circulation before administration of the radiolabeled biotin limit the application of this system (14). For the DNA-complementary DNA binding pretargeting system, no immunogenic issues or complications due to the presence of competing endogenous species are reported. However, the oligonucleotides need to be modified to prevent rapid degradation by DNases and/or RNases (11). The pretargeting concept based on the use of bsAb directed against both a target antigen and a radiolabeled hapten is carried out in the following
two steps. In the first step, the bsAb is injected. After the bsAb has accumulated in the tumor due to the specific binding to the tumor-associated antigen and cleared from the blood, the radiolabeled peptide carrying the hapten is administrated. This small molecule will be trapped in the tumor by binding to the bsAb or cleared rapidly from the body via the kidneys. A schematic overview of this pretargeting strategy is shown in Figure 1. Depending on the radionuclide used this pretargeting approach can be used to visualize, or treat tumor lesions, for example, using $^{111}$In and $^{99m}$Tc for single-photon emission computed tomography (SPECT) imaging, $^{68}$Ga or $^{18}$F for positron emission tomography (PET) imaging, or $^{131}$I, $^{90}$Y, and $^{177}$Lu for pretargeted radioimmunotherapy (PRIT). A significant advantage of this system over the biotin/avidin-based approaches is that the primary pretargeting agent, the bsAb, can be humanized to reduce its immunogenicity. Additionally, the lower affinity of the bsAb-hapten binding compared to the avidin-biotin binding results in the rapid dissociation of the bsAb from the hapten in the circulation, thereby omitting the need for a clearing agent. In the past decade, several improvements on this system have been introduced, which resulted in a flexible universal and efficient pretargeting system. However, the non-covalent binding between the radiolabeled hapten and the bsAb at the tumor cell surface limit the retention of the radiolabeled hapten-peptide in the tumor. Recent developments in the use of bioorthogonal chemistry (cf. the rapid and selective formation of a covalent bond between the pretargeting agent and the radiolabeled agent in vivo) are very promising and show the potential of this system for efficient pretargeting of tumors. Thus, this chemical pretargeting system is an attractive alternative for bsAb-based pretargeting.

In this review, we will focus on the pretargeting concept based on the use of bsAb and additionally discuss the potential and the limitations of this novel bioorthogonal chemistry pretargeting approach.

**BISPECIFIC ANTIBODY-BASED PRETARGETING SYSTEMS**

The first studies on the use of a two-step system to target tumors were described by Reardan et al. (15). This group used antibodies against radiolabeled EDTA. This study showed that by separating the antibody tumor targeting from radionuclide targeting improved tumor-to-non-tumor ratios could be obtained (16). These antibodies had no affinity to the tumor and accumulated passively due to the enhanced permeability and retention effect in the tumor (4, 17, 18). For specific pretargeting of tumors, bsAb directed against both the tumor-associated antigens and the radiolabeled agent are required.

**FIRST GENERATION; ANTI-TUMOR Fab′ × ANTI-CHELATE-METAL Fab′ FRAGMENTS**

The first generation of bsAb for specific pretargeting methods was chemically conjugated anti-tumor Fab′ × anti-chelate-metal Fab′ fragments (6). Several preclinical studies revealed good tumor uptake (19, 20). Stickney et al. linked the sulfhydryl groups of Fab′ fragments of anti-CEA to those of an anti-benzyl-EDTA to form an F(ab′)$_2$ bifunctional antibody (19). The potential of this anti-CEA × anti-benzyl-EDTA F(ab′)$_2$ was evaluated in mice bearing colon tumor xenografts. The anti-CEA × anti-benzyl-EDTA Fab′ × Fab′ was injected 24 h prior to the i.v. administration of $^{111}$In-labeled benzyl-EDTA. One day later, the biodistribution revealed a tumor uptake of 18.5%ID/g, whereas the blood level was 1.3%ID/g. A clinical study was performed to assess the use of this approach in patients with recurrent colon carcinoma. In 14 patients, 20 of the 21 known tumor lesions were detected by scintigraphic imaging. Additionally, nine unknown lesions were detected of which eight could be confirmed.
SECOND GENERATION; THE USE OF A DIVALENT RADIOLabeled HAPten-PEPTIDE

The pretargeting system using anti-tumor Fab' × anti-chelate-metal Fab' fragments was based on monovalent binding to the chelate–radiometal complex at the tumor. The stability of such a complex is limited and rapid release of the radiolabeled chelate from the tumor may occur (21). Le Doussal et al. reported improved stability of the complex using divalent hapten-peptide (22). In BALB/c mice i.v. injection of bsAb directed against the lyb.8.2 antigen and the hapten dinitrophenyl (DNP) followed by either the mono \( [N\varepsilon-(2,4-dinitrophenyl)-l-lysyl-diethylenetriaminepentaacetic acid (DNP-DTPA)] \) or divalent (di-DNP-DTPA).

The \( ^{125}\text{I} \)-divalent DNP derivative exhibited a significantly higher binding as compared to the monovalent derivative to the pretargeted cells (for monovalent 23 vs. 65%ID/g for the divalent derivative). It was hypothesized that at the cell surface the divalent hapten could cross-link two bsAbs thereby stabilizing not only the binding to the bsAb but also the bsAb binding to the tumor. This so-called affinity enhancement system (AES) improved tumor targeting and retention of the radiolabeled di-hapten-peptide (20, 22). This AES was confirmed by different groups in several animal models, in which enhanced tumor uptake and improved retention of the radiolabeled di-hapten-peptide was observed (23–28).

For example, the use of a bsAb in combination with a divalent \( ^{111}\text{In} \)-DTPA-peptide resulted in a tumor uptake of 3.5%ID/g whereas the tumor uptake of the monovalent DTPA was 2.8%ID/g in mice with s.c. A375 melanoma xenograft. A pronounced AES effect was observed in nude mice with RCC xenografts using bsAb directed against the renal cell carcinoma-associated antigen G250, in combination with a divalent hapten-peptide, Phe–Lys(DTPA–\(^{111}\text{In})\)–Tyr–Lys(DTPA–\(^{111}\text{In}), \(^{111}\text{In}-\text{di-DTPA} (16). At 1 h p.i. of the hapten, an almost 10-fold higher tumor uptake of \(^{111}\text{In}-\text{di-DTPA} (80\%\text{ID/g}) \text{was observed as compared to the monovalent} \(^{111}\text{In}-\text{DTPA} (9\%\text{ID/g}; \text{Figure 2). Moreover, the retention of} \text{the radiolabel in the tumor at 72 h p.i. was significantly higher for the} \(^{111}\text{In}-\text{di-DTPA} (93 \pm 41\%\text{ID/g}) \text{compared to} \(^{111}\text{In}-\text{DTPA} (0.9 \pm 0.1\%\text{ID/g}).}

Based on the promising preclinical studies, a clinical trial was performed to evaluate the pretargeting approach in patients with primary colorectal cancers. In 11 patients with primary colorectal carcinoma tumors, anti-CEA × anti-DTPA Fab'–Fab' antibody was injected followed 2–8 days later by \(^{111}\text{In}-\text{labeled N-\alpha-(In-DTPA)-tyrosyl-N-\varepsilon-(In-DTPA)-lys} (^{111}\text{In-di-DTPA}) (29). For comparison, six patients with similar clinical status were injected with \(^{111}\text{In-anti-CEA F(ab')2. At 1–4 days p.i. of} \(^{111}\text{In-di-DTPA, the biodistribution results revealed a tumor uptake of 1.8–17.5\%\text{ID/kg and were similar to the tumor uptake of} \(^{111}\text{In-anti-CEA F(ab')2 (5.5–30.2\%ID/kg). However, the tumor-to-blood (T/B) and the tumor-to-liver (T/L) ratios were significantly improved in the pretargeting approach: 7.8 vs. 4.2 and 2.8 vs. 0.8, respectively (29).}

Besides using a pretargeting approach to detect tumor lesions, the pretargeting approach was also tested to treat tumor lesions. A clinical trial was conducted to estimate the dose delivered to tumor targets and normal tissues using a pretargeting approach consisting of bsAb anti-CEA × anti-DTPA and \(^{131}\text{I-di-DTPA} (30).

In the clinical trial patients with recurrent medullary thyroid carcinoma (MTC; five patients) and small-cell lung cancer (SCLC; five patients) were pretargeted with a bsAb anti-CEA × anti-DTPA Fab'–Fab' (0.1–0.3 mg/kg) and 4 days later 6 nmol (5.8–9.8 mCi) of \(^{131}\text{I-di-DTPA} \text{was administrated. The patients were included
based on the CEA expression as evaluated by immunohistochemical analysis of the biopsy of their primary tumor. The tumor uptake of \( ^{131} \)I-di-DTPA and the activity dose to the tumor was significant higher in MTC (average uptake 0.116%ID/g; dose ranged from 4.8 to 135 cGy/mCi) than in SCLC (average uptake 0.009%ID/g; dose ranged from 1.9 to 8 cGy/mCi), indicating that the MTC is a more suitable tumor type for PRIT. The therapeutic efficacy and toxicity of PRIT with escalating doses of bsAb anti-CEA \( \times \) anti-DTPA (20–50 mg) and 4 days later followed by \( ^{131} \)I-di-DTPA (1.48–3.7 GBq) was evaluated in 26 patients with recurrent MTC (24). The dose-limiting toxicity was hematologic and an activity dose of 1.78 GBq/m² could be injected safely in this group of patients. The maximum tumor uptake ranged from 0.003 to 0.26%ID/g and the tumor doses ranged from 7.9 to 500 Gy/MBq (2.94–184 cGy/mCi). From the 26 patients, 17 patients were included to evaluate the tumor response. The maximal tolerated dose (MTD) of 48 mCi/m² was administrated to 13 patients. A decrease in tumor mass, serum thyrocalcitonin, and CEA levels was observed in six of the patients receiving the MTD. The tumors that showed a response were generally small (maximum diameter of 37 mm).

Additionally, the anti-(In)DTPA had a much lower affinity for DTPA with other radiometals such as \(^{90} \)Y or \(^{177} \)Lu. Therefore, it appeared that imaging radionuclides require different antibodies directed against the specific anti-chelate-metal complex than the radionuclides of therapeutic interest.

**Third Generation; Bispecific Fab’2 Anti-CEA \( \times \) Anti-Histamine-Succinyl-Glycine**

A more universal/ flexible pretargeting system was developed by using an anti-hapten antibody not directed against the chelated radiometal, but directed against the histamine-succinyl-glycine (HSG) hapten (22, 32, 33). The HSG hapten itself is not involved in binding the radionuclide and thus other chelates suitable for binding radionuclides can be added, for example, the HSG peptides can be conjugated with various chelating moieties such as DTPA, DOTA, or N3S chelates for radio-labeling with \(^{111} \)In, \(^{177} \)Lu-177/Y-90, or Tc-99m/Re-188, respectively or with other agents/moieties such as fluorophores for fluorescence imaging. Sharkey et al. showed the potential of this system by using the bispecific anti-CEA F(ab’\( ^{2} \)) \( \times \) anti-HSG Fab in combination with HSG peptides labeled with either \(^{99m} \)Tc or \(^{188} \)Re via an N3S chelate or with \(^{111} \)In, \(^{177} \)Lu, and \(^{90} \)Y via a DOTA moiety (32). In nude mice with human colon tumor xenografts, this trivalent anti-CEA \( \times \) anti-HSG bsAb was injected 24–48 h prior to the i.v. administration of the divalent radiolabeled HSG peptides, \(^{111} \)In-IMP241, \(^{177} \)Lu-IMP241, and \(^{99m} \)Tc-IMP243. The tumor uptake of radiolabeled HSG peptides after pretargeting with the bsAb was significantly higher than with the peptides alone (for \(^{111}\)In-IMP241 0.03 ± 0.02 vs. 2.54 ± 0.44%ID/g, for \(^{177}\)Lu-IMP241 0.03 ± 0.01 vs. 2.59 ± 0.30%ID/g, and for \(^{99m}\)Tc-IMP243 0.1 ± 0.02 vs. 7.36 ± 3.19%ID/g). The tumor-to-normal tissue ratio exceeded 8:1 within 3 h p.i. of the hapten-peptide. Consequently, this provides the opportunity to use the same HSG peptide for different purposes depending on the radionuclide used, for example, using \(^{111}\)In and \(^{99m}\)Tc-labeled HSG peptide for SPECT imaging, \(^{124} \)I, \(^{68} \)Ga, or \(^{18} \)F-labeled HSG peptide for PET imaging or \(^{131} \)I, \(^{90} \)Y, and \(^{177} \)Lu labeled HSG peptide for PRIT (32–38). Besides the flexibility of the system, Sharkey et al. showed that the hapten–peptide structure can alter the biodistribution and clearance of the construct. The liver and kidneys uptake of \(^{99m}\)Tc-hapten peptide consisting of a N3S chelate and DOTA (IMP245) was significantly lower compared to the \(^{99m}\)Tc-hapten bearing only the N3S chelate whereas the tumor uptake and retention did not change. Therefore, when less background is essential around the kidneys and/or urinary bladder, the preferable renal elimination can be altered to a more hepatic elimination by modifying the hapten-peptide construct.

Further improvements were obtained using a recombinant bispecific trivalent construct, hBS14, with bivalent CEA binding and a monovalent HSG binding (39). The trivalent antibody was produced by myeloma cells transfected with hBS14-pdHL2 DNA vector and purified to near homogeneity in a single step using a novel HSG-based affinity chromatography system. In nude mice bearing a CEA-expressing GW-39 human colon tumor xenograft the efficacy of the \(^{125} \)I-hBS14 (0.4 nmol) in combination with the bivalent \(^{111}\)In-HSG-hapten (0.04 nmol; 27 h interval), IMP241, was evaluated. At 3 h p.i., the \(^{111}\)In-IMP241 tumor uptake was
19.1 ± 8.7% ID/g and the blood levels were only 0.25 ± 0.08% ID/g corresponding with a tumor-to-blood ratio of 83 ± 44% ID/g. Technology that exploits fusing two hybridoma Ab-producing cells using quadroma technology did not produce adequate yields in mammalian cell cultures. Therefore, a new strategy capable of preparing a trivalent bsAb was developed. Humanized trivalent Fab bsAb constructs were produced at a higher yield by the dock-and-lock (DNL) technology. The DNL technology is based on the natural association between protein kinase A (PKA; cyclic AMP-dependent protein kinase) and A-kinase anchoring proteins. The natural association between protein kinase A (PKA; cyclic AMP-dependent protein kinase) and A-kinase anchoring proteins is used to anchor the trivalent bsAb to a target cell.

FIGURE 3 | Schematic representation of how a trivalent bispecific antibody is formed by the DNL method. Component A links a cysteine-modified dimerization and docking domain (DDD) to a Fab of an anti-tumor antibody, which results in spontaneous formation of component A2. Component B links an anchoring domain to a Fab of an anti-hapten antibody. The AD is modified with cysteine on each end. AD will naturally “dock” with the DDD when components A and B are mixed, which brings the two molecules together in a well-defined orientation and also results in disulfide bonds across the two proteins. This research was originally published in Goldenberg et al. (42).

Using the DNL technology, several recombinant humanized bsAbs with a divalent antigen specificity and with a monovalent specificity to HSG were produced including an anti-CEA bsAb (TF2) (43, 44), an anti-Trop2 (TF12) (45), an anti-CD20 (46), and an anti-MUC1 (47) bsAb. An effective detection and treatment of tumor lesions using a pretargeting system is best illustrated by the work done on TF2. The ability to detect small micrometastatic human colon cancer nodules (<0.3 mm in diameter) in the lungs of nude mice using this system was evaluated and compared to 18F-FDG-PET imaging and to TF6, an irrelevant anti-CD22-based bsAb. The tumors were first pretargeted with TF2 and 21–24 h later the 124I or 131I radiolabeled hapten-peptides were injected at a bsAb:hapten molar ratio of 10:1. The TF2-pretargeted tumors in the lungs could be localized at 1.5 h p.i. of radiolabeled HSG peptide whereas the peptide alone, TF6, or 18F-FDG failed. This showed the high potential of the system for sensitive and specific imaging of tumor lesions.

The specificity of the TF2 based pretargeting system was further evaluated using di-HSG peptides radiolabeled with 68Ga, a positron emitting radionuclide with a more suitable half-life (68 min) for pretargeted imaging purposes than 124I (4.2 days) (48). Nude mice were s.c. implanted with CEA-expressing LS174T human colonic tumor, a CEA-negative tumor, or an inflammation model (49). Nude mice were s.c. implanted with CEA-expressing LS174T human colonic tumor, a CEA-negative tumor, or an inflammation model. In line with the previous study, the 18F-FDG uptake in all the present tumors was sufficient, however, also an uptake was observed in various normal tissues such as the intestines.

To evaluate the use of the TF2 and 177Lu-IMP288 in PRIT, the therapeutic efficacy and toxicity of the system was evaluated in mice with s.c. LS174T tumors (50). The median survival of mice treated with 1, 2, or 3 cycles of PRIT was 24, 45, and 65 days, respectively, whereas in the untreated mice the median survival was 13 days. Additionally, PRIT effectively delayed the tumor growth with limited hematologic toxicity, indicating that PRIT using TF2 and 177Lu-IMP288 might be an effective treatment against colon cancer. Furthermore, the biodistribution of 111In-IMP288 and 177Lu-IMP288 in mice with intraperitoneal LS174T tumors was identical as observed by ex vivo counting as well as by pretargeted immuno-SPECT imaging (51). This indicates that pretargeted immuno-SPECT with TF2 and 111In-IMP288 can be used for the non-invasive monitoring of the therapeutic efficacy of PRIT with TF2 and 177Lu-IMP288 in mice with LS174T lesions.

The preclinical pretargeting studies using TF2 in combination with radiolabeled di-HSG peptides indicated that PRIT can induce tumor growth inhibition. Therefore in the feasibility, safety and therapeutic efficacy of TF2/Lu-177-I-IMP288 for the treatment of CEA-expressing tumor lesions was investigated in a first-in-man phase I study in patients with advanced colorectal carcinoma. Four dose schedules in cohorts of five patients were evaluated (52). First, the effect of the time interval between administration of TF2 (75 mg) and IMP288 (100 μg) was evaluated: 5 days (cohort 1) and 1 day (cohort 2). Additionally, the effect of a higher bsAb
dose (150 mg TF2, 1-day interval, 100 µg IMP288, cohort 3) and a lower IMP288 dose (75 mg TF2, 1-day interval, 25 µg IMP288, cohort 4) was evaluated. Reducing the time interval and lowering the IMP288 dose resulted in improved tumor targeting. Although it is reported that higher bsAb doses results in improved tumor uptake, the wash-out of the tumor was observed after 24 h. The patients could tolerate 7.4 GBq of 177Lu-IMP288 without experiencing dose-limited toxicity, even though some patients (8 out of 20 patients) experienced some level of hematologic toxicity. The TF2 bsAb induced human anti-human antibodies (HAHA) in 11 out of 21 patients. Most likely, the best PRIT regime will be a fractionated multi-dose treatment regimen (e.g., multiple cycles) as observed in preclinical pretargeting experiments (50) and recent clinical trials using 90Y-labeled antibodies in combination with gemcitabine (53). However, further clinical studies regarding the pretargeting conditions and protocol are needed.

A DIFFERENT APPROACH; PRETARGETING BASED ON BIOORTHOGONAL CHEMISTRY

The combination of chemistry and biology has resulted in many innovations and has contributed to our understanding of many biological processes. Nevertheless, many biomolecules including lipids, glycans, and nucleic acids as well as various posttranslational modifications cannot be monitored with genetically encoded reporters as manipulations can interfere with the structure and function of the molecules or the molecules are not genetically encoded. Therefore, new methods were investigated to covalently modify biomolecules in vivo. This has led to the discovery of bioorthogonal reactions to detect, track, or visualize various biomolecules. Using bioorthogonal chemistry, as defined by Bertozzi et al., reactions can occur inside a living organism, which do not interfere or cross-react with naturally occurring functionalities, are reactive under mild and physiological conditions and should not induce cellular toxicity (54). Over the years, several bioorthogonal chemical ligation strategies have been developed including the tetrazine ligation, Staudinger ligation, oxime/hydrazone formation from aldehydes and ketones, and quadricyclane ligation [for reviews on these bioorthogonal chemical reactions see in Ref. (13, 55, 56)]. The archetypical bioorthogonal reaction is the copper-catalyzed 1,3-dipolar Huisgen "click" cycloaddition between azides and alkynes (55). Using this click chemistry, units are joined with heteroatom links (C-X-C) in a modular, rapid, and easy manner without the generation of toxic byproducts (57).
and flexibility as well as the bioorthogonality and speed of click chemistry make it an ideal method for the creation of radiopharmaceuticals, especially 18F-radiolabeled peptides (58). The binding of a radiolabeled probe to the tumor-targeted antibody might also be obtained using bioorthogonal reactions. In general, this chemical approach of pretargeting is less likely to be immunogenic and could provide a universal approach for tagging and in vivo tracking of Abs. However, the need for a copper catalyst in coupling of azide and terminal alkyne to generate a triazole limits the use of the 1,3-Huisgen cycloaddition reaction in biological systems due to the toxicity of copper in vivo. Various bioorthogonal reactions have been developed that do not need the presence of a Cu catalyst. For example, copper-free click chemistry can be achieved via the relief of strain (strain-promoted azide-alkyne cycloadditions, SPAAC). Alternatively, Blackman et al. reported the very fast reaction kinetics and in vitro bioorthogonality of the inverse-electron-demand Diels–Alder (IEDDA) reaction between trans-cyclooctene (TCO) and electron deficient tetrazines (59). Subsequently, Devaraj et al. tagged the anti-EGFR antibody cetuximab with trans-cyclooctene succinimidy carbonate and combined with a fluorescent tetrazine probe was able to target A549 cancer cells in serum at 37°C (60). A schematic overview depicting the use of TCO-modified mAb and probe was able to target A549 cancer cells in serum at 37°C (60). A schematic overview depicting the use of TCO-modified mAb and probe was able to target A549 cancer cells in serum at 37°C (60).

The fast reaction kinetics \[ k_2 = 13,090 \text{ M}^{-1}\text{s}^{-1} \] (60) of the IEDDA reaction between the trans-cyclooctene and tetrazines are very promising, however, are still significantly lower compared to the association constants of the non-covalent high affinity interactions used in humans \( (3 \times 10^5 \text{ up to } 7.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}) \). In addition, Rossin et al. reported that the TCO can be deactiviated through isomerization to the unreactive cis-cyclooctene (CCO) isomer via copper-containing proteins (e.g., transcuprein, mouse serum albumin, ceruloplasmin) (61). TCO tags that are linked to the antibody through an axial substituent afforded a marked increase of the reactivity (61). In conjunction, the stability of the TCO tag could be improved by removal of the PEG linker between the TCO and the lysine residue on the Ab, increasing the steric hindrance on the TCO thus hampering interaction with serum protein-bound copper. The increased reactivity (up to \( k_2 = 2.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) in PBS (61) and improved stability of the IEDDA between a tetrazine and TCO-tagged antibody resulted in an improved tumor-to-blood ratio (61). Devaraj et al. (64) reported that the in vivo reaction yield of a moderately reactive IEDDA system \( (k_2 = 6 \times 10^3 \text{ M}^{-1}\text{s}^{-1} \text{ in PBS}) \) could be improved.

![FIGURE 6](https://www.frontiersin.org/)

**FIGURE 6** | Schematic overview of tumor pretargeting by using the inverse-electron-demand Diels–Alder reaction. This research was originally published in Rossin et al. (60).
by altering the pharmacokinetics of the radiolabeled probe by polymer conjugation (64). In mice with LS174T xenografts TCO-tagged A33 antibody (30 µg) was administrated and followed 24 h later by the $^{18}$F-labeled, dextran-based tetrazine probe (30 µg; 150 µCi). PET imaging 3 h p.i. of the $^{18}$F-labeled tracer revealed a significant higher tumor accumulation in TCO-tagged antibody pretargeted mice compared to mice pretargeted with Ab lacking TCO. However, the tumor-to-blood ratio still remained low due to the relative high amount of free-circulating CC49-TCO. For effective tumor targeting, a clearing agent was developed that could clear the TCO-tagged antibody from the circulation prior to administration of the radiolabeled tetrazine (61). In mice with LS174T tumor xenografts, $^{125}$I-CC49-TCO was injected after which a single or double dose (30 h or 30 and 48 h after mAb injection) of the clearing agent, followed 2 h later by $^{177}$Lu-tetrazine. Three hours p.i., the blood level of $^{125}$I-CC49-TCO was lowest after the double dose of clearing agent (0.19 ± 0.04%ID/g), followed by the single dose (1.16 ± 0.43%ID/g) and highest in the group without clearing agent injection (8.47 ± 4.12%ID/g). The biodistribution of $^{177}$Lu-tetrazine showed that the elimination of the free CC49-TCO in combination with a reduced amount of tetrazine (from 17 to 6.7 nmol) significantly improved the tumor uptake from 3.12 ± 0.87%ID/g (60) to 7.45 ± 1.46%ID/g for the single dose and to 6.13 ± 1.09%ID/g for the double dose of clearing agent. Both single as well as the double dose approach significantly increased the tumor-to-muscle and tumor-to-blood ratios compared to the previous approach without clearing agent. In fact, a 125-fold improvement of the tumor-to-blood ratio at 3 h after tetrazine injection was achieved with a double dose of clearing agent. Corresponding mouse dosimetry experiments suggested that at MTD for bone marrow (dose-limiting organ for both approaches), this should allow for an eightfold higher tumor dose than is possible with non-pretargeted RT. The estimated dose to the tumor in mice with directly $^{177}$Lu-labeled CC49 is significantly higher compared to the pretargeted approach using CC49-TCO and $^{177}$Lu-Tz (9573 vs. 479 mGy/MBq). However, also the normal tissues receive high doses, for example, the bone marrow receive 1136 mGy/MBq, whereas in the pretargeting approach the bone marrow dose is 7 mGy/MBq. Subsequently, this system was further improved by modifying the pharmacokinetics of the tagged antibody and by increasing the stability of the tag. The use of a more hydrophilic tag with an increased in vivo tag stability ($t_{1/2} = 10$ days) afforded a slower clearing CC49-TCO conjugate with increased tumor targeting, resulting in a 50% increased tumor uptake and T/NT ratios of the tetrazine probe (65). So far, the reaction of TCO with 1,2,4,5-tetrazines is the...
fastest bioorthogonal reaction (61). While others were unsuccessful in enlisting the slower SPAAC for antibody-based pretargeting in mice (62). Lee et al. reported a pretargeting system consisting of azido-dibenzocyclooctynes modified mesoporous silica nanoparticles in combination with 18F-azide and achieved tumor uptake in mice, possibly due to the high loading capacity of the mesoporous particles (66). Further work is required to establish whether the fastest cyclooctynes enable efficient SPAAC-based pretargeting with antibodies as well.

CONCLUSION
Clinical studies revealed that the pretargeting approach based on bsAb directed against a tumor-associated antigen and against HSG in combination with radiolabeled (divalent) peptides containing the HSG residue resulted in high contrast images. Therefore, the use of this pretargeting system is very promising for non-invasive imaging of tumors before, during, and after therapy. Additionally, pretargeted RIT using this system is capable to inhibit tumor growth with minor toxicity. However, to fully develop this pretargeting approach in the clinic research should focus on the best regime and protocol. Although several comparative studies between a pretargeting approach and directly radiolabeled antibodies have been performed, further quantitative comparative studies are needed to evaluate the potential of the pretargeted imaging and therapy. Furthermore, preclinical studies using bioorthogonal chemical pretargeting based on the inverse Diels–Alder cycloaddition revealed that tumors can be imaged and treated with this new approach. This approach is still being further optimized and clinical studies are warranted to determine the potential of this new strategy.

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