Monoclonal antibodies (mAbs) have long been recognized as effective tumor-targeting vehicles. Targeted delivery of radionuclides to tumor-associated antigens for imaging and therapy of cancer is an active research area in the preclinical as well as the clinical domain [2]. The U.S. Food and Drug Administration’s (FDA) approval at the beginning of this century of the first two radiolabeled monoclonal antibodies (Zevalin® and Bexxar®) for the treatment of non-Hodgkin’s lymphoma patients is a landmark event in the history of radioimmunotherapy (RIT, Figures 1 and 2). Targeted radiopharmaceuticals are well-suited for the therapy of this disease, as the tumors are very accessible to antibodies, and the lymphocytes are very sensitive to radiation.

The absorbed dose in the tumor vs. that in normal tissue is an important predictor for therapeutic success and the occurrence of side effects in RIT. In contrast to lymphohematopoietic cancers, solid tumors such as colorectal cancer have been much less responsive to RIT. Solid tumors show a very low uptake (0.001–0.01% injected dose per gram tumor) of the targeting antibody, resulting in a cumulative tumor dose of 1500 cGy, while typically 5000 cGy is needed to achieve therapeutic responses in most cancers. When the tumor uptake is limited, the dose needed to achieve a therapeutic response cannot be administered because of the need to restrict the dose to other, normal tissue. For example, the slow plasma clearance properties of radiolabeled antibodies result in prolonged exposure of the highly radiosensitive bone marrow to ionizing radiation.

Despite the high clinical need and large potential markets, solid tumors have remained out of reach of RIT. This challenge has led to a number of approaches to improving the tumor-to-nontumor radiation dose ratios to a level that will allow effective tackling of the tumor while sparing normal tissues.

The tumor-to-nontumor dose ratio can be boosted by a two-step targeting approach known as pretargeting (see Intermezzo and Figure 1), which essentially separates the delivery of the radioactive isotope from that of the antibody [3]. In this approach, a non-radiolabeled antibody modified with a tag is injected and allowed to reach maximum tumor uptake and sufficient clearance from blood. The clearance from blood may be accelerated by injecting a clearing agent, also known as a chase, which binds to the residual antibodies in blood and rapidly directs these to the liver. Then, a relatively small and fast-clearing molecule carrying a radionuclide is administered that quickly binds to the tumor-localized antibody via the tag, while non-bound probe is rapidly cleared from the body.

Pretargeting combines the excellent tumor-seeking properties of antibodies with the fast distribution and clearance of low molecular weight probe, and has demonstrated an ability to provide substantially higher tumor-to-nontumor ratios of radioactivity than those achievable through the standard one-step radioimmunotherapy approach [3].

Two pretargeting approaches have been evaluated in the clinic:

- Procedures that use the interaction between biotin-streptavidin
- Procedures that use bispecific antibodies with affinity for the tumor and for a radiolabeled small molecule [3].

A phase I pretargeting study in 15 non-Hodgkin’s lymphoma patients using an anti-CD20/ streptavidin fusion protein and an 90Y-labeled biotin probe afforded a high mean tumor-to-whole-body radiation dose ratio of 49 and two complete responses [4]. A similar approach in nine colorectal cancer patients gave an 8- to 11-fold increase of tumor-to-normal organ dose ratios compared to conventional RIT [5].

**Note:** This contribution is partly based on an article previously published in Angewandte Chemie International [1].
**INTERMEZZO**

**Tumor-to-nontumor dose ratios in RIT and pretargeted RIT**

The absorbed dose in the tumor vs. that in normal tissue is an important predictor for therapeutic success and the occurrence of side effects in RIT. Figure I visualizes the substantial reduction in nontumor radiation dose by using pretargeted RIT as opposed to conventional RIT in which the antibody is radiolabeled before administration. In pretargeted RIT the antibody is radiolabeled in vivo at a moment that it has already reached a favorable tumor-to-nontumor ratio. This improved ratio allows for an increased radioactive dose and corresponding therapeutic efficacy, while nontumor radiation exposure of the patient is minimized.

**Dosimetry**

To optimize RIT, and future pretargeted RIT, the administered radioactivity dose can be tailored to each patient individually based on the internal dose distribution calculated from a PET or SPECT study using the same antibody radiolabeled with a diagnostic isotope. In clinical practice, however, image-based dosimetry is not yet routinely applied, leading to an under-treatment of a large proportion of the RIT patient population. Philips Research activities in this area are addressed elsewhere in this issue of Medicamundi [1].

**Reference**


However, to date not one pretargeting technology has won FDA approval. The superior image contrast and the ability to administer higher (therapeutic) radiation doses compared to directly labeled antibodies is offset by the drawbacks of the current biological pretargeting systems, such as immunogenicity (streptavidin systems) or the need to reengineer the parent antibody (bispecific antibodies). To address this, we sought to design a pretargeting system that combines a straightforward, rapid and cost-efficient antibody modification with only very limited perturbation of its in vivo properties and a low likelihood of immunogenicity. For this we turned towards a special class of organic reactions known as bioorthogonal reactions [6]. These precious and rare reactions have the unique characteristic that they occur in water and biological environments (e.g. pH 7, 37 °C) and are unperturbed by the wide range of constructs that are present in such complex mixtures.

The exquisite selectivity of these reactions has been exploited for the tagging and visualization of biomolecules in cells, zebrafish and mice for chemical biology research purposes [7-10]. However, the reactivity of the reactions used requires a high dose of probe to achieve detectable...
We therefore designed a novel tumor pretargeting approach based on the Diels–Alder reaction between tetrazine-DOTA derivative 1 radiolabeled with 111In and anti-TAG72 antibody CC49 (2) conjugated to TCO through the lysine residue (Figure 2). The TAG72 antigen was selected because of its limited internalization and shedding as well as its overexpression in a wide range of solid tumors, including colorectal cancer [12].

**Methods**

All animal experiments were approved by the ethical review committee of the Maastricht University Hospital (the Netherlands), and were performed according to the principles of laboratory animal care (NIH publication 85–23, revised 1985), and the Dutch national law “Wet op dc Dierproeven” (Stb 1985, 336).

**Imaging experiments**

Tumor-bearing mice were injected with 111In-tetrazine (21 μg/75 μL, 20-50 MBq) 24 h after administration of 100 μg mAb (CC49-TCO, CC49 or Rtx-TCO). Single photon emission computed tomography (SPECT) was
tumor, liver, kidney and thigh muscle. A phantom filled with a known amount of $^{111}$In was used to calibrate the scanner for tissue radioactivity quantification.

**Biodistribution experiments**

Dual isotope biodistribution experiments were performed by injecting tumor-bearing mice ($n=3$) intravenously with $^{125}$I-labeled mAbs (CC49-TCO, CC49 or Rtx-TCO, 100 μg/100 μL, ca. 0.2 MBq) and, 24 h later, with $^{111}$In-tetrazine (21 μg/75 μL, ca. 0.8 MBq). Three hours after tetrazine administration, the animals were anesthetized with isoflurane and sacrificed by cervical dislocation. Blood was withdrawn by heart puncture and organs and tissues of interest were harvested, blotted dry and weighed. The radioactivity of the samples was measured in a $\gamma$-counter along with standards to determine the %ID/g.

**Results and discussion**

The investigation commenced with determination of the stability and reactivity of the two components in biological media. In vitro assays in phosphate buffered saline (PBS), serum, and blood (Table 1) showed that $^{111}$In-1 should be stable for the duration of its presence in mice (blood clearance half-life of 9.8 minutes, see Figure 4). $^{111}$In-1 and CC49-TCO displayed high reactivity towards one another in vitro in semi-equimolar conditions and at low concentration (3.3 μM) within 10 minutes in PBS, serum and blood (Table 2). During the completion of our studies, a lower in vitro reactivity for a more stable system was reported, see Devaraj et al. [13].

The yields obtained from the reactions with 10 and 15 equivalents of $^{111}$In-1 indicated the presence of an average of 7.4 reactive TCO moieties per antibody. Furthermore, the nonspecific binding of $^{111}$In-1 to unmodified CC49 antibody or other media constituents was found to be very low. To assess the stability of

![Figure 2. Principle of pretargeted RIT using the inverse-electron-demand Diels–Alder reaction.](image-url)
the antibody-bound TCO moiety in vivo mice were treated with CC49-TCO and the extracted blood samples were then reacted ex vivo with an excess of tetrazine 1. The reaction yield, corrected for CC49-TCO blood clearance, revealed that 75% of the CC49-bound TCO present in blood was still reactive after 24 hours circulation, thus indicating good in vivo stability.

To test the Diels–Alder reaction in living animals we administered CC49-TCO to mice bearing colon cancer xenografts, followed one day later with injection of 3.4 equivalents of 111In-1 with respect to TCO. The chemically tagged tumors reacted rapidly with 111In-1, resulting in pronounced localization of radioactivity in the tumor, as demonstrated by single photon emission computed tomography/computed tomography (SPECT/CT) imaging of live mice three hours after injection (Figure 5a). The SPECT quantification of the tumor gave 4.2% injected dose per gram (%ID/g) and a tumor-to-muscle ratio (T/M) of 13.1. We also observed limited uptake in blood and nontarget tissues, such as the liver, which we attributed to reaction with residual circulating CC49-TCO.

Besides the tumor, the bulk of the radioactivity was found in the bladder, and some residual activity was visible in the kidney (Figures 5a and 6a). Importantly, the tumor could not be discriminated from the surrounding tissue in mice treated with unmodified CC49 (Figure 5b, e; 0.3%ID/g, T/M = 0.5). Almost no radioactivity was retained in the blood and nontarget organs, as the probe was again rapidly eliminated through the urinary tract, thus signifying its bio-orthogonality.

Mice treated with TCO-modified rituximab (Rtx), which lacks specificity for TAG72, showed the expected retention of 111In-1 in blood and non-target organs, and a significantly reduced accumulation in the tumor (Figure 5c, f; 1.0%ID/g, T/M = 2.1). The high-resolution postmortem image (Figure 6b) shows 111In activity in the aorta and carotid arteries as well as in the retroorbital regions. The rim of the tumor is also visible, because of extensive vascularization, whereas the rest of the tumor is devoid of radioactivity.

Next, we studied the distribution and specific co-localization of both pretargeting components through corresponding dual isotope biodistribution experiments with 125I-labeled antibodies (CC49-TCO, CC49, or Rtx-TCO) and 111In-1 (Figure 7). Residual 125I-antibodies were detectable in blood and in blood-rich organs such as the heart and lung 27 hours after injection, and showed the typical distribution pattern of long-circulating antibodies (Figure 7a) [14].

Both CC49 and CC49-TCO accumulated efficiently in tumors, with tumor-to-blood ratios (T/B) around 3 (Figure 7c) and high T/M ratios (Figure 7d). Considerably lower accumulation in the tumor, accompanied by a T/B ratio lower than 1 was found for Rtx-TCO (Figure 8a), thereby supporting the antigen-specific binding of CC49-TCO. The 111In-1 biodistribution data confirmed the 111In-1 SPECT images.

In the mice pretreated with CC49-TCO or Rtx-TCO, the distribution of 111In-1 mirrored that of the antibodies (Figure 7a–d). For example, a fivefold higher uptake of 111In-1 was found in the tumors containing 125I-CC49-TCO compared to 125I-Rtx-TCO. Almost no 111In uptake was detected in the blood and in most tissues of the group pretreated with unmodified 125I-CC49.

Importantly, while the uptake of 125I-CC49 by the tumors was the highest among the three groups, the 111In uptake by the tumors in this
It is noteworthy that this high reaction yield and corresponding tumor contrast were achieved in the complex interior of a mouse in a reaction time of minutes, limited by the 9.8 minute circulation half-life of $^{111}$In.

**Conclusion**

We have demonstrated the first use of a bioorthogonal chemical reaction between two exogenous moieties in living animals for the non-invasive imaging of low abundance targets under clinically relevant conditions. Intravenous administration of a small, semi-equimolar amount (nanomol) of a rapidly excreted probe convincingly delineated a tumor-bound antibody in a high chemical yield, despite the challenging pharmacokinetic constraints of a mammalian disease model. The inverse-electron-demand Diels–Alder reaction, therefore, has the potential to improve the state of the art of pretargeting, because it circumvents the use of immunogenic streptavidin systems and the protein engineering techniques used for bispecific antibodies. This pretargeting system can be applied to a range of antibodies (and fragments) due to its universal and straightforward conjugation chemistry.

*Corrected for non-specific accumulation; not corrected for TCO degradation.
addition, it combines this rapid and cost-efficient antibody modification with only very limited perturbation of the in vivo properties and a low likelihood of immunogenicity. The latter would potentially allow for repeat treatments, resulting in a higher efficacy.

Current efforts are directed towards reducing the amount of free circulating CC49-TCO to increase the tumor-to-blood ratio of $^{111}$In-1 in preparation for radiotherapy studies with tumor-bearing mice. Finally, this approach may re-introduce full length antibodies to nuclear imaging.
References


