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DOTA-tetrazine probes with modified linkers for tumor pretargeting

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ABSTRACT

Introduction: Pretargeted radioimmunoimaging and -therapy approaches building on the bioorthogonal inverse-electron-demand Diels–Alder (IEDDA) reaction between strained *trans*-cyclooctenes (TCO) and electron-deficient tetrazines (Tz) have yielded impressive results in recent years and have proven a vital alternative to biological pretargeting systems. After improvement of the TCO–antibody conjugates, we here report on our evaluation of a new series of radiolabeled Tz-probes.
Methods: Four new Tz-probes were synthesized, radiolabeled with lutetium-177, and characterized *in vitro* in terms of lipophilicity, reactivity, and stability in PBS and mouse serum. The *in vivo* biodistribution profile and tumor-targeting potential of the probes were evaluated in LS174T tumor-bearing mice pretargeted with TCO–antibody conjugates using non-pretargeted mice as control.
Results: Radiolabeling of all probes proceeded in high yields providing the ^{177}Lu-labeled tetrazines in >95% radiochemical purity without any further purification. In mouse serum, half-lives of the probes varied between 8 and 13 h, with the exception of the most lipophilic probe, [^{177}Lu]**1b**, with a serum half-life of less than 1 h. This probe also showed the fastest blood clearance ($t_{1/2}$ = 5.4 min), more than 2-fold faster than PEG-linked probes [^{177}Lu]**3** and [^{177}Lu]**4**, and even 3-fold faster than the other small probes without the PEG-linker, [^{177}Lu]**1a** and [^{177}Lu]**2**. In the pretargeting experiments, tumor uptake of the lead probe [^{177}Lu]**4** (~6 %ID/g) was most closely approached by [^{177}Lu]**2**, followed by [^{177}Lu]**3** and [^{177}Lu]**1a**. While all the smaller and more lipophilic probes suffered from increased liver uptake, the PEG-linked probe [^{177}Lu]**3** with its additional negative charge surprisingly showed the highest kidney uptake among all of the probes.
Conclusion: The *in vitro* performance of some of the new tetrazine probes turned out to be comparable to the established lead probe [^{177}Lu]Lu-DOTA-PEG$_{11}$-Tz ([^{177}Lu]**4**). However, tumor pretargeting studies *in vivo* showed lower tumor uptake and increased uptake in non-target organs.

1. Introduction

After FDA approval of the first agents ibritumomab tiuxetan (Zevalin) and iodine tositumomab (Bexxar) more than a decade ago, radioimmunotherapy (RIT) is well established as a second-line therapy for treatment of refractory non-Hodgkin's lymphoma [1]. One of the major limitations of conventional RIT with radiolabeled monoclonal antibodies (mAbs) is the high radiation dose to non-target tissues resulting from the combination of prolonged blood clearance and slow target uptake (reviewed in Ref. [2]). Pretargeted radioimmunotherapy (PRIT) addresses this issue by combining the superior targeting properties of antibodies with the favorable pharmacokinetics of smaller molecules (reviewed in Refs. [3,4]). The method relies on tumor pretargeting with engineered mAbs having both affinity for a tumor-associated antigen and the capability to bind to a radioligand. The fast-clearing radioligand is then injected in a second step, after allowing sufficient time for accumulation of the mAbs in the tumor and clearance from blood. Currently, two pretargeting approaches have been clinically validated: bispecific antibodies with affinity for both the tumor and the radiolabeled small hapten [5], and antibody-conjugates making use of the streptavidin–biotin interaction [6]. While the first system requires extensive reengineering and perturbation of the parent mAb, streptavidin–biotin systems frequently suffer from immunogenicity, thereby precluding repeated treatment cycles [7]. Recently developed chemical pretargeting strategies relying on antibody modification with

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small tags may hold the potential to circumvent these issues (reviewed in Refs. [8,9]). Among several bioorthogonal chemical reactions evaluated for this purpose, the inverse-electron-demand Diels–Alder (IEDDA) reaction between strained *trans*-cyclooctenes (TCO) and electron-deficient tetrazines (Tz) has yielded promising results for pretargeted radioimmunoimaging and -therapy in preclinical studies [10–19], whereas the Staudinger ligation and the strain-promoted alkyne-azide cycloaddition reaction (SPAAC) suffered from a number of limitations [20–22]. Following our initial report on pretargeted tumor imaging in live mice using a TCO–antibody conjugate and an ^{111}In-labeled Tz-probe (Fig. 1) [10], we and others have continuously improved the system by developing more reactive TCO-tags with increased *in vivo* stability [14] and dedicated Tz-probes for radiolabeling with copper-64 [12], gallium-68 [23], fluorine-18 [17,24], carbon-11 [25,26], technetium-99m [27], and iodine-125 [28]. In the meantime, Tz-probes with reduced gastrointestinal clearance have shown promise for pretargeted immuno-PET of colorectal cancer [16,18] and radioimmunotherapy of pancreatic ductal adenocarcinoma [19] in preclinical studies. To further increase tumor-to-blood ratios and maximize tumor dose in pretargeted radioimmunotherapy, we have introduced a rapid bioorthogonal chemical clearing approach for removal of residual circulating TCO-conjugated antibodies from blood prior to injection of the radiolabeled probe [13]. Finally, our efforts resulted in an optimized pretargeting protocol employing antibody constructs with a more reactive and less hydrophobic TCO-tag with higher *in vivo* stability, and improved tumor accumulation [15].

Having optimized the reactivity and pharmacokinetics of the TCO-tagged antibody, in this work, we set out to explore the parameters that govern *in vivo* performance of the tetrazine probe, which may in the future enable the design of a probe with improved tumor uptake and reduced uptake in the kidney, the organ retaining most of the activity apart from the tumor [13]. Inspired by earlier investigations suggesting that an increase of negative charges in DOTA- and DTPA-conjugated peptides resulted in reduction of renal uptake [29,30], we designed a Tz-probe **3** (Fig. 2), in which the Tz-moiety is attached via a PEG_{10}-thioureabenzyl-linker to the carbon backbone of the DOTA, allowing for as many as four carboxymethyl functionalities and resulting in a net negative charge of the ^{177}Lu-labeled probe at physiologic pH. Aiming at probes with better tumor penetration and a more homogenous uptake, we created the shorter Tz-DOTA derivatives **1a** and **1b** linked by straight chain C_6- and C_{11}-amidoalkyl groups, respectively, and probe **2**, which in fact corresponds to the original probe **4** without the PEG-spacer (Fig. 2). The new probes were evaluated *in vitro* and *in vivo* employing our previously developed clearing agent strategy [13] and pretargeting components (Fig. 2), and benchmarked against lead probe **4**.

2. Experimental

2.1. Chemistry

Synthetic procedures and spectral data for the new Tz-probes **1a/b**, **2**, **3**, and **4** are described in §1 and §2 of the Supplemental Information associated with this article. Preparation of CC49-TCO (**6**) and galactose–albumin–tetrazine (**5**) has been reported elsewhere [10,13].

2.2. Radiochemistry

Radioiodination of CC49-TCO (**6**) was performed with the Bolton–Hunter method, followed by purification and quality control according to a published procedure [13]. A detailed procedure including results is also reported in § 3.1 of the Supplemental Information. For animal experiments, the specific activity of the [^{125}I]I-CC49-TCO was adjusted to 2–5 kBq/μg by adding nonradioactive CC49-TCO.

The DOTA-conjugated Tz-probes **1a/b**, **2**, **3**, and **4** were dissolved in 0.2 M ammonium acetate at pH 7.0 at concentrations of 1–2 mg/mL and stored at −80 °C before use. Radiolabeling for the *in vitro* stability studies was performed by combining suitable volumes of probe stock solutions (corresponding to 10 μg for tetrazine **1a/b** and **2**, 15 μg for tetrazine **3** and **4**) with [^{177}Lu]$LuCl_3$ (20–35 MBq; PerkinElmer) in 0.2 M ammonium acetate at pH 5.5 (total volume of the reaction mixture was 20 μL) and incubating at 60 °C for 5 min in a thermomixer (350 rpm). Following the addition of 5 μL of 10 mM diethylenetriaminepentaacetic acid (DTPA) and incubation for another 5 min at 60 °C, radiochemical yield and purity were assessed by radio-iTLC and radio-HPLC, respectively. Molar activities (A_m) for the ^{177}Lu-labeled Tz-probes **1a**, **1b**, **2**, **3**, and **4** were 2.28 ± 0.21, 2.54 ± 0.65, 2.02 ± 0.42, 2.85 ± 0.28, and 2.62 ± 0.40 MBq/nmol, respectively ($n = 6$ for each probe). For investigation of the *in vitro* reaction kinetics, labeling was performed according to the same procedure, but at higher specific activity (50–60 MBq [^{177}Lu]$LuCl_3$, 5 μg tetrazine, i.e., A_s ~ 10–12 MBq/μg), and gentisic acid (20 μL of a 20 mg/mL solution in 0.9% saline—1.0 M Na_2CO_3, 9:1) was added post-labeling to prevent autoradiolysis.

For the *in vivo* studies, the tetrazines were labeled to a molar activity (A_m) of 0.11–0.15 MBq/nmol by combining a suitable volume of the Tz-stock solutions with [^{177}Lu]$LuCl_3$ in 1.0 M NH_4OAc at pH 5.0 (10 μL) and incubating at 60 °C for 5 min, followed by the addition of gentisic acid (50 μL of a 20 mg/mL solution in 0.9% saline—1.0 M Na_2CO_3, 9:1) and 10 mM DTPA (5 μL). After incubation at 60 °C for another 5 min, aliquots of the labeling mixture were analyzed by radio-TLC and radio-HPLC, and the reaction mixture was diluted with sterile 0.9% saline for animal experiments, with each dose (80 μL, ca. 1 MBq) containing 6.67 nmol of tetrazine and 100 μg of gentisic acid.

2.3. In vitro characterization of radiotracers

2.3.1. Distribution coefficient $LogD_{7.4}$ in 1-octanol/PBS at pH 7.4

$LogD_{7.4}$ values were measured using the shake flask method. Tetrazines were radiolabeled according to the protocol for the *in vitro* studies, however, apart from [^{177}Lu]$LuCl_3$, also non-radioactive $LuCl_3$ (0.8 eq. with respect to probe) was added to the labeling mixture. The labeled tetrazines were additionally purified by passing through a C_8 Sep-Pak® cartridge and eluting with ethanol, mainly to remove traces of highly polar [^{177}Lu]LuDTPA. To 1-octanol (0.5 mL) and PBS at pH

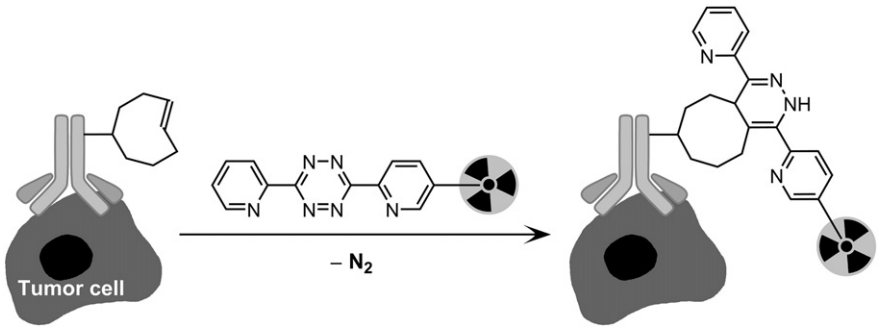

Fig. 1. Principle of tumor pretargeting with the inverse-electron-demand Diels–Alder (IEDDA) reaction between a *trans*-cyclooctene-tagged mAb and a radiolabeled tetrazine.

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