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Development of ^{170}Tm-DOTA-cetuximab for radioimmunotherapy

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Thulium-170 [$T_{1/2}$ = 128.4 days, E_β(max) = 968 keV, E_γ = 84 keV (3.26%)] has radionuclidic properties suitable for use in therapy. ^{170}Tm can be produced by a relatively feasible route involving thermal neutron bombardment on natural Tm $(NO_3)_3$ (100% ^{169}Tm) in medium flux research reactors. The combination of beta-particle emission of Tm-170 with therapeutic properties of C225 monoclonal antibody (cetuximab) as well as optimization studies for future Tm-167 labeling was targeted in this study. Conjugated cetuximab was obtained by the addition of 0.5 ml of a cetuximab pharmaceutical solution (1 mg, in phosphate buffer, pH 7.8) to a glass tube pre-coated with *in situ* prepared 1,4,7,10-tetraazacyclododecane-*N*,*N*,*N*,*N*-tetraacetic acid mono-(*N*-hydroxysuccinimidyl) ester (DOTA-NHS) (~5 mg) at 25°C. Cetuximab was labeled with ^{170}Tm-Thulium chloride (100 MBq) after conjugation with DOTA-NHS in 2–3 h (radiochemical purity >99%, instant thin-layer chromatography, specific activity = 77–385 TBq/mmol). Biodistribution studies in wild-type rats for free Tm-170 and the radioimmunoconjugate were performed to determine the distribution up to 72 h. A comparative time-frame study was performed for critical organs for both radiochemical species. The major organs of accumulation were shown to be the lung, liver, and spleen, respectively.

Keywords: cetuximab; Thulium-170; biodistribution; radioimmunotherapy

Introduction

Cetuximab (IMC-C225—marketed under the name Erbitux) is a chimeric (mouse/human) monoclonal antibody, an epidermal growth factor receptor (EGFR) inhibitor, given by intravenous infusion for treatment of metastatic colorectal cancer and head and neck cancer. It received Food and Drug Administration approval in 2006 for use in combination with radiation therapy for treating squamous cell carcinoma of the head and neck or as a single agent in patients who received prior platinum-based therapy.[1]

Many radiolabeled cetuximab compounds have been developed to visualize and monitor EGFR tumors in animals and humans using copper-64,[2] technetium-99m,[3] and yttrium-86 [4] or for therapeutic purposes including ^{177}Lu and ^{90}Y in tumor-bearing nude mice [5] as well as I-131.[6]

Thulium-170, having both gamma and beta emissions (E_β = 968 keV and E_γ = 84 keV), is a new candidate for labeling of immunoconjugates, which guarantees both applications (radioimmunotherapy and imaging studies). Thulium-170 can be prepared by neutron irradiation of natural target (natural Tm $(NO_3)_3$) using (n, γ) reaction in reasonable amounts.[7] The cation $^{170}Tm^{3+}$ as a lanthanide has suitable chelating properties with 1,4,7,10-tetraazacyclododecane-*N*,*N*,*N*,*N*-tetraacetic acid (DOTA); thus, in this work we chose DOTA as the chelating agent, which can be linked to the antibodies via *N*-hydroxysuccinimidyl (NHS). Activation of the DOTA molecule, resulting in 1,4,7,10-tetraazacyclododecane-*N*,*N*,*N*,*N*-tetraacetic acid mono-(*N*-hydroxysuccinimidyl) ester (DOTA-NHS), allows conjugation to cetuximab antibody.

Very few reports are available on the development of Tm-170-labeled compounds for therapeutic applications including ^{170}Tm-ethylenediamine tetra(methylene phosphonic acid),[8] and to the authors' knowledge no reports are available on developing Tm-170-labeled immunomolecules. Radiopharmaceuticals prepared by Thulium-170 have the advantage of comparatively longer half-life of 128.4 days, which makes it possible to supply the radiopharmaceuticals worldwide.

In this work, we report radiolabeling, quality control, and biodistribution studies for ^{170}Tm-DOTA-cetuximab.

Experimental

Thulium nitrate (spectroscopic grade N99.99% pure) was obtained from E. Merck (Darmstadt, Germany). Sephadex G-50, sodium acetate, phosphate buffer components, and chloroform were purchased from Sigma–Aldrich Company Ltd., Poole, England. Cetuximab (erbitux) was a pharmaceutical sample purchased from Merck UK and was used without further purification. DOTA-NHS ester was freshly synthesized in our laboratory on the basis of the conventional method and kept under a blanket of dry N_2. Radiochromatography was performed by counting Whatman No. 2 TLC paper, using a thin-layer chromatography scanner, Bioscan AR2000, Paris, France. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd Ed. All

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of the rats were male NMRI purchased from Pasteur Institute of Iran, weighing 180–200 g. In each group/interval, five rats were used and kept at routine day/night light program and under common rodent diet pellets.

Production and quality control of ^{170}Tm

^{170}Tm was produced by thermal neutron bombardment on natural Tm $(NO_3)_3$ (100% ^{169}Tm) at Tehran Research Reactor (TRR) for a period of 7 days at a flux of 3–4 $\times$ 10^{13} neutrons/cm^2 s. In a typical procedure, the amount of target that was equal to 1 mg of Tm was dissolved in HNO_3 and transferred to a quartz ampoule followed by evaporating the liquid. The ampoule was sealed and put in an aluminum can and sent for irradiation. The irradiated powder was dissolved in 100 μl of 0.1 M HCl, and this radiochemical form was used for radionuclidic purity determination using beta and gamma spectroscopy for the detection of various interfering beta and gamma emitting radionuclides.

Preparation of 1,4,7,10-tetraazacyclododecane-*N*,*N*,*N*,*N*-tetraacetic acid mono-(*N*-hydroxysuccinimidyl) ester

This compound was prepared according to methods previously given in the literature with slight modifications.[9,10] For a single run, a mixture of DOTA in acidic form (4.04 mg, 0.01 mmol), *N*-hydroxysuccinicimide (1.15 mg, 0.01 mmol), and dicyclohexyl carbodiimide (DCC) (2 mg, 0.01 mmol) were dissolved in anhydrous CH_2Cl_2 (300–500 μl) under a blanket of N_2 in a glass vial. The mixture was vortexed for 30 s and stirred at 25°C for 15 h. Thin-layer chromatography using ethyl acetate : hexane (1:1) mixture as mobile phase and silica gel as stationary phase was performed to monitor the reaction progress.

Conjugation of DOTA-NHS with the cetuximab

The DOTA-NHS prepared above was conjugated to the antibody using a small modification of the DOTA-NHS method.[11] The residue mixture was evaporated under a flow of N_2 gas in a glass tube producing a thin film coat of DOTA-NHS. The commercially available cetuximab (2 mg/ml pH 7.8) was added to the coated glass tube while gently mixing at room temperature for 30 s. The mixture was then incubated in a water bath at 25°C for 15–18 h. The conjugation mixture was then passed through a Sephadex G-50 (fine) column (2 $\times$ 15 cm, prepared by soaking 3 g of the resin in 50 ml milli-Q water for 4 h), and 1-ml fractions were collected and checked for the presence of protein using visible Lowry colorimetric assay by mixing freshly prepared Folin–Colciteau reagent (prepared by mixing 5 μl of Folin and 25 μl of fresh Cu-tartrate solution) and 10 μl of the eluted fractions using UV absorbance at 280 nm or visible Folin-phenol reagent.[12] The fraction containing the highest concentration of immonoconjugate was chosen and kept at 4°C for radiolabeling.

Radiolabeling of the antibody conjugate with ^{170}Tm

The antibody conjugate was labeled using an optimization protocol according to the literature.[13] Typically, 80–100 MBq of ^{170}Tm-chloride (in 0.1 N HCl) was added to a conical vial and dried under a flow of nitrogen. Double-distilled water was added to the vial containing ^{170}Tm activity (2.32 mCi, 85.8 MBq) followed by drying the vial using nitrogen flow (two times). The protein-containing fraction with the maximum protein content in 1 ml of phosphate buffer (0.1 M, pH 8) was added to the vial and mixed gently for 30 s. The resulting solution was incubated at 37–40°C for 2–3 h. After radiochemical purity determination using instant thin-layer chromatography (ITLC) (Whatman No. 2 paper, 1 mM diethylene triamine pentaacetic acid (DTPA), pH 5), the mixture can be further purified using size-exclusion chromatography in case of low radiochemical purity.

Briefly, the radiolabeled antibody conjugate was purified from free ^{170}Tm by size-exclusion chromatography on a Sephadex G-50 (fine) column (15–20 ml bed volume) and eluted with phosphate-buffered saline (PBS). Fractions (1 ml) were collected, and the radioactivity of each fraction was measured by a recently calibrated radioisotope dose calibrator. The protein presence in each fraction was determined using a fast protein assay method described above.[12]

The fractions containing the proteins (visible blue color by naked eye) with the maximum radioactivity (fraction No. 7) was tested for purity by ITLC using a radio-TLC scanner.

Control labeling experiments were also performed using $^{170}TmCl_3$, and DOTA with $^{170}TmCl_3$. Both reaction mixtures were passed through separate size-exclusion chromatography columns and eluted with PBS. The fraction number 5-7 showed the presence of protein. Fraction 6 was used in the other experiments ($n = 3$).

Stability testing of the radiolabeled compound in final formulation

Stability of ^{170}Tm-DOTA-cetuximab in PBS was determined by storing the final solution at 4°C for 14 days and performing frequent ITLC analysis to determine radiochemical purity. The stability of the conjugated DOTA-cetuximab stored at 20°C for more than 1 month was also investigated. ITLC analysis of the conjugated product was performed to monitor for degradation products or other impurities. After subsequent ^{170}Tm-labeling of the stored conjugated product, both labeling efficiency and radiochemical purity were determined.

Stability testing of the radiolabeled compound in presence of human serum

Briefly, a 1:3 mixture of radiolabeled antibody to freshly prepared human serum was incubated at 37°C while it was gently stirred. At each time interval, 1 ml sample of the mixture was assessed by size-exclusion chromatography on a Sepharose column (1 $\times$ 30 cm), which was previously equilibrated with PBS and eluted at a low rate of 0.5 ml/min at room temperature; 1-ml fractions were collected. Each eluted fraction was checked for the presence of protein using Lowry method and radioactivity content. Control experiments were also performed separately for human serum sample, antibody, and free Tm-170 cation.

Biodistribution of ^{170}Tm-acetate and ^{170}Tm-DOTA-Cetuximab to wild-type rats

To perform the biodistribution studies, ^{170}Tm-acetate and ^{170}Tm-DOTA-cetuximab were administered to wild-type rats. A volume (100 μl) of final ^{170}Tm-DOTA-cetuximab solution containing 100 μCi (3.7 GBq) of radioactivity was injected intravenously into rats via their tail vein. The animals were euthanized at the exact time intervals (2, 4, 24, and 48 h, 1 and 2 months) for ^{170}Tm-acetate and 2, 4, 24, and 168 h for the radioimmunoconjugate. The activity in different organs was calculated as a percentage of injected dose per gram using a dose calibrator.

Result and discussion

Production and quality control of ^{170}Tm

Irradiation of natural $Tm(NO_3)_3$ was performed at a thermal neutron flux of $3–4\times10^{13}$ neutrons/cm^2 s for 5 days at TRR, and the radionuclide was prepared according to standard methods with a range of specific activity 10–15 mCi/mg for radiolabeling use. However, because of long physical half-life and presence of carrier Tm-169, the specific activity is rather low. Gamma ray spectrum of the appropriately diluted $^{170}TmCl_3$ solution showed a major peak at 84 keV, which is the photopeak of ^{170}Tm, as well as two minor peaks at 52 and 59 keV, which are the X-ray peaks associated with the ^{170}Tm decay. The radioisotope was dissolved in acidic media as a starting sample and was further diluted and evaporated to obtain the desired pH and volume followed by sterile filtration. The absence of any other photo-peaks in the gamma ray spectrum indicated that the ^{170}Tm was produced with a radionuclidic purity of >99.99%. The radiochemical purity of the ^{170}Tm solution was checked in a solvent system containing DTPA 1 mM and Whatman No. 2 TLC paper, as stationary phase; Tm^{3+} cation is complexed to more lipophilic ^{170}Tm-DTPA ligand and migrates to higher R_f.

Preparation and structure conformation of DOTA-NHS

Various solvents can be used in the conjugation reaction of DOTA and NHS, such as dimethylformamide, dimethylsulfoxide, etc. Because of the high boiling point of such solvents and the possibility of protein denaturing caused by these solvents, we tried to use dichloromethane on the basis of the previous reports for DCC-mediated conjugation reactions. This reaction is performed at room temperature and can be done overnight as reported.[9]

Most of the reaction takes place in the first few hours. Considering the size-exclusion chromatography performed in the next step of the process, all the starting materials can be separated. A more lipophilic species in the reaction mixture (monitored by TLC) is related to the formation of the ester **3** (Figure 1). The exact stoichiometry of NHS : DOTA (1:1) is mandatory in order to avoid the formation of the di-succinimidyl ester. As mentioned, thin-layer chromatography using ethyl acetate : hexane (1:1) mixture as mobile phase and silica gel as stationary phase was performed to monitor the reaction progress. The formation of mono-succinimidyl ester at R_f of 0.36 was observed. In case of longer reaction times, another species at R_f = 0.56–0.6 can be observed and is related to di-succinimidyl ester. All reactants remain at the R_f of 0.0–0.1.

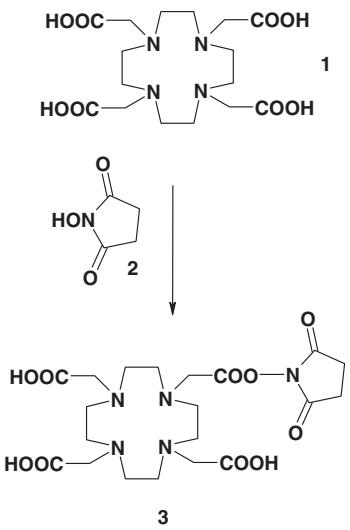

Figure 1. Synthesis of DOTA-NHS active ester for conjugation.

Conjugation of cetuximab with DOTA-NHS and radiolabeling of cetuximab with ^{170}Tm

The conjugated DOTA-cetuximab fractions containing the maximum protein content were mixed with ^{170}Tm-$TmCl_3$ solution, vortexed, and kept at room temperature. Small fractions were taken from this mixture and tested by Radio-thin layer chromatography (RTLC) to find the best time scale for labeling. After 2–3 h, the radiochemical purity of reaction reached almost 100%. The mixture was then passed through another Sephadex G-50 size-exclusion chromatography column to remove unbound ^{170}Tm cation and/or other low molecular weight impurities.

The eluted fractions were checked by Folin–Colciteau® reagent and for the presence of radioactivity to determine the ^{170}Tm-DOTA-Cetuximab containing fractions. Fraction number 7 was chosen as the suitable final product with appropriate specific activity for animal tests. Instant thin-layer chromatography using 1 mM DTPA as mobile phase and Whatman No. 2 paper strips as stationary phase was performed to ensure the existence of only the desired radiolabeled antibody. Radiolabeled antibody stayed at the origin, whereas free Tm-170 ions migrated to higher R_fs (Table. 1).

Stability of radiolabeled protein in presence of human serum

After incubation of ^{170}Tm-DOTA-cetuximab (3.7 MBq) with freshly prepared human serum at 37°C for up to 72 h, 96–98% of all of the radioactivity eluted in the same position as ^{170}Tm-DOTA-cetuximab, using size-exclusion chromatography. Thus, there was no evidence for either degradation or trans-chelation of ^{170}Tm to other serum proteins over a time period consistent with the normal blood clearance time of cetuximab.

Biodistribution studies in rats

The animals were sacrificed by CO_2 asphyxiation at selected times post injection (2, 4, 24, and 48 h, 1 and 2 months). Dissection began by drawing blood from the aorta followed by removing the heart, spleen, muscle, brain, bone, kidneys, liver, intestine, stomach, lungs, and skin samples. The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g). The biodistribution of ^{170}Tm cation was determined in wild-type animals for 2–48 h and 1–2 months post injection (Figure 2). The blood content is low at all time intervals, and this shows the rapid removal of activity in the circulation. The heart, lung, muscle, intestine,

Table 1. R_f values for various radiochemical species using 1 mM DTPA, pH 5 eluent on Whatman No. 2 paper

Radiochemical species	R_f
$^{170}Tm^{3+}$	0.8
^{170}Tm-DOTA	0.3
^{170}Tm-DOTA-cetuximab	0.0

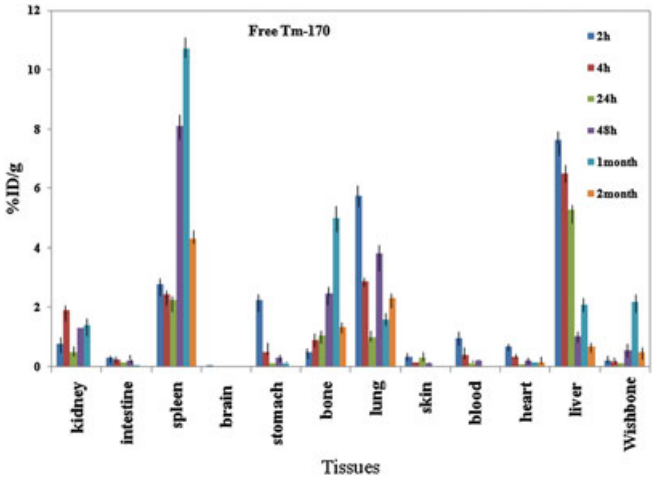

Figure 2. Percentage of injected dose per gram (ID/g %) of ^{170}Tm-acetate in wild-type rat tissues at 2, 4, 24, and 48 h, and 1 and 2 months post injection ($n = 3$).

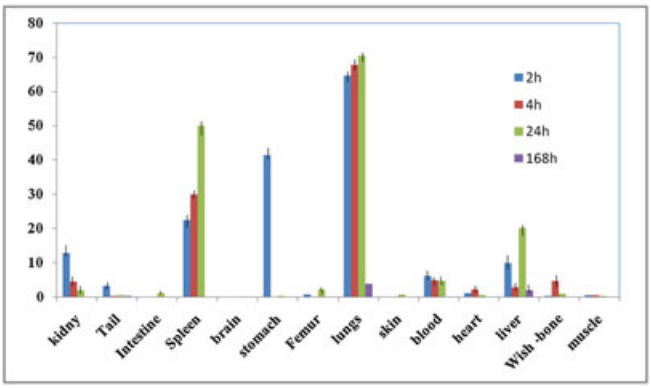

Figure 3. Percentage of injected dose per gram (ID/g %) of ^{170}Tm-DOTA-cetuximab in wild-type rat tissues at 2, 4, 24, and 168 h post injection ($n = 3$).

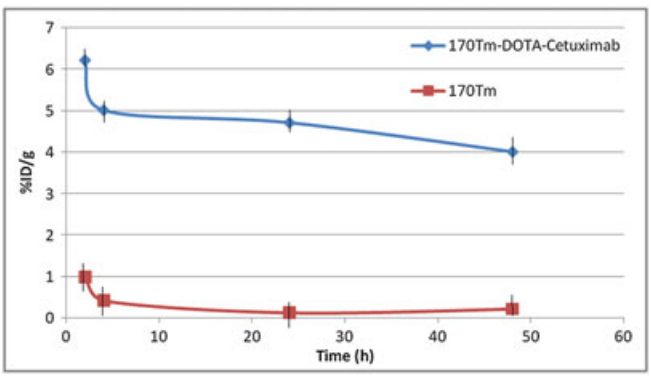

Figure 4. Comparative %ID/g in the blood for ^{170}Tm-DOTA-rituximab (blue) and $^{170}TmCl_3$ (red) in wild-type rats ($n = 3$).

and also skin do not demonstrate significant uptake, which is in accordance with accumulation of other cations. Bone uptake for the cation goes up to 6% up to 1 month and then is reduced to 1% in the second month. Spleen also has significant uptake possibly related to reticuluendothelial uptake. Tm^{3+} is a water soluble cation; therefore kidney plays an important role in excretion via urine.

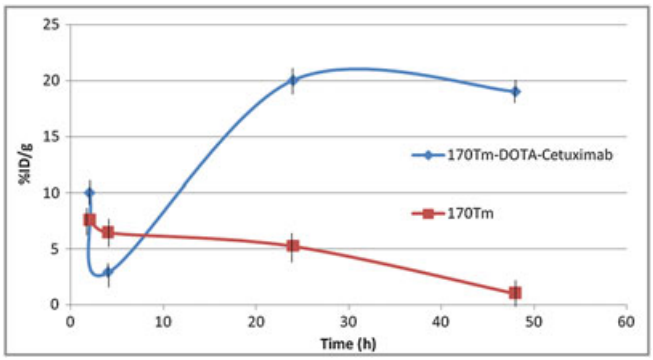

Figure 5. Comparative %ID/g in the liver for ^{170}Tm-DOTA-rituximab (blue) and $^{170}TmCl_3$ (red) in wild-type rats ($n = 3$).

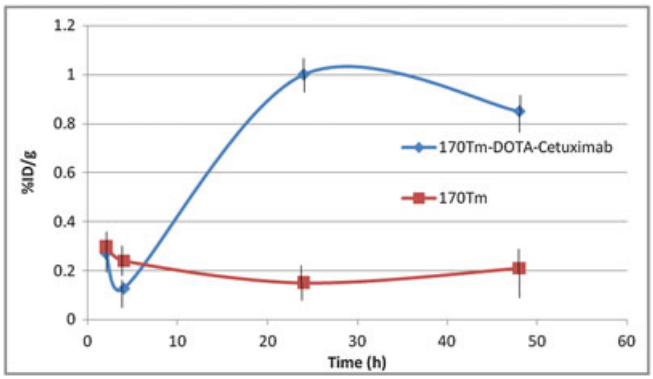

Figure 6. Comparative %ID/g in the intestine for ^{170}Tm-DOTA-rituximab (blue) and $^{170}TmCl_3$ (red) in wild-type rats ($n = 3$).

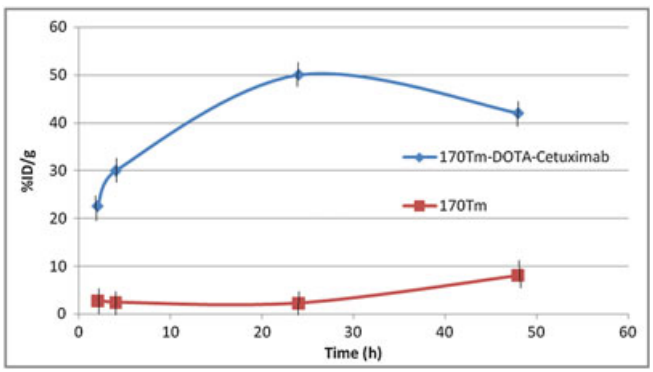

Figure 7. Comparative %ID/g in the spleen for ^{170}Tm-DOTA-rituximab (blue) and $^{170}TmCl_3$ (red) in wild-type rats ($n = 3$).

The distribution of ^{170}Tm-DOTA-cetuximab among tissues was determined for normal rats. A volume (100 µl) of final [^{170}Tm]-DOTA-cetuximab solution containing 3.7 MBq (100 µCi) radioactivity was injected into the dorsal tail vein. The total amount of radioactivity injected into each rat was measured by counting the 1-ml syringe before and after injection in a dose calibrator with a fixed geometry. The animals were sacrificed by ether asphyxiation at selected times post injection (2, 4, 24, and 168 h).

Dissection began by drawing blood from the aorta, followed by collecting heart, spleen, kidneys, liver, intestine, stomach, lung, brain, femur, muscle, and skin samples. The samples were

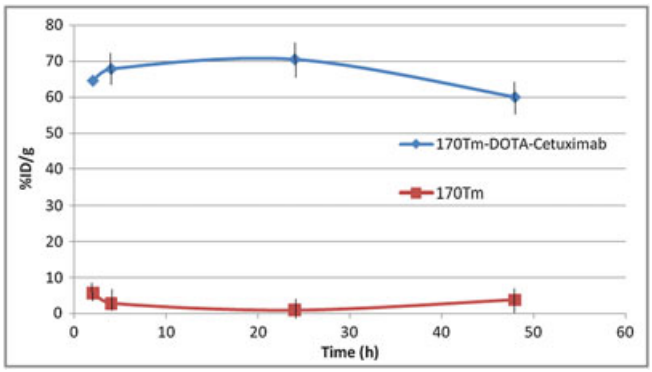

Figure 8. Comparative %ID/g in the lung for ^{170}Tm-DOTA-rituximab (blue) and $^{170}TmCl_3$ (red) in wild-type rats ($n = 3$).

weighed and their specific activities determined with a gamma spectrometer using an HPGe detector counting the area under the curve of the 84-keV peak. The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g) (Figure 3).

The highest activity concentrations were visualized in the lungs, spleen, and the liver (~15% ID/g).[2] Comparison of vital organs uptake for ^{170}Tm-DOTA-rituximab and ^{170}Tm-acetate demonstrates kinetic pattern difference between both species.

Both species are removed from the blood circulation rapidly via different mechanisms, but in case of free Tm cation, the blood amount is much lower and reaches minimum after 48 h (Figure 4).

In case of liver, a different pattern is observed for free cation. The uptake decreased after 48 h possibly because of the billiary excretion, whereas for the conjugate the liver uptake increases in 24 h followed by the retention of the activity in the liver (Figure 5). This has been already shown for other radiolabeled cetuximab conjugates in the literature with 10–18% liver uptake.[5]

The intestine activity content is comparable with the liver because the intestine is an organ receiving hepatobilliary excretion contents (Figure 6). The liver activity reaches its maximum after 24 h and stays constant, whereas the major part of the free Tm^{3+} activity is not excreted from the liver and possibly from the kidneys especially before 24 h.

In case of the spleen, a different pattern is observed for free cation (Figure 7). The uptake was low at all time intervals, whereas for the conjugate, the spleen uptake increased at 24 h followed by the retention of the activity. This has been previously shown for other radiolabeled cetuximab conjugates in the literature with 10–15% spleen uptake.[5]

The lung is the major site of accumulation with rapid onset and then remains constant, whereas the lung uptake of the free Tm activity is not significant (Figure 8). It has been already shown that the EGFR are commonly overexpressed in lung malignancies.[14] Thus, using the radiolabeled anti-EGFR conjugates including ^{170}Tm-DOTA-cetuximab can lead to lung uptake, as shown in this study.

Conclusion

Tm-170 was prepared by irradiation of natural thulium nitrate sample by 4×10^{13} neutrons/cm^2 s neutrons (radionuclide purity >99%, radiochemical purity >99%). One of the most important advantages of thulium over some other lanthanides is that a relatively high radionuclidic purity could be produced from mono-isotopic Tm-169 target. Cetuximab was conjugated with DOTA chelating agent, followed by ^{170}Tm-radiolabeling. The radiochemical purity of the ^{170}Tm-DOTA-cetuximab was determined by the use of ITLC (>99%). The biodistributions of radiopharmaceutically acceptable $^{170}TmCl_3$ and ^{170}Tm-DOTA-cetuximab formulations were checked in wild-type rats up to 72 h post injection. ^{170}Tm-DOTA-cetuximab accumulated mostly in the liver and spleen as shown for other radiolabeled cetuximab immunoconjugates. The distinct accumulation of the activity in lungs for the radiolabeled antibody and liver and spleen for the free cation was observed. The development of other ^{170}Tm-labeled monoclonal antibodies for ultimate radioimmunotherapy based on DOTA conjugates is possible.

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Conflict of Interest

The authors did not report any conflict of interest.

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