Synthesis and application of a novel cysteine-based DTPA-NCS for targeted radioimmunotherapy

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ABSTRACT

Introduction: For the development of safe and effective protein-based radiolabeled complexes such as radioimmunotherapy (RIT), the selection of the radionuclides and the chelating agents used for the radiolabeling of tumor-targeting molecules is a critical factor. We aim to synthesize a novel bifunctional chelating agent containing the isothiocyanate group for easy conjugation with antibodies having the characteristics of high stable chelation with therapeutic radionuclides.
Methods: We have synthesized the DTPA analogue retaining L-cysteine as a core ligand of the thiol group. The chelating power of cysteine-based DTPA-NCS (cys-DTPA-NCS) was compared with that of commercial ρ-SCN-Bn-DTPA. In an application, the cetuximab was radioimmunoconjugated with ^{177}Lu using cys-DTPA-NCS. The affinity was tested in a cell line overexpressing EGFR. A therapy study was conducted in nude mice with subcutaneous HT-29 xenografts.
Results: The cys-DTPA-NCS presents an excellent ability to chelate as compared to the ρ-SCN-Bn-DTPA. For mean ratio chemical labeling yields of 95%, the result was 0.97. ^{177}Lu-cys-DTPA-NCS-cetuximab was prepared under ambient condition with a high radiolabeling yield and the radiochemical purity was sustained for at least 6 days. The IC_{50} value of the ^{177}Lu-labeled cetuximab was 10 nM (95% confidence). The stability and therapeutic efficacy of the candidate radiopharmaceutical were verified.
Conclusion: The new DTPA derivative, cys-DTPA-NCS, is a good bifunctional chelating agent that can be used for protein-based radiopharmaceutical using lanthanides such as ^{177}Lu and ^{90}Y. The prepared ^{177}Lu-cys-DTPA-NCS-cetuximab can be used for the diagnosis and treatment of human colorectal tumor.

1. Introduction

Since the successful development and commercialization of Yttrium-90(^{90}Y) labeled ibritumomab (Zevalin) for the treatment of non-Hodgkin's lymphoma (NHL), radioimmunotherapy (RIT) has been used to treat malignant tumors by delivering a sufficient radiation dose to specific receptor antigens that are overexpressed in tumor cells [1,2].

For the development of new protein-based radiolabeled complexes such as RIT, the effectiveness of radiolabeling with radionuclides is essential through the introduction of a chelator into the protein because antibodies should be prepared without any degradation of their affinity during the radiolabeling process. Many bifunctional chelator systems such as 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-acetic acid (DOTA) and diethylenetriamene pentaacetate (DPTA) have been designed and applied [3–5]. The use of macrocyclic chelating agents such as DOTA provides stable complexes with metals and forms radiometal complexes with high *in vivo* stability. However, the rate of conjugation is relatively slow and the complexation should be reacted by incubation at an elevated temperature [5–7].

In contrast, DTPA is an efficient candidate when radiolabeling with a therapeutic radioisotope such as ^{177}Lu or ^{90}Y [3,7–9]. Specially, ^{177}Lu is an ideal radionuclide for RIT due to its favorable decay characteristics. It decays with a half-life of 6.73 days by the emission of beta particles with maximum energy levels of 497 keV (78.6%) and gamma photons with energy levels of 208 keV (11%) which allow simultaneous scintigraphic studies [3,6–9]. ^{90}Y does not emit gamma photons

and is therefore suitable for the treatment of relatively large solid tumors [3,7–9]. A high radiolabeling yield can be accomplished by the simple mixing of a radionuclide with a chelating solution containing DTPA within 15 min under most under ambient conditions [3,7,8]. Furthermore, the attachment of a hydrophilic DTPA chelate to biomolecules increases urinary excretion, which reduces abdominal radioactivity levels in non-targeted organs [10]. Among the various DTPA derivatives, p-isothiocyanatobenzyl DTPA is the most commonly used acyclic bifunctional chelator for labeling proteins and other biomolecules for radiopharmaceutical or MRI applications [3,9]. The SCN groups react very efficiently and selectively with primary amino group. However, the ρ-SCN-benzyl DTPA is prepared from ρ-nitrophenyl alanine in a multistep synthesis process [9–12]. Therefore, we aim to synthesis a cysteine-based DTPA derivative containing isothiocyanate in fewer and easier steps by producing 4-ethylaniline-DTPA-L-cysteine. The cysteine-based DTPA-NCS (cys-DTPA-NCS) possesses an isothiocyanate and five free carboxylic acids. It also provides coordination geometry similar to that of DTPA without any side reactions and easily conjugates with an amine of biomolecules. Here, we show that the cys-DTPA-NCS presents an excellent ability to chelate.

As an application, we chose cetuximab which is a chimeric IgG1 and that binds to the epidermal growth factor receptor (EGFR) [13,14]. This receptor is overexpressed in cancers of the pancreas, colon, head and neck, lung (non-small-cell), kidney, breast, prostate, ovary, bladder, and brain (glioma). Its expression is associated with the inhibition of apoptosis, cell cycle progression, angiogenesis, cell motility, and metastasis. Cetuximab was approved in 2004 by the US FDA. However, cetuximab therapy has not been shown to extend patients' lives. Especially when used as a combination treatment, it has been shown to shrink tumors in some patients and delay tumor growth [15–17]. Therefore, this antibody has been of interest for the purpose of imaging studies. In this study, we describe the in vitro and in vivo properties of ^{177}Lu-labeled cetuximab using cys-DTPA-NCS to determine its potential for radioimmunotherapeutic applications.

2. Materials and methods

All chemicals and reagents used in the present study were obtained from commercial suppliers and were used without any further purification.

The progress of the chemical reaction was monitored by TLC glass sheets pre-coated with silica-gel, (G-25 UV254, Macherey-Nagel Inc). The NMR spectra were recorded with Bruker Avance 500 (500 MHz, 1H, KRICT, Daejeon) spectrometers. Mass spectra were recorded with the Hewlett Packard HP 1100 series LC/MSD (Peptron Inc., Daejeon).

^{177}Lu was purchased from Perkin-Elmer (Massachusetts, USA) and the radioactivity was measured using an ionizing chamber (Atomlab 200, Bio-dex, New York, USA) by setting the calibration value for ^{177}Lu as corrected and calibrated by the manufacturer.

3. Synthesis of S-(4-isothiocyanatophenylethyl)-Cys-DTPA

3.1. N,N-Bis[(tert-butoxycarbonyl)methyl]-2-ethanolamine

To tert-butyl bromoacetate (17.02 mL, 115 mmol) in DMF (100 mL), potassium carbonate (12.514 g, 125 mmol) was added. The reaction mixture was cooled to 0 °C followed by a dropwise addition of ethanolamine (2.98 mL, 49.4 mmol) in a nitrogen atmosphere. After continuously stirring for 30 min at 0 °C, the reaction was left overnight under ambient conditions.

After evaporation, the concentrated sodium-carbonate solution (100 ml along with a diethylether (150 ml) was added. The organic layer was then separated and washed with a concentrated sodium-carbonate solution (50 ml) and with brine (100 ml). The solvent was evaporated to give 1 in an oil form. ^{1}H NMR (CDCl$_3$), δ(ppm): 1.46 (s,

18H), 3.09 (t, 2H), 3.44 (s, 4H), 3.65 (t, 2H) (LC/MSD M + 1): cald. for 290.19 found 290.2.

3.2. N,N-Bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine

To compound 1 in dichloromethane (100 ml), triphenylphospine (15.90 g, 60 mmol) was added and the mixture was cooled to 0 °C. N-bromo-succinimide (10.68 g, 60 mmol) was added at small levels for 5 min. After stirring for 2 h at 0 °C, the solvent was evaporated to yield a semisolid product. The desirable compound was separated by adding a diethylether (300 ml). After collecting the ether layer, it was evaporated in vacuo and purified by a silica gel column chromatography (Diethylether:Hexane = 4:6) to give 2 (14.5 g, 83%) as a colorless oil. ^{1}H NMR (CDCl$_3$), δ(ppm): 1.46 (s, 18H), 3.12 (t, 2H), 3.43 (t, 2H), 3.47 (s, 4H) (LC/MSD M + 1): cald. for 352.10 found 352.1.

4. 2-(4-N-Boc-aminophenyl)ethanol

To a solution of 2-(4-aminophenyl)ethanol (2 g, 14.6 mmol) in methanol (15 ml), (Boc)2O (3.5 g, 16.0 mmol) in methanol (10 ml) and in triethylamine (10 ml) was added and reacted at 40 °C for 8 h. After evaporation, dichloromethane (50 mL) was added to the reaction mixture and washing with c-NaCl (20 mL) and H$_2$O (20 mL) was conducted. After evaporation in vacuo, the mixture was purified by column chromatography (MC:MeOH = 20:1) to yield 3 (2.91 g, 84%) as a colorless oil.

^{1}H NMR (CDCl$_3$), δ(ppm): 1.54 (s, 9H), 2.84 (t, 2H), 3.84 (t, 2H), 6.50 (br s, 1H, NH), 7.17 (d, 2H), 7.32 (d, 2H) (LC/MSD M + 1): cald. for 237.14 found 260.2 (M + Na) +.

5. 1-(4-N-Boc-aminophenyl)-2-bromoethane

To a solution of 3 (2.9 g, 12.2 mmol) in dichloromethane (30 ml), triphenylphospine (3.21 g, 12.3 mmol) and N-bromo-succinimide (2.18 g, 12.3 mmol) were added consecutively under a nitrogen condition at 0 °C. The resulting solution was stirred for additional 2 h at 0 °C. After the solvents were evaporated in vacuo, the resulting crude was treated with diethyl ether and filtered off. It was concentrated and purified by silica gel column chromatography (Diethylether:Hexane = 1:5) to give 4 (3.30 g, 90%) as a white powder.

^{1}H NMR (CDCl$_3$), δ(ppm): 1.54 (s, 9H), 3.12 (t, 2H), 3.55 (t, 2H), 6.52 (br s, 1H, NH), 7.15 (d, 2H), 7.34 (d, 2H) (LC/MSD M + 1): cald. for 300.05 found 300.0.

6. S-((4-N-Boc-aminophenyl)-1-ethyl)-cysteine methylester

Cysteine methylester (343.0 mg, 2.0 mmol) was activated with 0.5 M sodium methoxide (8 ml, 4 mmol). The prepared compound 4 (600.0 mg, 2.0 mmol) in methanol (20 ml) was then added in portion. After stirring for 5 h at room temperature, the solvent was removed and purified by column chromatography (MC:MeOH = 15:1) to give 5 (653 mg, 92%) as yellowish oil.

^{1}H NMR (CD3OD), δ(ppm): 1.53 (s, 9H), 2.81 (m, -CH$_2$-S-CH$_2$-), 2.92 (t, 2H), 3.66 (t, 1H), 3.75 (s, 3H), 6.50 (br s, 1H, NH), 7.13 (d, 2H), 7.31 (d, 2H) (LC/MSD M + 1): cald. for 355.2 found 355.0.

7. S-(N-Boc-aminophenyl)-Cys(tBu4-DTPA) methylester

To compound 5 (300 mg, 0.85 mmol), two equivalent measures of compound 2 in acetonitrile (10 ml) were added in a dropwise manner with 2 M of phosphate buffer (pH = 8, 5 ml) in portions.

After stirring for one day at room temperature, the buffer layer was changed and charged with one equivalent amount of compound 2 to process the reaction continuously. This was then stirred for 48 h. After

stirring for 48 h and isolating the acetonitrile layer, the solvent was evaporated and partitioned using water and ethyl acetate. The organic layer was dried and purified with column chromatography (Ethyla-cetate:Hexane = 1:9) to give 6 (610 mg, 80%) as a pale yellow oil.

^{1}H NMR (CDCl$_3$), δ(ppm): 1.44 (s, 36H), 1.50 (s, 9H), 2.80 (br m, -CH$_2$-CH$_2$-S-CH$_2$-CH-N-(C$_2$H$_4$)$_2$-), 3.42 (s, 8H), 3.68 (s, 3H), 6.50 (br s, 1H, NH), 7.11 (d, 2H), 7.27 (d, 2H) (LC/MSD M + 1): cald. for 897.52 found 897.3.

8. S-aminophenylethyl-Cys-DTPA

To a solution of 6 (300 mg, 0.33 mmol) in methanol (20 ml), conc-hydrochloric acid solution (36%, 10 ml) was added and heated for 1 h at 70 °C. After evaporation, it was precipitated with diethylether to afford 7 as a slightly yellowish powder.

^{1}H NMR (D2O), δ(ppm): 2.76 (br m, -CH$_2$-CH$_2$-S-CH$_2$- CH-N-(C$_2$H$_4$)$_2$-), 2.91 (t, 4H), 3.33 (t, 4H), 3.95 (s, 8H), 7.19 (m, aromatic, 4H) (LC/MSD M + 1): cald. for 559.2 found 559.3.

9. S-isothiocyanato-phenylethyl-Cys-DTPA (Cys-DTPA-NCS)

S-aminophenylethyl-Cys-DTPA (100 mg) was dissolved in 3 ml of double-distilled water. To the solution, 1.2 equivalent of 1 M CSCl$_2$ in chloroform was added and reacted for 2 h at room temperature under a nitrogen atmosphere.

After isolation of the aqueous layer, it was lyophilized to give a white powder. The freeze-dried compound was stored at −78 °C before use.

10. Radiolabeling with [177]Lu

Different concentrations of cys-DTPA-NCS (10^{-6} to 10^{-12} M) in 50 mM sodium acetate buffer (pH 5.5) were mixed with 3.7 MBq of LuCl$_3$ solution and allowed to stand for 10 min at room temperature. The radiolabeling yield was analyzed by Instant Thin-Layer Chromatography (ITLC). The ITLC was developed with silica gel paper (Gelman Science Inc., Michigan, USA) using saline as the mobile phase and was determined with a Cyclone Storage Phosphor System (PerkinElmer Life Science, Massachusetts, USA). The same procedure was applied for the radiolabeling of ρ-SCN-Bn-DTPA (Macrocyclic Inc. Dallas, USA).

11. Biodistribution assay

The *in vivo* behavior of the radioimmunoconjugate was studied in female BALB/c nude mice (Orient Co., Daejeon, Republic of Korea). 0.185 MBq (5 μCi) of ^{177}Lu-cys-DTPA-NCS was injected intravenously into the tail vein. The mice (n = 5) were sacrificed 24, 96 and 168 h after the injection, and the radioactivity levels in the blood, kidney, liver, heart, intestine and lung were determined using a γ-scintillation counter (PerkinElmer Life Science, Massachusetts, USA). These were expressed as a percentage of the injected dose per gram of tissue (% ID/g).

12. Preparation of cys-DTPA-NCS-cetuximab

For the preparation of the immunoconjugate, cetuximab (Merck KGaA, Darmstadt, Germany) was reacted with cys-DTPA-NCS at molar ratio of 1:1 for 10 min at room temperature in a 1× PBS buffer solution. The un-conjugated cys-DTPA-NCS was cleared using a Centricon filter system (Millipore, Netherlands) at a molecular cut-off level of 50 kDa. To measure the un-conjugated portion of Cys-DTPA-NCS, the filtrate was analyzed at 254 nm using a microplate spectrophotometer (Molecular Devices, California, USA). The purified immunoconjugate, cys-DTPA-NCS-cetuximab, was stored at −20 °C before use.

13. Stability assay

For assessment of the stability, ^{177}Lu-cys-DTPA-NCS-cetuximab was stored at room temperature in saline. After 6 days, aliquot was taken and analyzed by ITLC as above.

14. Cell affinity assay

HT-29 human colon cancer cells were obtained from the Korea Cell Line Bank and were cultured in RPMI1640 (LONZA, Walkersville, MD, USA), supplemented with 10% fetal bovine serum, 300 mg/L L-glutamine, 25 mM HEPES, 25 mM NaHCO$_3$, 100 U/ml penicillin, and

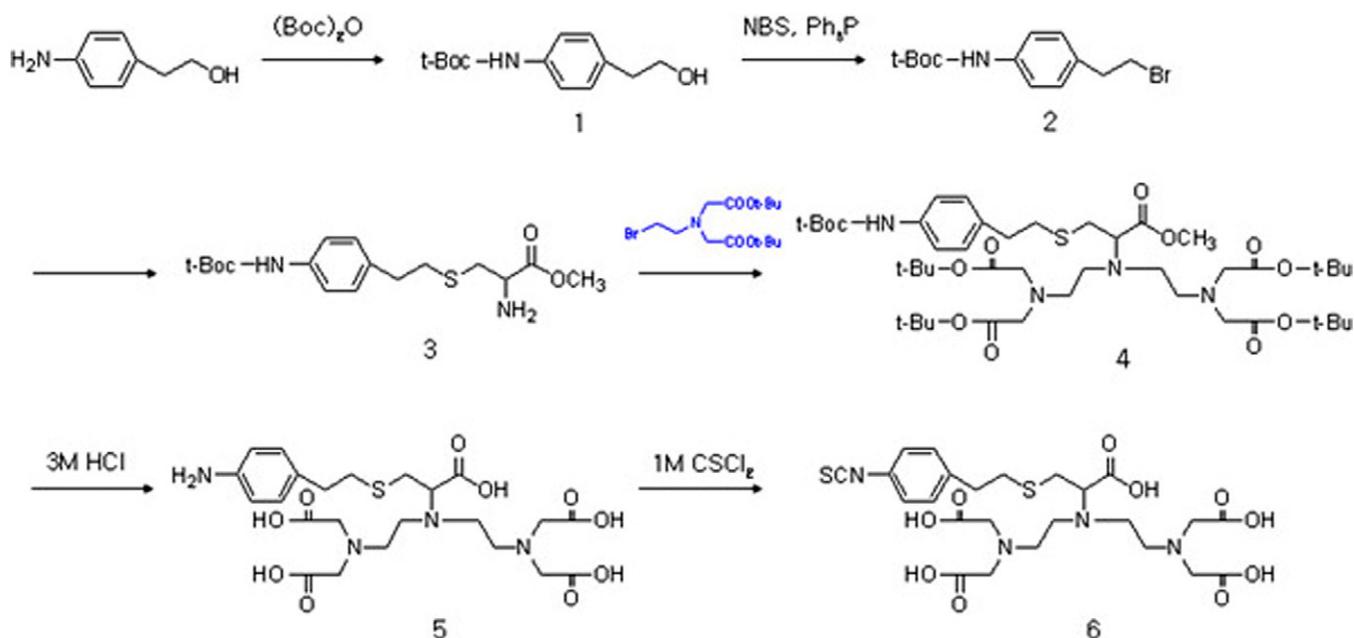Fig. 1. Synthesis pathway for cys-DTPA-NCS.

Table 1
Comparison of radiolabeling yields of ^{177}Lu-cys-DTPA-NCS and ^{177}Lu-ρ-SCN-Bn-DTPA at different concentrations.

Concentration [M]	ρ-SCN-Bn-DTPA	Cys-DTPA-NCS
10^{-6}	95.53	98.66
10^{-7}	80.42	98.82
10^{-8}	83.54	99
10^{-9}	25.14	42.97
10^{-10}	20.45	6.81
10^{-11}	0.81	0
10^{-12}	0.52	1.18

100 g/ml streptomycin (Sigma, Milan, Italy) in an atmosphere of 5% CO_2 in air at 37 °C. 5×10^5 HT-29 cells were seeded into 12-well plates and were incubated at 37 °C for 12–16 h. After washing once with a binding medium (the minimum essential medium with 0.2% bovine serum albumin), the cells were incubated at room temperature for 2 h with 500,000 cpm of ^{177}Lu-cys-DTPA-NCS-cetuximab in a binding medium with increasing concentrations of unlabeled cetuximab (10^{-5} to 10^{-13} M). The cells were rinsed with PBS (phosphate buffered saline) at a pH of 7.4 twice and lysed in 0.2 ml of 1 N NaOH for 5 min. The percentage of radioactivity was determined with a Wallac 1470 automated gamma counter (PerkinElmer Life Science, Massachusetts, USA). IC_{50} values for the peptides were calculated through a non-linear regression analysis using the GraphPad Prism5 computer fitting program.

15. Radioimmunotherapy

Female Balb/C athymic (*nu/nu* genotype) mice were bred and maintained in negative pressure isolators. The mice were housed in a barrier facility with 12-h light/dark cycles and provided with sterilized food and water *ad libitum*. All procedures were performed on mice at least 7 weeks of age. HT-29 tumor xenografts were established in the hind flank by SC injections of 1×10^6 cells in 100 μl of 50:50 Matrigel (BD Bioscience, NJ, USA) and a serum-free media. The tumor volume was assessed by bilateral Vernier caliper measurements, using the formula; Volume $= 0.5(\text{width})^2 \times \text{length}$, where the length was the longest diameter across the tumor, and the width was the corresponding perpendicular value. HT-29 human colon tumor cells were grown as subcutaneous xenografts in nude mice. When a tumor volume of approximately 600–700 mm^3 was obtained, saline or 25 μg of cetuximab or 9.25 MBq/25 μg of ^{177}Lu-cys-DTPA-NCS-cetuximab was administered. Tumor volumes were recorded once a week until the tumor volume quadrupled from that at the start of treatment (relative tumor volume × 4; RTV 4).

16. Result and discussion

The synthetic route for cysteine based DTPA-NCS (cys-DTPA-NCS) is shown in Fig. 1. 1-(4-N-Boc-aminophenyl)-2-bromoethane was prepared by treating N-protection with Boc, followed by bromination with N-bromo-succinimide. Cysteine derivative 3 was obtained by the reaction of 2 and cysteine methylester under sodium methoxide. The coupling of 3 and N,N-Bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine in a buffer system afforded the desired S-aminophe-

nylethyl-Cys-DTPA with a good yield. Selective hydrolysis was carried out using 3 N HCl per 0.1 mmol of reactant. The final product, cys-DTPA-NCS, was obtained via a reaction with thiophosgene. Finally, we easily synthesized this DTPA derivative containing isothiocyanate using cysteine as a core ligand.

In addition, we expected improved chelating power resulting from the chelation of the thiol group of the cysteine backbone with radioisotopes. To demonstrate this, the chelating power of cys-DTPA-NCS and commercial ρ-SCN-Bn-DTPA with the same amount of ^{177}Lu was qualitatively investigated for comparison. ρ-SCN-Bn-DTPA has received considerable attention for producing immunoconjugates labeled with trivalent radiometals [3,7]. 10^{-6} to 10^{-12} M of cys-DTPA-NCS or ρ-SCN-Bn-DTPA was radiolabeled with 3.7 MBq of ^{177}Lu and the radiolabeling yields were determined. Table 1 shows the labeling efficiency, showing that a high labeling yield (≥95%) can be achieved when using over quantity 1 nM of cys-DTPA-NCS. The concentrations requiring a 50% yield of radiolabeling cys-DTPA-NCS and ρ-SCN-Bn-DTPA were calculated as 1.2 nM and 2.1 nM, respectively. Specifically, radiolabeling using cys-DTPA-NCS resulted in higher yields and more specific activity than the radiolabeling of the ρ-SCN-Bn-DTPA conjugate. Presumably, the reason for this was that Lu-O_5N_3S in cys-DTPA-NCS is more rapid and more stable than Lu-O_5N_3 in ρ-SCN-Bn-DTPA (Fig. 2). The rapid formation of the radiometal complex of the bifunctional chelator is required to minimize radiolytic damage to the protein conjugate, which can occur during the radiolabeling of an antibody conjugate with high activity for therapeutic applications [5,18]. Superior chelation chemistry allows for the scaled-up production of stable ^{177}Lu- or ^{90}Y-radioimmunoconjugates under ambient condition which is necessary for effective and safe RIT applications. The results indicated that cys-DTPA-NCS presents an excellent ability to chelate as compared to ρ-SCN-Bn-DTPA.

The mean molar ratio of ^{177}Lu for the cysteine-based DTPA-NCS (cys-DTPA-NCS) was studied using different concentrations of cys-DTPA-NCS. In all cases, we started from the same concentration of ^{177}Lu at 4 nmol. The molar concentration of ^{177}Lu was calculated on the basis of the specific activity of 5.18 GBq/mg (according to the manufacturer) for labeling 714 ng of ^{177}Lu (3.7 MBq). The limitation of a 95% radiolabeling yield was calculated as 3.96 nmol. From the results, the initial Lu/cys-DTPA-NCS molar ratio was 1.02. Therefore, for mean ratio chemical labeling yields of 95%, the result was 0.97.

The result of the biodistribution study for ^{177}Lu-cys-DTPA-NCS is shown in Fig. 3. The accumulated radioactivity in the organs at 24 h post injection is negligible for the radiolabeled cys-DTPA-NCS. Radioactivity accumulated in the kidney was $1.28\% \pm 0.15\%$ of the injected dose per gram (%ID/g) at 24 h and $0.44 \pm 0.08\%$ID/g at 168 h. The relatively high uptake in the kidney shows the route of excretion through the urinary system. The radiolabeled BFCA had no specific accumulation in any organs indicating the *in vivo* stability of this BFCA.

For the development of tumor-targeting radiopharmaceuticals, a number of factors, such as the nature of the tumor-associated target and the physical properties of the radionuclide, the labeling chemistry factors require a careful consideration. Radioimmunotherapy studies have mainly used monoclonal antibodies as a targeting agent. Cetuximab is a chimeric mouse/human monoclonal antibody of the IgG1 subclass that targets the human epidermal growth factor
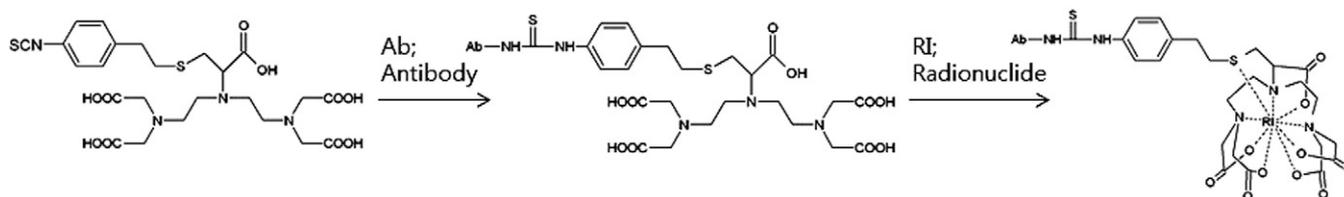
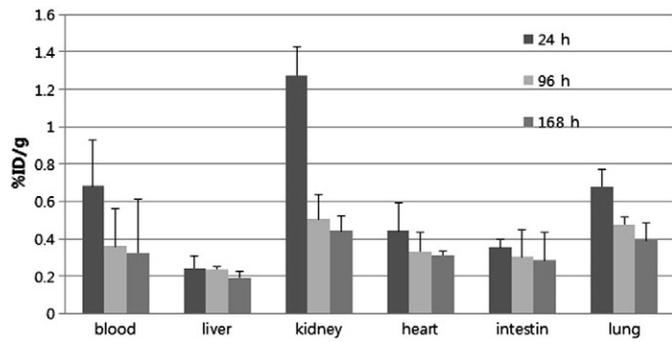**Fig. 2.** Schematic figure of the radioimmunoconjugation of cys-DTPA-NCS.

Fig. 3. *In vivo* biodistribution of ^{177}Lu-cys-DTPA-NCS in normal mice (%ID/g, n = 5, intravenous injection).

receptor (EGFR) [13–17]. EGFR is overexpressed in about one third of all human cancers and is expressed in normal cells, particularly those of epithelial origin, ranging in density from 40,000 to 100,000 receptors per cell, whereas in cancer cells, up to 2×10^6 receptors per cell have been reported [18]. For these reasons, the potential of EGFR as a target for both imaging and therapy has been an active area of investigation. In particular, cetuximab has been investigated in imaging studies for the purpose of monitoring disease and EGFR expression as well as for patient selection or dosimetry calculations. Radiolabeled cetuximab with ^{64}Cu, ^{89}Zr and ^{111}In has been investigated as an imaging agent for PET for the purposes of quantitating EGFR expression and to select patients for immunotherapy [15–17]. In this study, we evaluated the *in vitro* and *in vivo* properties of radiolabeled cetuximab and determined its potential for RIT applications.

For the radioimmunoconjugation of cetuximab with a therapeutic radioisotope, ^{177}Lu, 250 μg of purified cys-DTPA-NCS-cetuximab in $1 \times$ PBS was labeled with 370 MBq of ^{177}Lu in 50 mM HCl for 15 min at room temperature. A high radiolabeling yield (>98%) of ^{177}Lu-cys-DTPA-NCS-cetuximab was obtained (Figs. 4 (A) and (B)), and the stability was investigated while stored for 6 days at room tempera-

ture. The ITLC profiles showed that the radiolabeled cetuximab was very stable without other byproducts or free ^{177}Lu (Figs. 4 (C) and (D)). Important for the success of the RIT application is a chelating agent that rapidly forms a stable radiometal complex with a therapeutic radioisotope such as ^{90}Y, ^{177}Lu. Until now, ^{131}I is widely used for RIT because the procedures related to it are relative easy to perform and it is inexpensive. However, ^{131}I is not an ideal radionuclide for RIT because it binds to a tyrosine moiety, which prevents binding to the binding site of a target antigen [19,20]. Radiometal and radiolanthanides such as ^{90}Y and ^{177}Lu, are potentially more suitable for RIT. The 11 mm tissue penetration range of the β-emissions of ^{90}Y is suitable for the treatment of relatively large solid tumor masses, and the 2 mm range of ^{177}Lu is well suited for small lesions. In particular, ^{177}Lu γ photon is able to scintigraphically visualize and monitor the progress scintigraphically after a treatment [21,22].

To determine the inhibitory concentration of the 50% (IC$_{50}$) value of ^{177}Lu-labeled cetuximab, we carried out a competitive binding assay. Milenic et al. reported that HT-29 xenografts had the highest uptake of ^{111}In-labeled cetuximab [13]. HT-29 human colon carcinoma cells were incubated for 3 h at 37 °C with 500,000 cpm of ^{177}Lu-cys-DTPA-NCS-cetuximab and increasing concentrations of unlabeled cetuximab (10^{-5} to 10^{-13} M). The ^{177}Lu-cys-DTPA-NCS-cetuximab exhibited an IC$_{50}$ value of 10 nM (95% confidence) on HT-29 cells (Fig. 5). Zhang et al. reported values for cetuximab at IC$_{50}$ values of 0.22–8.20 nM for seven head and neck SCC cell lines [14]. The typical binding profile indicates that radioimmunoconjugation using cys-DTPA-NCS has a minor influence on the antigen recognition site of cetuximab to maintain specific affinity against cell-surface receptors.

For RIT we chose a one-time dose of 9.25 MBq of ^{177}Lu labeled cetuximab in this study. With this ^{177}LuCl$_3$ dose, health mice showed no observable toxicity, as indicated by unchanged mouse body weights (data not shown). HT-29 human colon tumor cells were grown as subcutaneous xenografts in nude mice. When a tumor volume of approximately 600–700 mm^3 was obtained, saline or 25 μg of cetuximab or 9.25 MBq/25 μg of ^{177}Lu-cys-DTPA-NCS-cetuximab was administered. Tumor volumes were recorded once a week until
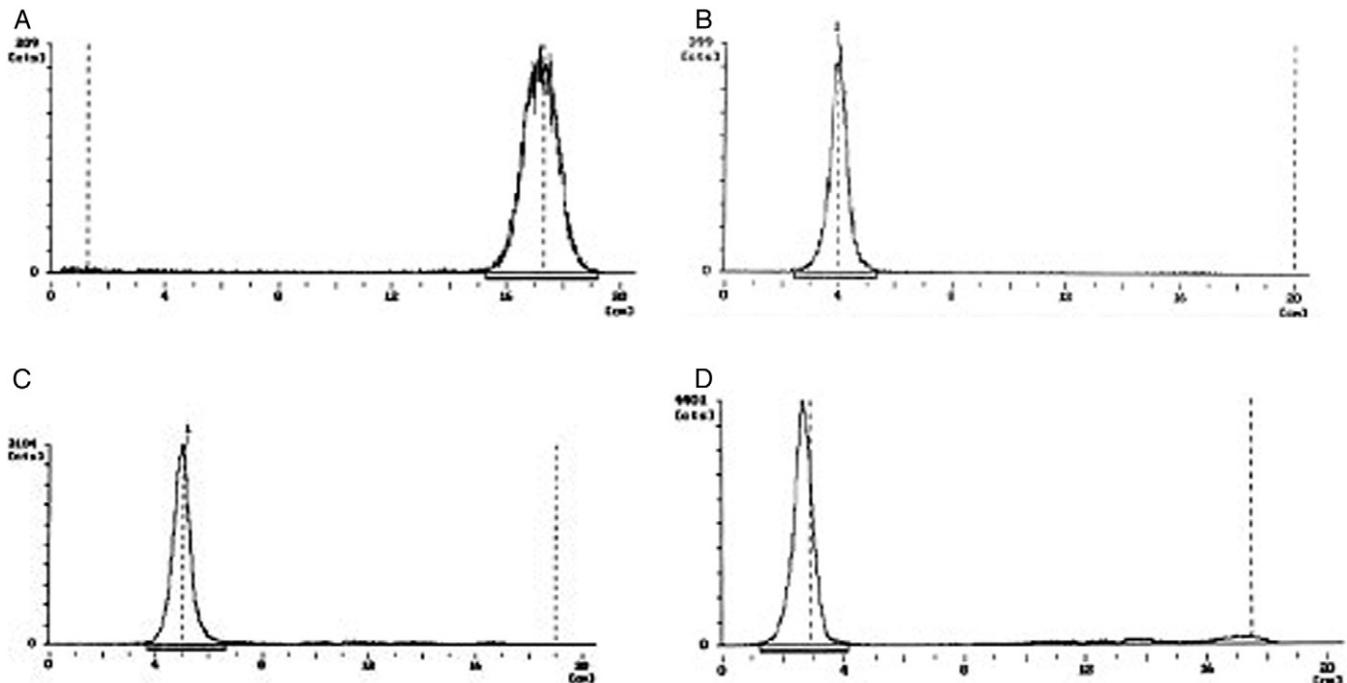

Fig. 4. ITLC profiles of ^{177}Lu (A) and ^{177}Lu-cys- DTPA-NCS-cetuximab at 0 day (B), 2 days (C) and 6 days (D) after conjugation. The *x-axis* shows the distance in cm from the origin (*left*) and the *y-axis* shows the proportional activity count.
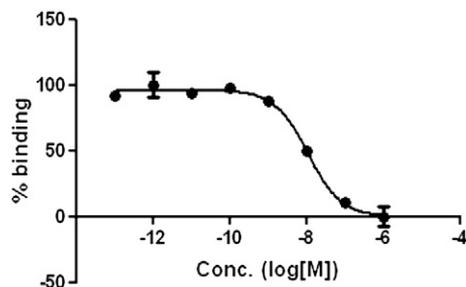
Fig. 5. Binding plot of ^{177}Lu-cys-DTPA-NCS-cetuximab and HT-29 cells.

the tumor volume quadrupled from that at the start of the treatment (relative tumor volume × 4; RTV 4). With this dose, HT-29 tumor growth was significantly inhibited. The average growth delay achieved was 15.7 (±4) and 18.6 (±3.3) days for saline and unlabeled cetuximab, while it was more than 27 days for the group treated with ^{177}Lu-cys-DTPA-NCS-cetuximab (Fig. 6). Solid tumor models for efficacy determination were devised for tumor growth delays or for tumor growth inhibition. Tumor growth delay study began when an established tumor nodule was present. Activity in a tumor growth delay activity is stronger evidence of the clinical potential of a treatment as compared to tumor growth inhibition activity [23,24]. The therapeutic effect indicates that ^{177}Lu-cys-DTPA-NCS remains stable *in vivo* and that the tumor targeting of ^{177}Lu-cys-DTPA-NCS-cetuximab can be improved by selecting an adequate EGFR-expressing tumor model. We also believe that most of the therapeutic efficacy of ^{177}Lu-labeled cetuximab will come from the optimal therapeutic dose via the maximum tolerated dose and a dose–response analysis.

The use of cys-DTPA-NCS as bifunctional chelator for radio-immunoconjugation has several advantages. First, the DTPA derivative has coordination geometry similar to that of DTPA and can be radiolabeled with lanthanide by a simple mixing process under ambient condition. Second, it possesses isothiocyanate that can stably react with amines in an aqueous solution. Finally, its superior chelating power allows rapid and stable formation of the radiometal complex, which is necessary for effective and safe RIT.

17. Conclusion

For the development of new protein-based radiolabeled complexes for the treatment of various diseases, we synthesized cysteine-based DTPA deri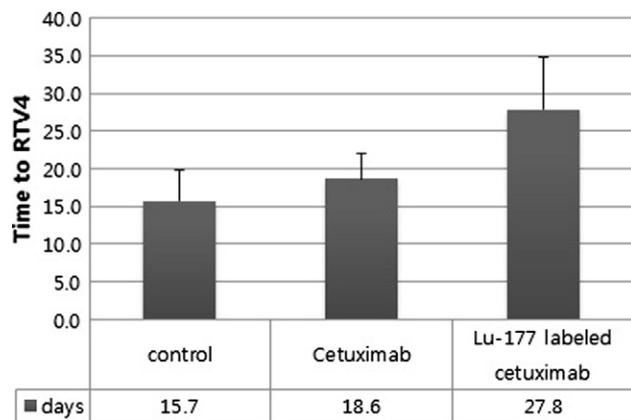vatives containing an isothiocyanate moiety for selective conjugation with an amine in bio-active molecules. This cys-DTPA-NCS was easily radiolabeled with ^{177}Lu in an ambient condition, and the formation rate of the radiometal complex was rapid. ^{177}Lu-labeled cetuximab using cys-DTPA-NCS sustained its radiochemical and biological stability and demonstrated therapeutic efficacy in an experimental animal model. The new DTPA derivative, cys-DTPA-NCS, is a good bifunctional chelating agent for radioimmunoconjugation, and the prepared ^{177}Lu-cys-DTPA-NCS-cetuximab is feasible as a form of targeted radioimmunotherapy.

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Fig. 6. Therapeutic efficacy of ^{177}Lu-cys-DTPA-NCS-cetuximab in HT-29 human colon carcinoma cells xenografts. Female athymic nude mice bearing tumors were injected with a one-time dose of saline, cetuximab and 9.25 MBq of ^{177}Lu-cys-DTPA-NCS-cetuximab. The growth delay was calculated as the mean time to the relative tumor volume (RTV 4) *P*<0.05.