

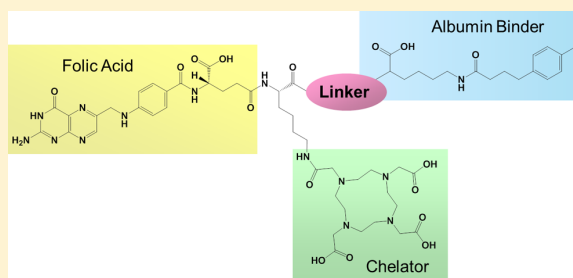
Preclinical Comparison of Albumin-Binding Radiofolates: Impact of Linker Entities on the in Vitro and in Vivo Properties

Klaudia Siwowska,[†] Stephanie Haller,[†] Francesca Bortoli,[†] Martina Benešová,^{†,‡} Viola Groehn,[§] Peter Bernhardt,^{||,⊥} Roger Schibli,^{†,‡} and Cristina Müller^{*,†,‡,§}[†]Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Paul Scherrer Institut, 5232 Villigen-PSI, Switzerland[‡]Department of Chemistry and Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland[§]Merck & Cie Schaffhausen, 8200 Schaffhausen, Switzerland^{||}Department of Radiation Physics, The Sahlgrenska Academy, University of Gothenburg, SE-413 45 Gothenburg, Sweden[⊥]Department of Medical Physics and Medical Bioengineering, Sahlgrenska University Hospital, SE-413 45 Gothenburg, Sweden

Supporting Information

ABSTRACT: Tumor targeting with folic acid radioconjugates has been proposed as a promising strategy for radionuclide therapy of folate receptor α (FR)-positive cancer. Recently, it was shown that modification of radiofolates with an albumin-binding entity increased the tumor-to-kidney ratios of accumulated radioactivity in mice. The goal of this study was to evaluate the lead compound cm10 and compare it with new albumin-binding folate conjugates. Compound cm12 was designed with a long spacer consisting of a PEG-11 entity, and compound cm13 contained a short alkane chain between the albumin-binding moiety and folic acid. All of the derivatives were labeled with ^{177}Lu ($t_{1/2} = 6.65$ days, $E_{\beta^- \text{ average}} = 134$ keV; $E_{\gamma} = 113$ keV, 208 keV), a clinically established radionuclide for therapeutic purposes. The evaluation revealed that all of the albumin-binding radiofolates exhibited increased in vitro stability compared with the reference compound (^{177}Lu -cm14) without albumin binder. Serum protein binding, determined with an ultrafiltration assay, was high (>88%) for the derivatives with albumin-binding entities. The FR-binding affinity was in the same range ($K_D = 4.0$ – 7.5 nM) for all of the radiofolates, independent of the albumin-binding entity and spacer length. FR-specific uptake was proven in vitro using FR-positive KB tumor cells. In vivo studies with KB-tumor-bearing mice were performed in order to assess the tissue distribution profile of the novel radiofolates. ^{177}Lu -cm13 showed high tumor uptake at late time points ($13.3 \pm 2.94\%$ IA/g, 48 h p.i.) and tumor-to-kidney ratios (0.59 ± 0.03 , 48 h p.i.) in the same range as ^{177}Lu -cm10 (0.55 ± 0.07 , 48 h p.i.). However, the tumor-to-kidney ratio of ^{177}Lu -cm12 (0.28 ± 0.07 , 48 h p.i.) was reduced compared with ^{177}Lu -cm10 and ^{177}Lu -cm13. The results of this study indicate that the spacer entity between folic acid and the albumin binder is of critical importance with regard to the tissue distribution profile of the radiofolate. The PEG spacer compromised the beneficial effects of the lead compound, but the design with a short alkane spacer appeared to be promising. Future studies will focus on the design of radiofolates with lipophilic and more rigid spacer entities, which may allow a further improvement of their tissue distribution profiles.

KEYWORDS: radionuclide therapy, ^{177}Lu , folate radioconjugate, albumin-binding entity, linker, spacer, PEG spacer, alkane spacer



INTRODUCTION

Over the past years, the concept of tumor targeting based on specific tumor-associated markers has been developed for imaging and therapy of many cancer diseases.¹ Such strategies exploit ligands that accumulate preferentially in the malignant cells expressing the target of interest. Folate receptor α (FR) is one of the tumor targets that has been extensively investigated in this regard.² It is expressed in a variety of carcinomas of epithelial origin (including ovarian, endometrial, lung, and kidney cancers),^{3,4} but in healthy tissues FR expression is limited to only a few sites, including the kidneys.^{5,6} The vitamin folic acid has been extensively studied as a targeting agent for

selective delivery of attached imaging probes and therapeutics to FR-positive tumor cells.² A large number of folate conjugates have been synthesized for coordination of radionuclides suitable for positron emission tomography (PET) or single photon emission computed tomography (SPECT) imaging, which is commonly combined with computed tomography (CT).^{7–10} Two such radiofolate imaging agents, ^{111}In -DTPA-

Received: November 7, 2016

Revised: December 7, 2016

Accepted: December 28, 2016

Published: January 17, 2017

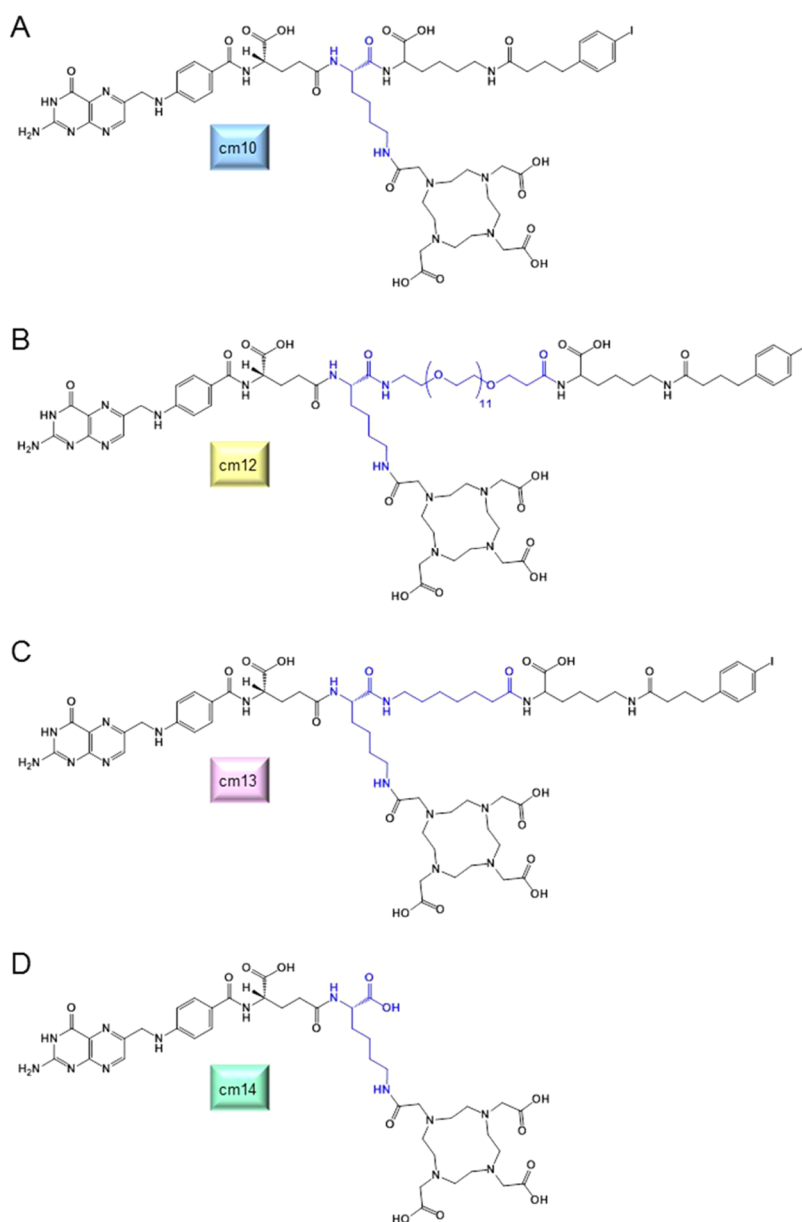


Figure 1. Chemical structures of the albumin-binding folate conjugates (A) cm10, (B) cm12, (C) cm13, and (D) the control compound cm14, which does not contain an albumin-binding entity. The lysine residue, connecting folic acid and the DOTA chelator, as well as different spacer entities between folic acid and the albumin-binding entity are shown in blue.

folate and ^{99m}Tc -EC20 (Etarfolatide, Endocyte Inc.), have been used in the clinics for imaging of FR-positive cancer.^{11,12} In terms of a therapeutic approach, promising examples include folate conjugates of toxins and chemotherapeutics such as mitomycin, maytansinoid, and vinca alkaloids.^{13,14} Some of them have already been advanced to clinics, including EC145 (Vintafolide, Endocyte Inc.), a folate conjugate of a vinca alkaloid.^{15,16}

Our research activities have been focused on the development of FR-targeted radionuclide therapy in which folic acid is used for selective delivery of β^- -particle-emitting radionuclides.¹⁷ The concept of radionuclide therapy has been successfully employed in clinics using other targeting ligands such as tumor-seeking hormone peptides (e.g., somatostatin analogues) and low-molecular-weight molecules (e.g., prostate-specific membrane antigen ligands).^{18,19} Despite the promising potential of folic acid radioconjugates for therapeutic purposes,

there is one major concern: folate radioconjugates accumulate to a significant degree in the kidneys. This is due to the expression of the FR in the renal proximal tubule cells, to which radiofolates bind during urinary excretion.^{5,6} Common strategies employed in combination with therapeutic radioconjugates, including the application of positively charged amino acids (e.g., lysine) and diuretic agents (e.g., furosemide), remain ineffective in reducing renal uptake of radiofolates because of their specific binding to FRs.²⁰

Recently, we set out to face the issue of low tumor-to-kidney ratios of accumulated radioactivity by the strategy of chemical modification of the radioconjugates. Binding to serum albumin has been proposed for enhancing the blood circulation time of low-molecular-weight molecules that are otherwise rapidly cleared through the kidneys.²¹ Dumelin et al.²² have identified a number of small albumin-binding moieties based on the lead structure, 4-(*p*-iodophenyl)butyric acid, using a DNA-encoded

chemical library. Previously, we included such an albumin-binding moiety into the structure of a folate conjugate with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator.²³ This novel trifunctional conjugate was thoroughly investigated in vitro and in vivo, and the results were very promising, as they showed a several-fold-increased tumor-to-kidney ratio of accumulated radioactivity and allowed for the first time the performance of preclinical therapy studies using a radiofolate that effectively reduced the tumor growth in mice.²³ Further optimization of the tissue distribution profile would be desirable, however, in order to prevent damage to the kidneys, which was observed when therapeutic doses of the albumin-binding ¹⁷⁷Lu-folate were applied.²⁴

The goal of this study was to investigate the properties of new albumin-binding folate conjugates with longer spacer entities in order to better understand the structural requirements to modulate binding to serum proteins (Figure 1). It was believed that increasing the distance between folic acid and the albumin-binding entity would facilitate the interaction of the albumin binder with serum proteins. The lead compound, denoted as cm10, was designed with a lysine residue to link folic acid with a DOTA chelator and the albumin-binding entity.²⁵ On the basis of the same synthesis concept, a long spacer consisting of either a PEG-11 entity (folate conjugate cm12) or a short spacer consisting of an alkane chain (folate conjugate cm13) was introduced between the albumin-binding entity and folic acid. A folate conjugate without an albumin-binding moiety (folate conjugate cm14) served as the reference compound.

For the evaluation of the folate derivatives, they were labeled with ¹⁷⁷Lu ($t_{1/2} = 6.65$ days, E_{β^-} average = 134 keV, $E_{\gamma} = 113$ keV, 208 keV), a radionuclide that has been clinically established for therapeutic purposes. The radiofolate conjugates were evaluated extensively in vitro and by the performance of post-mortem biodistribution studies and preclinical SPECT imaging using tumor-bearing mice.

MATERIALS AND METHODS

Organic Synthesis of the Folate Conjugates. The chemical synthesis of the folate compounds is described in Figures S1–S4 in the [Supporting Information](#).

Preparation of ¹⁷⁷Lu-Folate Conjugates. Radiolabeling of cm10, cm12, cm13, and cm14 with ¹⁷⁷Lu was carried out at a specific activity up to 50 MBq/nmol. ¹⁷⁷LuCl₃ in HCl (0.05 M, obtained from Isotope Technologies Garching ITG GmbH) was mixed with sodium acetate (0.5 M, pH 8) in a 5:1 ratio to obtain a solution of pH ~4.5. After the addition of the folate conjugate (1 mM stock solution), the reaction vial was incubated for 10 min at 95 °C. The labeling of cm14 was performed in the presence of 3 mg of ascorbic acid, which was added from an aqueous stock solution (140 mg/mL). Quality control was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis (Merck LaChrom HPLC) using a C-18 column (XTerra MS, 5 μ m, 4.6 mm $\times$ 150 mm, Waters). The eluent consisted of acetonitrile (solvent A) and 0.1% trifluoroacetic acid in Milli-Q water (solvent B). For quality control, a gradient of 5–80% A and 95–20% B over 15 min with a flow rate of 1.0 mL/min was used. For in vitro and in vivo applications, 10 μ L of 5 mM sodium diethylenetriaminepentaacetate (NaDTPA) was added to the reaction mixture for complexation of potential traces of unreacted ¹⁷⁷Lu(III) prior to dilution.

Determination of Distribution Coefficients (LogD Values). The *n*-octanol/PBS distribution coefficient (logD) values of the ¹⁷⁷Lu-labeled folate conjugates (cm10, cm12, cm13, and cm14) were determined using a previously published procedure.²⁶ In brief, the compounds were labeled at a specific activity of 25 MBq/nmol. For each radioconjugate, a sample containing ~0.5 MBq of ¹⁷⁷Lu-labeled compound (25 μ L) was mixed with 1475 μ L of phosphate-buffered saline (PBS) (pH 7.4) and 1500 μ L of *n*-octanol. The vials were vortexed vigorously for 1 min and then centrifuged (2500 rpm) for 6 min for phase separation. The activity concentration in a defined volume of each layer was measured in a γ counter (PerkinElmer Wallac Wizard 1480). The distribution coefficient was expressed as the logarithm of the ratio of counts per minute (cpm) measured in the *n*-octanol phase to the cpm measured in an equal volume of the PBS phase. The values reported are the mean $\pm$ standard deviation (SD) of the results of three to five independent experiments, each performed with five replicates. The significance of the data was determined using a one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test (GraphPad Prism software, version 5.01). A *p* value of <0.05 was considered statistically significant.

Determination of Albumin-Binding Properties. An ultrafiltration assay was employed to determine the binding of the ¹⁷⁷Lu-folate conjugates (labeled at 25 MBq/nmol) to human plasma proteins as previously reported.²³ Centrifuge ultrafiltration devices (30 000 Da nominal molecular weight limit, methylcellulose micropartition membranes, Millipore) were used to separate the free fraction of the radiofolates from the plasma-protein-bound fraction. Each ¹⁷⁷Lu-folate conjugate (0.5 MBq, 25 μ L) was added to plasma samples of 250 μ L and incubated for 15 min at room temperature. The same procedure was performed additionally with PBS instead of plasma as a control experiment. Aliquots (250 μ L) of the radioactive samples were loaded onto the ultrafiltration devices and centrifuged at 2500 rpm for 40 min. The filtered solutions were measured in a γ counter (PerkinElmer Wallac Wizard 1480). The counts of the filtered solutions were calculated as a fraction of the radioactivity in the corresponding loading solutions, which was set to 100%. The values reported are the mean $\pm$ SD of three to four independent measurements, each performed with two replicates. The significance of the data was analyzed using a one-way ANOVA with Bonferroni's multiple comparison post-test (GraphPad Prism software, version 5.01). A *p* value of <0.05 was considered statistically significant.

Cell Lines and Cell Culture. KB cells (cervical carcinoma cell line, subclone of HeLa cells, ACC-136) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). IGROV-1 cells (human ovarian carcinoma cell line) were a kind gift of Dr. Gerrit Jansen (Department of Rheumatology, Free University Medical Center, Amsterdam, The Netherlands). Cells were cultured in folate-deficient RPMI medium (FFRPMI, Cell Culture Technologies GmbH, Gravesano, Switzerland) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. Routine cell culture was performed twice a week using trypsin for detachment of the cells.

Determination of FR Affinities (K_D Values). All of the folate conjugates (cm10, cm12, cm13, and cm14) were labeled with ¹⁷⁷Lu at a specific activity of 25 MBq/nmol. IGROV-1 tumor cells were seeded in 48-well plates ($\sim 0.25 \times 10^6$ cells in 0.5 mL of FFRPMI medium per well), allowing cell adhesion

and growth overnight at 37 °C. During the experiment, cells were kept on ice and all reagents were ice-cold. After removal of the supernatant, cells were washed once with PBS prior to the addition of FFRPMI medium without supplements (450 μ L/well). The ^{177}Lu -folate conjugate was added to each well in a volume of 50 μ L at different concentrations (0.1–500 nM, corresponding to 2 kBq–10 MBq). In some wells, tumor cells were coincubated with excess folic acid (100 μ M) to block FRs on the cell surface for determination of unspecific binding of the ^{177}Lu -folate conjugate. After incubation of the well plates for 1 h with shaking on ice at 4 °C, cells were washed twice with ice-cold PBS to remove unbound radiofolates, allowing determination of FR-bound ^{177}Lu -folate conjugates. Sodium hydroxide (1 M, 0.5 mL) was added to each well to lyse the cells. The cell suspensions were then transferred to tubes for measurement in a γ counter (PerkinElmer Wallac Wizard 1480). The values reported are the mean $\pm$ SD obtained from four independent experiments, each performed with three replicates. The significance of the data was analyzed using a one-way ANOVA with Bonferroni's multiple comparison post-test (GraphPad Prism software, version 5.01). A p value of <0.05 was considered statistically significant.

Cell Internalization Studies. Cellular uptake and internalization studies with ^{177}Lu -labeled folate conjugates (cm10, cm12, cm13, and cm14) were performed according to a previously published procedure.²³ KB cells were seeded in 12-well plates ($(\sim 0.5\text{--}1.0) \times 10^6$ cells in 2 mL of FFRPMI medium per well), allowing cell adhesion and growth overnight at 37 °C. After removal of the supernatant, cells were washed once with PBS prior to the addition of FFRPMI medium without supplements (975 μ L/well). The folate radioconjugate (labeled at 25 MBq/nmol) was added to each well in a volume of 25 μ L (1.5 pmol, 38 kBq). In some wells tumor cells were coincubated with excess folic acid (100 μ M) to block FRs on the cell surface. After incubation of the well plates for 0.5, 2, or 4 h at 37 °C, cells were washed twice with ice-cold PBS to determine total uptake of the radiofolates. In order to assess the internalized fraction, cells were additionally washed with a stripping buffer (an aqueous solution of 0.1 M acetic acid and 0.15 M NaCl, pH 3)²⁷ to release FR-bound radiofolates from the cell surface. Cell samples were lysed by addition of NaOH (1 M, 1 mL) to each well. The cell suspensions were transferred to tubes for measurement in a γ counter (PerkinElmer Wallac Wizard 1480). After homogenization of the cell suspensions by vortexing, the concentration of proteins was determined for each sample using a Micro BCA Protein Assay Kit (Pierce, Thermo Scientific) in order to standardize the measured radioactivity to the average content of protein (0.2 mg) in a single well. The results were expressed as the percentage of total added radioactivity. Graphs were prepared using GraphPad Prism software (version 5.01).

Stability of ^{177}Lu -Folate Conjugates. The folate conjugates (cm10, cm12, cm13, and cm14) were labeled with ^{177}Lu (500 MBq) at a specific activity of 50 MBq/nmol. The labeling solution was divided into two samples (A and B) containing ^{177}Lu -folate conjugate diluted in PBS (~ 250 MBq in 500 μ L). Sample A was investigated without additives, while ascorbic acid (3 mg) was added to sample B. Sodium acetate (130 μ L, 0.5 M, pH 8) was added to sample B to maintain a neutral pH (~ 7). In the case of ^{177}Lu -cm14, ascorbic acid had already been added for the labeling procedure. The radiochemical purity of the radiofolates was determined immediately after preparation ($t = 0$), and the integrity of the compounds of

samples A and B was investigated after incubation periods of 4 and 24 h at room temperature. The HPLC chromatograms were analyzed by determination of the peak area of the radiolabeled folate conjugates in relation to the area observed at $t = 0$, which was set to 100%.

In Vivo Studies. In vivo experiments were approved by the local veterinarian department and conducted in accordance with the Swiss law of animal protection. Athymic female nude mice (CD-1 Foxn-1/nu) at the age of 5–6 weeks were obtained from Charles River Laboratories (Sulzfeld, Germany). All of the animals were fed with a folate-deficient rodent diet (ssniff Spezialdiäten GmbH). Mice were inoculated with KB tumor cells (5×10^6 cells in 100 μ L of PBS) into the subcutis of each shoulder. For in vivo scans, mice were anesthetized with a mixture of isoflurane (1.5–2%) and oxygen.

Biodistribution Studies. Biodistribution studies were performed in triplicate 2–3 weeks after cell inoculation, when the tumor volume reached ~ 300 mm³. ^{177}Lu -folate conjugates (3 MBq, 0.5 nmol/mouse) were injected in a volume of 100 μ L into a lateral tail vein. The animals were sacrificed at 1, 4, 24, 48, 72, and 120 h after administration of the radioconjugates. Selected tissues and organs were collected and weighed, and the radioactivity was measured using a γ counter (PerkinElmer Wallac Wizard 1480). The results are reported as the percentage of the injected radioactivity per gram of tissue mass (% IA/g) using counts of a defined volume of the original injection solution measured at the same time, resulting in decay-corrected values.

Dosimetric Calculations. For dosimetric calculations, the biodistribution data were converted to non-decay-corrected values. The mean specific absorbed dose (Gy/MBq) to the tumor xenografts and kidneys was calculated by multiplication of the cumulated activity by the emitted electron energy per decay, the absorbed fraction of the emitted electron energy, and a conversion factor. The cumulated activity was estimated by calculating the area under the curve (AUC, in MBq·s) of the time–activity concentration curve. For the AUC between 0 and 24 h post injection (p.i.), linear integration of the first time points (0–24 h) was used. The AUC from 24 h to infinity was calculated by integration of a monoexponential function obtained by least-squares fitting of the time points from 24 h to the last time point ($r^2 > 0.88$). The absorbed fractions for the tumors (40–50 mg) at the time of injection and the kidneys (~ 125 mg) were assessed by Monte Carlo simulations using PENELOPE.²⁸ In the simulations, a uniform activity distribution within the kidneys and the tumors was assumed. The emitted electron energy per decay for ^{177}Lu was 147 keV (www.nndc.bnl.gov).

SPECT/CT Imaging Studies. Imaging studies were performed using a four-head, multiplexing, multipinhole small-animal SPECT camera (NanoSPECT/CT, Mediso Medical Imaging Systems, Budapest, Hungary). Each head was outfitted with a tungsten-based aperture of nine 1.4 mm-diameter pinholes and a thickness of 10 mm. The energy peaks for the camera were set at 56.1 keV ($\pm 10\%$), 112.9 keV ($\pm 10\%$), and 208.4 keV ($\pm 10\%$). For imaging experiments, the ^{177}Lu -labeled folate conjugates (cm10, cm12, cm13, and cm14) were injected (50 MBq, ~ 2 nmol/mouse) into the lateral tail vein of KB-tumor-bearing mice. SPECT/CT scans were performed 24 h after injection of the respective ^{177}Lu -folate conjugate followed by CT scans. The images were acquired using Nucline software (version 1.02, Mediso Ltd., Budapest, Hungary). The real-time CT reconstruction used a cone-beam-

filtered backprojection. The reconstruction of SPECT data was performed using HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany). Images were prepared using VivoQuant postprocessing software (version 2.5, inviCRO Imaging Services and Software, Boston, MA, USA). A Gauss postreconstruction filter (FWHM = 1 mm) was applied for the presentation of the SPECT images, and the scale was adjusted to allow the best visualization of tumors and kidneys in which radioactivity accumulated. Quantification of tumor-to-kidney ratios based on the acquired images was performed by determination of the accumulated radioactivity per volume of tumor and kidney tissue using the “3D ROI” tool of VivoQuant.

RESULTS

Preparation of ^{177}Lu -Folate Conjugates. The folate conjugates cm12, cm13, and cm14 were synthesized in moderate overall yields of 0.9–38% with purities of >94% according to the strategy previously reported for cm10 (Figures S1–S4).²⁵ Radiolabeling with ^{177}Lu was achieved at a radiochemical purity of >95%. The albumin-binding ^{177}Lu -folate conjugates were eluted with retention times of 9.6 min (^{177}Lu -cm10), 10.0 min (^{177}Lu -cm13), and 10.3 min (^{177}Lu -cm12) when the HPLC eluent consisted of ~55% acetonitrile. The control compound (without albumin binder) was eluted with ~33% acetonitrile 5.6 min after the start of the HPLC gradient.

Stability of ^{177}Lu -Folate Conjugates. The stability of the ^{177}Lu -folate conjugates at a relatively high activity concentration (500 MBq/mL) was investigated in the presence and absence of ascorbic acid. When ^{177}Lu -cm10 and ^{177}Lu -cm13 were incubated without the addition of ascorbic acid (samples A), they remained almost completely intact (>95%) over 4 h (Figure 2A), whereas the stability of ^{177}Lu -cm12 was slightly

reduced (~92% intact compound after 4 h). In the case of ^{177}Lu -cm14, only 75% intact compound was determined after 4 h. After an incubation time of 24 h, instability was observed for all of the investigated radiofolates, but the presence of ascorbic acid improved the stability of the radiofolates tremendously (samples B; Figure 2B). Under these conditions, the radiofolates were >96% stable over a period of 24 h, except in the case of ^{177}Lu -cm12, where only 84% intact compound was determined after 24 h (Figure 2B).

***n*-Octanol/PBS Distribution Coefficients.** All four ^{177}Lu -folate conjugates exhibited logD values below -4.0 (Table 1), indicating hydrophilic character. The logD value obtained for ^{177}Lu -cm10 (-4.4 ± 0.23) was significantly reduced ($p < 0.05$) compared with the logD values obtained for ^{177}Lu -cm12 (-4.1 ± 0.14) and ^{177}Lu -cm13 (-4.1 ± 0.20), which showed similar results ($p > 0.05$). The logD value of less than -5.5 for the control compound without the albumin-binding entity (^{177}Lu -cm14) was significantly lower ($p < 0.05$). These results indicated that the albumin-binding entity rendered the novel radiofolates somewhat more lipophilic compared with conventional folate radioconjugates.

Serum Protein Binding of the ^{177}Lu -Folate Conjugates. Plasma protein binding of the ^{177}Lu -folate conjugates (cm10, cm12, and cm13) was in all cases found to be >88% (Figure 3 and Table 1). The plasma-protein-bound fractions of ^{177}Lu -cm10 and ^{177}Lu -cm13 were in the same range ($93 \pm 1.5\%$ and $95 \pm 2.0\%$, respectively), whereas the bound fraction of ^{177}Lu -cm12 was slightly reduced ($88 \pm 5.2\%$). Only trace amounts of ^{177}Lu -cm14 ($\sim 1.0 \pm 1.5\%$) bound to plasma proteins (Figure 3 and Table 1). In a control experiment, the ^{177}Lu -folate conjugates were filtered after incubation in PBS instead of plasma. In this case the majority of the radioactivity (>90%) was found in the filtered fraction, indicating that the unbound radiofolates were readily filtered through the membrane.

FR-Binding Affinities of ^{177}Lu -Folate Conjugates. The FR-binding affinities of the ^{177}Lu -folate conjugates were found to be in the nanomolar range (4.0–7.5 nM) for all of the investigated radiofolates (Table 1). Representative saturation curves are given in Figure S5. The K_D values were in the same range for all of the ^{177}Lu -folate conjugates ($p > 0.05$).

Cell Uptake and Internalization Studies. Cellular uptake and internalization of the ^{177}Lu -folate conjugates were investigated over 4 h using KB tumor cells (Figure 4). The uptake was in the range of 13–22% 0.5 h after addition of the radiofolates and increased to 24–37% after an incubation period of 4 h. The internalized fraction was 3–6% after 0.5 h of incubation of the radiofolates and increased to 6–11% after 4 h. The results were comparable for all of the radiofolates except ^{177}Lu -cm12, which showed ~30% reduced internalization after 2 and 4 h compared with the other radiofolates. Coincubation of the cells with excess folic acid to block the FRs reduced the uptake to <0.1%, indicating FR-specific uptake of all of the investigated radiofolates (data not shown).

Biodistribution Studies. Biodistribution studies of the radiofolates were performed in KB-tumor-bearing mice over a period of 5 days (Figure 5 and Tables S1–S6 in the Supporting Information). The albumin-binding radiofolates showed significant retention of activity in the blood (6.5–10.5% IA/g, 1 h p.i.) and slow clearance over time (2.3–4.0% IA/g, 4 h p.i. and 0.2–0.6% IA/g, 24 h p.i.) (Figure 5 and Tables S1–S6). This was in clear contrast to compound ^{177}Lu -cm14 without

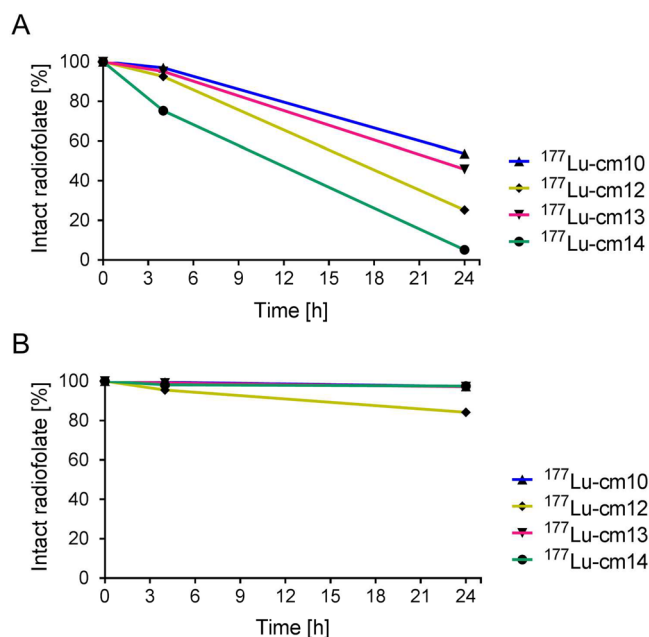


Figure 2. Intact fractions of ^{177}Lu -cm10, ^{177}Lu -cm12, ^{177}Lu -cm13, and ^{177}Lu -cm14 incubated at an activity concentration of 500 MBq/mL in PBS at room temperature over a time period of 24 h: (A) incubation in PBS only (samples A); (B) incubation in the presence of ascorbic acid (samples B).

Table 1. Summary of the in Vitro Properties of the ^{177}Lu -Labeled Folate Conjugates

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
LogD	-4.4 ± 0.23	-4.1 ± 0.14	-4.1 ± 0.20	$< -5.5^a$
K_D [nM]	5.1 ± 2.1	7.4 ± 1.2	4.0 ± 3.4	4.9 ± 2.7
serum protein binding [%]	93 ± 1.5	88 ± 5.2	95 ± 2.0	1.0 ± 1.5

^aIn the case of ^{177}Lu -cm14, the counts in the *n*-octanol phase were below the detection limit of the γ counter.

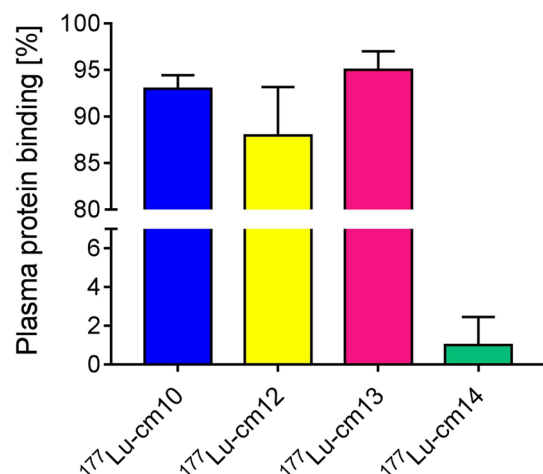


Figure 3. Fractions of radiofolates bound to human plasma proteins as determined using a filter assay. The results were calculated as percentages of standards, which were set to 100%.

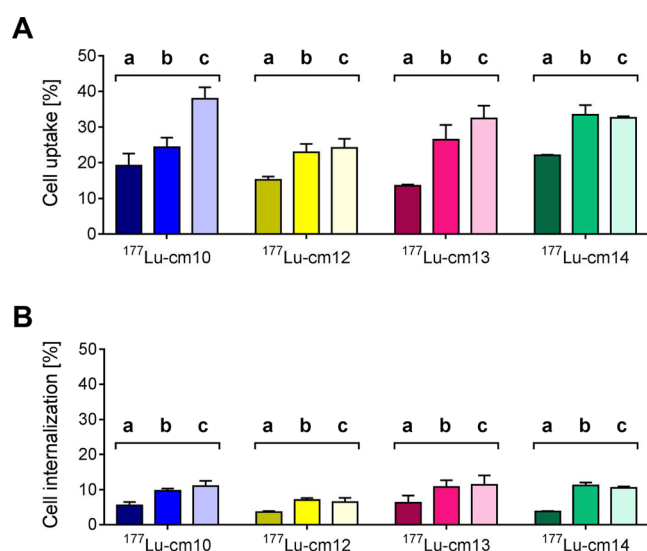


Figure 4. (A) Cell uptake and (B) internalization of ^{177}Lu -cm10, ^{177}Lu -cm12, ^{177}Lu -cm13, and ^{177}Lu -cm14 at (a) 0.5 h, (b) 2 h, and (c) 4 h after addition of the compounds to KB tumor cell monolayers. The results are given as mean $\pm$ SD of a representative experiment performed in triplicate, standardized to the amount of protein per well (0.2 mg).

the albumin-binding entity, which was cleared very rapidly, resulting in activity levels of $<0.5\%$ IA/g in the blood already at 1 h p.i.

All of the radiofolates showed distinct uptake in the tumor xenografts as well as in the kidneys (Figure 5). The accumulation of radioactivity in tumors increased within the first hours, presumably reaching a maximum between 4 and 24 h after injection. In this study, the lead compound ^{177}Lu -cm10 showed significant retention of activity in the blood, leading to

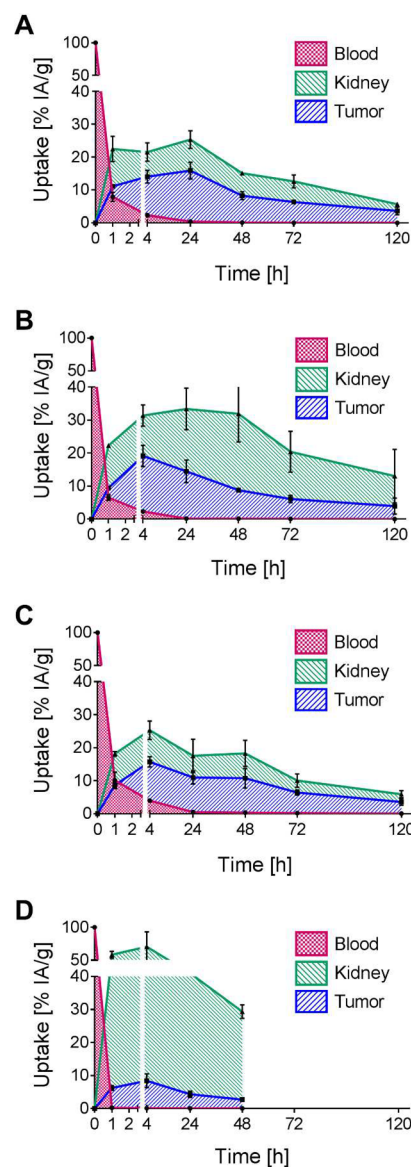


Figure 5. Time-dependent uptake and clearance of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12, (C) ^{177}Lu -cm13, and (D) ^{177}Lu -cm14 in KB-tumor-bearing mice. The albumin-binding compounds were investigated over 5 days, whereas the distribution of ^{177}Lu -cm14 was determined over the first 48 h.

maximum uptake of radioactivity in tumors ($17.6 \pm 2.58\%$ IA/g) and kidneys ($28.1 \pm 2.69\%$ IA/g) at 24 h after injection of the radiofolates. The retention of activity in the tumor tissue was high over several days ($8.59 \pm 0.56\%$ IA/g, 72 h p.i.), but the same held true also for the kidneys, where a significant amount of activity ($17.2 \pm 1.99\%$ IA/g) was retained 72 h after injection. Application of ^{177}Lu -cm13 resulted in a similar distribution profile with even slightly higher retention of radioactivity in the blood ($10.2 \pm 2.52\%$ IA/g vs $7.89 \pm 1.36\%$

IA/g) at 1 h after injection. The maximum uptake in the tumor ($16.0 \pm 1.58\%$ IA/g) was reached 4 h after administration of ^{177}Lu -cm13. The renal retention of this radioconjugate was high ($25.8 \pm 2.79\%$ IA/g, 4 h p.i.) resulting in similar tumor-to-kidney ratios for ^{177}Lu -cm10 and ^{177}Lu -cm13 over the first 24 h. At later time points, ^{177}Lu -cm13 showed slightly increased tumor-to-kidney ratios (~ 0.65 vs ~ 0.50 at 72 h p.i.). The in vivo behavior of ^{177}Lu -cm12, the compound with the PEG spacer, was similar to that of the other albumin-binding radiofolates at 1 h p.i., but at later time points the distribution revealed a different pattern. This was due to a significantly faster clearance of activity from the blood, resulting in an activity of $\sim 0.18\%$ IA/g at 24 h p.i., which was 3-fold lower than for the other albumin-binding radiofolates. Renal uptake of ^{177}Lu -cm12 was higher at 48 h p.i. ($\sim 40\%$ IA/g) compared with the renal retention of ^{177}Lu -cm10 and ^{177}Lu -cm13 ($18\text{--}22\%$ IA/g), resulting in a reduced tumor-to-kidney ratio (~ 0.28) in the case of ^{177}Lu -cm12 compared with ^{177}Lu -cm10 and ^{177}Lu -cm13 ($0.55\text{--}0.59$).

Relatively high accumulation of activity was also found in salivary glands after injection of the radiofolates with or without an albumin-binding entity (Figure 5 and Tables S1–S6). As a result of the high blood retention of albumin-binding radiofolates at early time points, increased levels of radioactivity were also detected in nontargeted organs and tissues, including liver and lungs as well as muscle and bone. In most of these tissues, radioactivity levels were reduced quickly over time to $<1\%$ IA/g at 48 h p.i., but in the liver the uptake remained at $\sim 2\text{--}3\%$ IA/g even at 48 h p.i. (Tables S1–S6).

A comparison of the tumor-to-kidney ratios for all of the albumin-binding radiofolates over time revealed similar values for ^{177}Lu -cm10 and ^{177}Lu -cm13, with a tendency of favorable ratios for ^{177}Lu -cm13 at later time points. However, significantly reduced values were observed for ^{177}Lu -cm12, in particular at later time points. As expected, ^{177}Lu -cm14 showed very low tumor-to-kidney ratios of ~ 0.1 over the whole 48 h period of investigation (Figure 6).

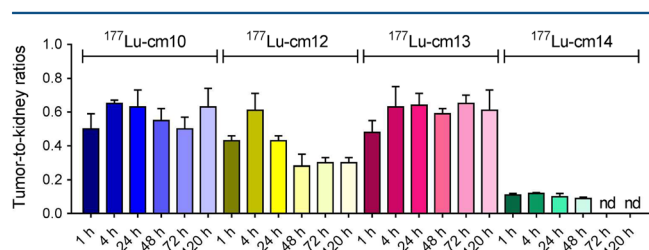


Figure 6. Time-dependent tumor-to-kidney ratios of ^{177}Lu -cm10, ^{177}Lu -cm12, ^{177}Lu -cm13, and ^{177}Lu -cm14 in KB-tumor-bearing mice. Data for ^{177}Lu -cm14 at 72 and 120 h post injection were not acquired (nd = not determined).

In order to determine potential degradation of the radiofolates in vivo, blood sampling was performed 4 and 24 h after injection of the albumin-binding radiofolates and 25 min after injection of ^{177}Lu -cm14 (see the Supporting Information). ^{177}Lu -cm10, ^{177}Lu -cm12, and ^{177}Lu -cm13 were entirely stable even 24 h after administration of the radiofolates as determined by HPLC analysis (Figure S6A–C). In the case of ^{177}Lu -cm14, free ^{177}Lu and degradation products were determined already 25 min after injection (Figure S6D). It remains unclear whether this was a result of in vivo degradation of ^{177}Lu -cm14 or processing of blood samples before analysis.

Dosimetry Studies. The biodistribution data for the radiofolates were applied to calculate the mean absorbed doses to the tumors and kidneys of KB-tumor-bearing mice. For the albumin-binding radiofolates, the mean absorbed tumor doses were in the range of $0.89\text{--}1.12$ Gy/MBq (Table 2). In

Table 2. Mean Absorbed Doses to the Tumors and Kidneys Calculated for KB-Tumor-Bearing Mice and Tumor-to-Kidney Dose Ratios^a

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
tumor	0.89	1.03	1.12	0.33
kidneys	1.70	3.54	1.92	3.64
tu-to-ki ratio	0.53	0.29	0.59	0.09

^aValues are given in Gy/MBq.

the case of the non-albumin-binding control compound ^{177}Lu -cm14, however, the mean absorbed dose to the tumor was only 0.33 Gy/MBq. The mean absorbed kidney dose of ^{177}Lu -cm10 (1.70 Gy/MBq) was in the same range as for ^{177}Lu -cm13 (1.92 Gy/MBq) but reduced in comparison with ^{177}Lu -cm12 (3.54 Gy/MBq). This value resembled more the value for the control compound ^{177}Lu -cm14 (3.64 Gy/MBq). The tumor-to-kidney ratios of the calculated mean absorbed doses were in the same range for ^{177}Lu -cm10 and ^{177}Lu -cm13 but reduced in the case of ^{177}Lu -cm12. The value obtained for ^{177}Lu -cm14 was about 6.5-fold smaller than the highest value for the albumin-binding radiofolates, which was obtained with ^{177}Lu -cm13 (Table 2).

SPECT/CT Imaging Studies. SPECT/CT images of KB-tumor-bearing mice were acquired 24 h after injection of the radiofolates (Figure 7). At this time point, $30\text{--}40\%$ of the

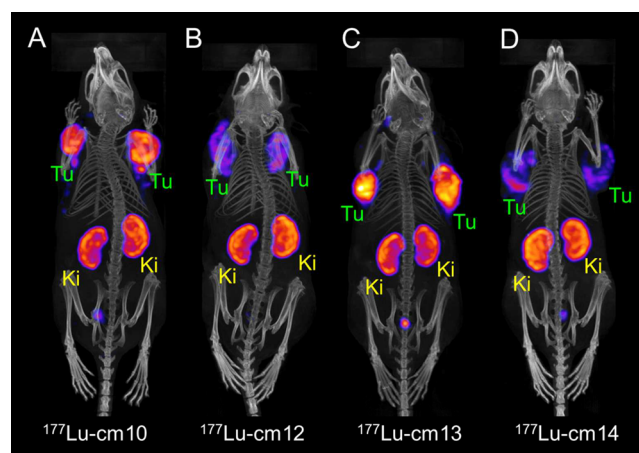


Figure 7. SPECT/CT images of KB-tumor-bearing mice 24 h after injection of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12, (C) ^{177}Lu -cm13, and (D) ^{177}Lu -cm14 (50 MBq, 2 nmol/mouse). The scale has been adjusted so that the accumulation of activity in the kidneys was comparable for all mice. (Tu = KB tumor xenograft; Ki = kidney).

injected activity was excreted in the case of albumin-binding radiofolates, whereas in the case of ^{177}Lu -cm14, already $>70\%$ of the activity was excreted. Accumulation of radiofolates was detected in the tumor xenografts as well as in the kidneys. The tumor-to-kidney ratios based on these SPECT acquisitions were also calculated. For this purpose, the counts per volume in the tumor and in the kidneys were determined and set into relation. This image analysis confirmed that ^{177}Lu -cm10 (~ 1.0) and ^{177}Lu -cm13 (~ 1.1) showed higher tumor-to-kidney ratios

than ^{177}Lu -cm12 (~ 0.7) and ^{177}Lu -cm14 (~ 0.7) 24 h after injection of the radiofolates.

Sections of these SPECT/CT images indicated inhomogeneous distributions of radioactivity in KB tumor xenografts, with higher accumulation at the outer rim of the tumors and little activity at the center of the xenografts (Figure S7). In the kidneys, the accumulation of radioactivity was limited to the renal cortex since FRs are expressed in the proximal renal tubules.

■ DISCUSSION

Small molecules, including folate conjugates, are rapidly cleared from circulation, which is considered as a favorable feature of radiopharmaceuticals applied for nuclear imaging purposes. An extended circulation time of radioligands and, as a consequence, an increased accumulation of activity at the site of target expression may, however, be desirable for therapeutic purposes. The fast excretion and high renal accumulation of activity of conventional radiofolates^{7,9} presents a drawback for therapeutic applications of this class of targeting agents due to the risk of damage to the kidneys by particle-emitting radiation. The concept of using an albumin-binding entity for modification of antibody fragments was revealed to be very promising.^{22,29} Recently, we employed such an albumin-binding entity for the modification of a folate radioconjugate, which improved its tissue distribution profile tremendously, and as a consequence, the risk of radionephrotoxicity could be reduced.^{23,24} This research has attracted the interest of researchers in radiopharmaceutical sciences who aim to use the same concept for the development of radiopharmaceuticals with improved in vivo properties.³⁰

In the present study, we were interested in investigating further the structural requirements in close proximity to the albumin binder, and therefore, we evaluated whether an extension of the radioconjugate's linker region would have an impact on the radioconjugate's tissue distribution profile. It did not appear reasonable, however, to just introduce alkane spacers of different lengths, as a long alkane spacer would have given the compound a lipophilic character. Therefore, a PEG-11 spacer was chosen to design ^{177}Lu -cm12 in order to evaluate the impact of a long spacer entity. ^{177}Lu -cm13 contained a short alkane spacer and was designed to test the effect of a small extension of the molecule to connect the albumin-binding entity.

In vitro stability experiments revealed that the albumin-binding entity had a positive impact on the compounds' stability, as shown by the fact that the albumin-binding radiofolates were less prone to degradation and radiolysis compared with ^{177}Lu -cm14. The hydrophilic properties of the radiofolates were slightly reduced by the introduction of an albumin-binding entity, but FR affinity and specificity were retained for all of the compounds independent of the albumin binder and length of the spacer entity. Comparison of the albumin-binding properties of ^{177}Lu -cm10 and ^{177}Lu -cm13 revealed almost identical results in vitro, whereas ^{177}Lu -cm12 showed slightly but not significantly reduced albumin-binding properties in vitro. Not surprisingly, the serum albumin-binding properties of ^{177}Lu -cm14 were negligible.

In vivo, the retention of the albumin-binding folates in the blood was >30-fold higher than that of ^{177}Lu -cm14 at 1 h after injection of the radiofolates. The tissue distribution profiles of ^{177}Lu -cm10 and ^{177}Lu -cm13 were similar, whereas the injection of ^{177}Lu -cm12 resulted in reduced tumor uptake and increased

retention of activity in the kidneys. As a result, the tumor-to-kidney ratios of ^{177}Lu -cm12 were reduced, in particular at later time points after injection. On the basis of the in vitro evaluation, the discrepancy between the kinetics of ^{177}Lu -cm12 and the two other albumin-binding radiofolates was not expected even though there were indications in vitro that the binding of ^{177}Lu -cm12 to albumin was somewhat reduced. Generally, however, the in vitro properties were comparable among the three albumin-binding compounds. Hence, the question of potential in vivo instability of ^{177}Lu -cm12 had to be addressed. An investigation of blood samples revealed that all of the albumin-binding radiofolates, including ^{177}Lu -cm12, were entirely stable in vivo over the investigated time period of 24 h (Figure S6). It thus remains unclear whether the slightly reduced albumin-binding properties of ^{177}Lu -cm12 shown in vitro affected the in vivo behavior so fundamentally that it compromised the beneficial tissue distribution demonstrated for ^{177}Lu -cm10 and ^{177}Lu -cm13.

The tumor-to-kidney ratios of ^{177}Lu -cm13 determined in post-mortem biodistribution studies were in the same range as those of ^{177}Lu -cm10. SPECT/CT images showed slightly improved tumor-to-kidney ratios of ^{177}Lu -cm13 compared with ^{177}Lu -cm10, but the results varied among individual animals (data not shown). The calculation of the mean absorbed doses to tumors and kidneys of the mice injected with the radiofolates unveiled that the tumor-to-kidney ratios of the estimated doses were in the same range for ^{177}Lu -cm13 and ^{177}Lu -cm10 (Table 2). ^{177}Lu -cm12 and ^{177}Lu -cm14 showed much smaller tumor-to-kidney ratios of the mean absorbed doses than ^{177}Lu -cm10, as expected on the basis of the unfavorable biodistribution data. In order to corroborate these findings and ensure that they have general validity, additional animal experiments were performed in a different tumor mouse model. For this purpose, IGROV-1 tumor xenografts were used as previously reported (see the Supporting Information).³¹ Again, ^{177}Lu -cm10 and ^{177}Lu -cm13 showed similar tumor-to-kidney ratios (1.03 ± 0.37 and 0.97 ± 0.07 , respectively), whereas the ratios of ^{177}Lu -cm12 (0.55 ± 0.10) and ^{177}Lu -cm14 (0.15 ± 0.03) were clearly reduced (Table S7). The same trend was observed in SPECT/CT imaging studies performed with IGROV-1-tumor-bearing mice, where the tissue distribution of ^{177}Lu -cm13 looked most promising (Figure S8). Overall, the results obtained in IGROV-1-tumor-bearing mice were in good agreement with those of the standard KB tumor mouse model. The generally higher tumor uptake in IGROV-1 tumor xenografts independent of the structure of the radiofolate was unexpected, as it is known that the KB tumor cells express higher levels of the FR compared with IGROV-1 tumor cells.³² However, the findings of this study may be explained by increased vascularization of the tumor xenografts in the case of IGROV-1 tumors, which led to a more homogeneous distribution of activity in this tumor type compared with KB tumor xenografts. This is visible on sections of SPECT/CT images (Figures S7 and S9).

On the basis of the lessons learned from this study, further albumin-binding folate radioconjugates will be designed with the aim of keeping the tumor uptake high but reducing the renal retention of radioactivity further. It remains to be mentioned that the advantages of increasing the tumor-to-kidney ratio as a consequence of an enhanced blood circulation time of folate radioconjugates have to be carefully traded off against the disadvantage of increasing the dose to the bone marrow. Future designs of radiofolates for therapeutic purposes have to take these facts into consideration and are therefore

aimed at finding an optimum between a sufficiently high accumulation of activity in the tumor tissue while keeping the renal dose and the dose to the bone marrow as low as possible.

CONCLUSION

This study indicates that the spacer entity is of critical importance with regard to the tissue distribution profile of albumin-binding radiofolates. It appears that an alkane spacer is well-tolerated with regard to the interactions of the compound with serum proteins and more promising than introducing a hydrophilic entity. It is likely that these findings are transferrable also to other targeting agents, and hence, the results of this study may facilitate the design of other albumin-binding radioligands.

As to what concerns the development of radiofolates with improved tissue distribution profiles, a potential option for future investigations may be the design of albumin-binding radiofolates with longer, and potentially more rigid, lipophilic spacer entities. This will be the topic of future investigations in our laboratories.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b01010.

Chemical synthesis of the folate conjugates, representative saturation curves for FR-binding affinity determination, biodistribution data obtained in tumor-bearing mice, investigations of the in vivo stability of the radiofolates, additional SPECT/CT imaging studies, and additional data on the radiofolates obtained in IGROV-1 tumor-bearing mice (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: cristina.mueller@psi.ch. Phone: +41-56-310 44 54. Fax: +41-56-310 28 49.

ORCID

Cristina Müller: 0000-0001-9357-9688

Notes

The authors declare the following competing financial interest(s): Patent applications on folate conjugates with albumin-binding entities have been filed by Merck & Cie.

ACKNOWLEDGMENTS

This work was financially supported by the Swiss Cancer League (KLS-02762-02-2011) and the Swiss National Science Foundation (Grants 310030_156803 and CRSII3_154490). K.S. was financially supported by a Swiss Government Excellence Scholarship and the Molecular Imaging Network (MINZ), University Hospital Zürich. P.B. was supported financially by the Swedish Cancer Society and Jubilee Clinic Cancer Research Foundation. The whole project was financially supported by Merck & Cie Schaffhausen. The authors thank Raffaella Schmid and Susan Cohrs for technical assistance at PSI and Dr. Konstantin Zhernosekov (ITG GmbH) for providing the ^{177}Lu for this study.

REFERENCES

- (1) Britz-Cunningham, S. H.; Adelstein, S. J. Molecular targeting with radionuclides: state of the science. *J. Nucl. Med.* **2003**, *44* (12), 1945–61.
- (2) Low, P. S.; Henne, W. A.; Doorneweerd, D. D. Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases. *Acc. Chem. Res.* **2008**, *41* (1), 120–9.
- (3) Low, P. S.; Kularatne, S. A. Folate-targeted therapeutic and imaging agents for cancer. *Curr. Opin. Chem. Biol.* **2009**, *13* (3), 256–62.
- (4) Parker, N.; Turk, M. J.; Westrick, E.; Lewis, J. D.; Low, P. S.; Leamon, C. P. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal. Biochem.* **2005**, *338* (2), 284–293.
- (5) Holm, J.; Hansen, S. I.; Hoiermadsen, M.; Bostad, L. A high-affinity folate binding-protein in proximal tubule cells of human kidney. *Kidney Int.* **1992**, *41* (1), 50–55.
- (6) Birn, H.; Spiegelstein, O.; Christensen, E. I.; Finnell, R. H. Renal tubular reabsorption of folate mediated by folate binding protein 1. *J. Am. Soc. Nephrol.* **2005**, *16* (3), 608–615.
- (7) Ke, C. Y.; Mathias, C. J.; Green, M. A. Folate-receptor-targeted radionuclide imaging agents. *Adv. Drug Delivery Rev.* **2004**, *56* (8), 1143–1160.
- (8) Segal, E. I.; Low, P. S. Tumor detection using folate receptor-targeted imaging agents. *Cancer Metastasis Rev.* **2008**, *27* (4), 655–64.
- (9) Müller, C.; Schibli, R. Folic acid conjugates for nuclear imaging of folate receptor-positive cancer. *J. Nucl. Med.* **2011**, *52* (1), 1–4.
- (10) Müller, C. Folate-based radiotracers for PET imaging - update and perspectives. *Molecules* **2013**, *18* (5), 5005–31.
- (11) Siegel, B. A.; Dehdashti, F.; Mutch, D. G.; Podoloff, D. A.; Wendt, R.; Sutton, G. P.; Burt, R. W.; Ellis, P. R.; Mathias, C. J.; Green, M. A.; Gershenson, D. M. Evaluation of ^{111}In -DTPA-folate as a receptor-targeted diagnostic agent for ovarian cancer: initial clinical results. *J. Nucl. Med.* **2003**, *44* (5), 700–707.
- (12) Fisher, R. E.; Siegel, B. A.; Edell, S. L.; Oyesiku, N. M.; Morgenstern, D. E.; Messmann, R. A.; Amato, R. J. Exploratory study of $^{99\text{m}}\text{Tc}$ -EC20 imaging for identifying patients with folate receptor-positive solid tumors. *J. Nucl. Med.* **2008**, *49* (6), 899–906.
- (13) Leamon, C. P.; Reddy, J. A. Folate-targeted chemotherapy. *Adv. Drug Delivery Rev.* **2004**, *56* (8), 1127–1141.
- (14) Vlahov, I. R.; Leamon, C. P. Engineering folate-drug conjugates to target cancer: from chemistry to clinic. *Bioconjugate Chem.* **2012**, *23* (7), 1357–69.
- (15) Li, J.; Sausville, E. A.; Klein, P. J.; Morgenstern, D.; Leamon, C. P.; Messmann, R. A.; LoRusso, P. Clinical pharmacokinetics and exposure-toxicity relationship of a folate-Vinca alkaloid conjugate EC145 in cancer patients. *J. Clin. Pharmacol.* **2009**, *49* (12), 1467–76.
- (16) Pribble, P.; Edelman, M. J. EC145: a novel targeted agent for adenocarcinoma of the lung. *Expert Opin. Invest. Drugs* **2012**, *21* (5), 755–61.
- (17) Müller, C.; Schibli, R. Prospects in folate receptor-targeted radionuclide therapy. *Front. Oncol.* **2013**, *3*, 249.
- (18) Brabander, T.; Teunissen, J. J.; Van Eijck, C. H.; Franssen, G. J.; Feelders, R. A.; de Herder, W. W.; Kwekkeboom, D. J. Peptide receptor radionuclide therapy of neuroendocrine tumours. *Best Pract. Res. Clin. Endocrinol. Metab.* **2016**, *30* (1), 103–14.
- (19) Lutje, S.; Heskamp, S.; Cornelissen, A. S.; Poeppel, T. D.; van den Broek, S. A.; Rosenbaum-Krumme, S.; Bockisch, A.; Gotthardt, M.; Rijpkema, M.; Boerman, O. C. PSMA ligands for radionuclide imaging and therapy of prostate cancer: clinical status. *Theranostics* **2015**, *5* (12), 1388–401.
- (20) Müller, C.; Vlahov, I. R.; Santhapuram, H. K.; Leamon, C. P.; Schibli, R. Tumor targeting using ^{67}Ga -DOTA-Bz-folate - investigations of methods to improve the tissue distribution of radiofolates. *Nucl. Med. Biol.* **2011**, *38* (5), 715–23.
- (21) Dennis, M. S.; Zhang, M.; Meng, Y. G.; Kadkhodayan, M.; Kirchhofer, D.; Combs, D.; Damico, L. A. Albumin binding as a

general strategy for improving the pharmacokinetics of proteins. *J. Biol. Chem.* **2002**, 277 (38), 35035–43.

(22) Dumelin, C. E.; Trüssel, S.; Buller, F.; Trachsel, E.; Bootz, F.; Zhang, Y.; Mannocci, L.; Beck, S. C.; Drumea-Mirancea, M.; Seeliger, M. W.; Baltes, C.; Muggler, T.; Kranz, F.; Rudin, M.; Melkko, S.; Scheuermann, J.; Neri, D. A portable albumin binder from a DNA-encoded chemical library. *Angew. Chem., Int. Ed.* **2008**, 47 (17), 3196–201.

(23) Müller, C.; Struthers, H.; Winiger, C.; Zhernosekov, K.; Schibli, R. DOTA conjugate with an albumin-binding entity enables the first folic acid-targeted ^{177}Lu -radionuclide tumor therapy in mice. *J. Nucl. Med.* **2013**, 54 (1), 124–31.

(24) Haller, S.; Reber, J.; Brandt, S.; Bernhardt, P.; Groehn, V.; Schibli, R.; Müller, C. Folate receptor-targeted radionuclide therapy: preclinical investigation of anti-tumor effects and potential radio-nephropathy. *Nucl. Med. Biol.* **2015**, 42 (10), 770–9.

(25) Müller, C.; Bunka, M.; Haller, S.; Köster, U.; Groehn, V.; Bernhardt, P.; van der Meulen, N.; Türler, A.; Schibli, R. Promising prospects for ^{44}Sc -/ ^{47}Sc -based theragnostics: application of ^{47}Sc for radionuclide tumor therapy in mice. *J. Nucl. Med.* **2014**, 55 (10), 1658–64.

(26) Müller, C.; Mindt, T. L.; de Jong, M.; Schibli, R. Evaluation of a novel radiofolate in tumour-bearing mice: promising prospects for folate-based radionuclide therapy. *Eur. J. Nucl. Med. Mol. Imaging* **2009**, 36 (6), 938–46.

(27) Ladino, C. A.; Chari, R. V. J.; Bourret, L. A.; Kedersha, N. L.; Goldmacher, V. S. Folate-maytansinoids: target-selective drugs of low molecular weight. *Int. J. Cancer* **1997**, 73 (6), 859–864.

(28) Uusijärvi, H.; Bernhardt, P.; Ericsson, T.; Forsell-Aronsson, E. Dosimetric characterization of radionuclides for systemic tumor therapy: influence of particle range, photon emission, and subcellular distribution. *Med. Phys.* **2006**, 33 (9), 3260–9.

(29) Trüssel, S.; Dumelin, C.; Frey, K.; Villa, A.; Buller, F.; Neri, D. New strategy for the extension of the serum half-life of antibody fragments. *Bioconjugate Chem.* **2009**, 20 (12), 2286–92.

(30) Meckel, M.; Kubicek, V.; Hermann, P.; Miederer, M.; Rösch, F. A DOTA based bisphosphonate with an albumin binding moiety for delayed body clearance for bone targeting. *Nucl. Med. Biol.* **2016**, 43 (11), 670–678.

(31) Müller, C.; Schibli, R.; Krenning, E. P.; de Jong, M. Pemetrexed improves tumor selectivity of ^{111}In -DTPA-folate in mice with folate receptor-positive ovarian cancer. *J. Nucl. Med.* **2008**, 49 (4), 623–9.

(32) Forster, M. D.; Ormerod, M. G.; Agarwal, R.; Kaye, S. B.; Jackman, A. L. Flow cytometric method for determining folate receptor expression on ovarian carcinoma cells. *Cytometry, Part A* **2007**, 71A (11), 945–50.

Supporting Information (SI)

Preclinical Comparison of Albumin-Binding Radiofolates: Impact of Linker Entities on the In Vitro and In Vivo Properties

Klaudia Siwowska¹, Stephanie Haller¹, Francesca Bortoli¹, Martina Benešová^{1,2}, Viola Groehn³, Peter Bernhardt⁴, Roger Schibli^{1,2}, Cristina Müller^{*,1,2}

¹ Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Paul Scherrer Institut, Villigen-PSI, Switzerland

² Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland

³ Merck & Cie, Schaffhausen, Switzerland

⁴ Department of Radiation Physics, The Sahlgrenska Academy, University of Gothenburg, and Department of Medical Physics and Medical Bioengineering, Sahlgrenska University Hospital, Gothenburg, Sweden

E-Mail addresses:

klaudia.siwowska@psi.ch, steffihaller@gmx.ch, bortolif@student.ethz.ch, martina.benesova@psi.ch, viola.groehn@merckgroup.com, peter.bernhardt@gu.se, roger.schibli@psi.ch, cristina.mueller@psi.ch

*** Correspondence to:**

PD Dr. Cristina Müller

Center for Radiopharmaceutical Sciences ETH-PSI-USZ

Paul Scherrer Institut

5232 Villigen-PSI

Switzerland

e-mail: cristina.mueller@psi.ch

phone: +41 56 310 44 54

fax: +41 56 310 28 49

1. Synthesis of DOTA-Folate Conjugates

Organic Synthesis of the Folate Conjugate cm10

Compound **cm10** was the prototype of a new generation of albumin-binding DOTA-folate conjugates where a L-lysine was used to link the albumin-binding entity with folic acid and the DOTA chelator (Supplemental Figure S1). The synthesis of compound **cm10** has been previously reported by Müller and co-workers.¹

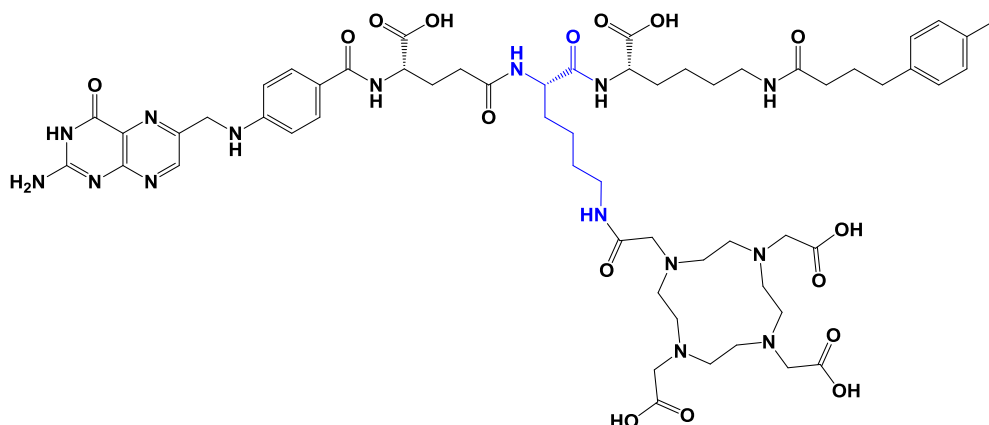


FIGURE S1. Chemical structure of the albumin-binding DOTA-folate conjugate **cm10**.

Organic Synthesis of the Folate Conjugate cm11

Compound **cm11** was supposed to be an albumin-binding DOTA- α -folate derivative, which means an isomer of **cm10** with the chelator/linker entity and the albumin-binding entity attached at the α -carboxyl group of the glutamate residue of folic acid (pteroyl-glutamic acid). However, the synthesis resulted in a mixture of α - and γ -isomers which led to discontinuation of the evaluation.

Organic Synthesis of the Folate Conjugate cm12

The albumin binding entity was synthesized by activation of 4-(*p*-iodophenyl)butyric acid (**S1**) with *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) in *N,N*-dimethylformamide (DMF) and subsequent reaction with *N* α -Boc-protected L-lysine methylester (Boc-Lys-OMe·HCl) in the presence of trimethylamine (Et₃N), followed by Boc-deprotection to yield compound **S2** (Supplemental Figure S2). Fmoc-NH-(PEG)11-COOH (purchased from Polypure AS.), was activated with HBTU in methylenechloride (CH₂Cl₂) and then reacted with compound **S2**. The Fmoc group was cleaved with diethylamine (Et₂NH) in methylenechloride. The resulting amine **S3** was coupled to Fmoc-L-Lys(Boc)-OH which was activated with HBTU. After cleavage of the Fmoc group with Et₂NH in methylenechloride the resulting amine **S4** was coupled to the γ -carboxyl group of *N*²-pivaloyl-*N*¹⁰-formylfolic acid α -methylester **S5** which was activated with HBTU. Cleavage of the Boc-protective group with TFA in methylenechloride in the presence of triisopropylsilane (TIPS) resulted in compound **S6**. The amine of the lysine side chain of **S6** was reacted with DOTA-tris(*t*-Bu

ester) in the presence of HBTU and triethylamine (Et_3N) resulting in the protected DOTA-folate conjugate **cm12**. Deprotection of **cm12** was achieved by treatment with TFA in methylenechloride and - after removal of solvents - with aqueous lithiumhydroxide solution (aq. LiOH) in the presence of THF. The product **cm12** was precipitated at pH 3.0 by addition of aqueous 1 M hydrochloric acid (HCl). The compound **cm12** was then isolated by centrifugation, washed with water and dried at 40 °C in vacuum (Supplemental Figure S2). The reactions were monitored by HPLC and LC-MS. The final product was characterized by LC-MS. The synthesis of compound **cm12** resulted in an overall yield of 0.9%. The compound was obtained in >94% purity as confirmed by HPLC. Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF MS): $[\text{M}+\text{H}]^+$ = calculated for $\text{C}_{84}\text{H}_{132}\text{IN}_{16}\text{O}_{29}$ 1955.8; found 1955.8, 1977.8 and 1999.8 (sodium aducts; $[\text{M}+\text{Na}]^+$ and $[\text{M}+2\text{Na}]^{2+}$).

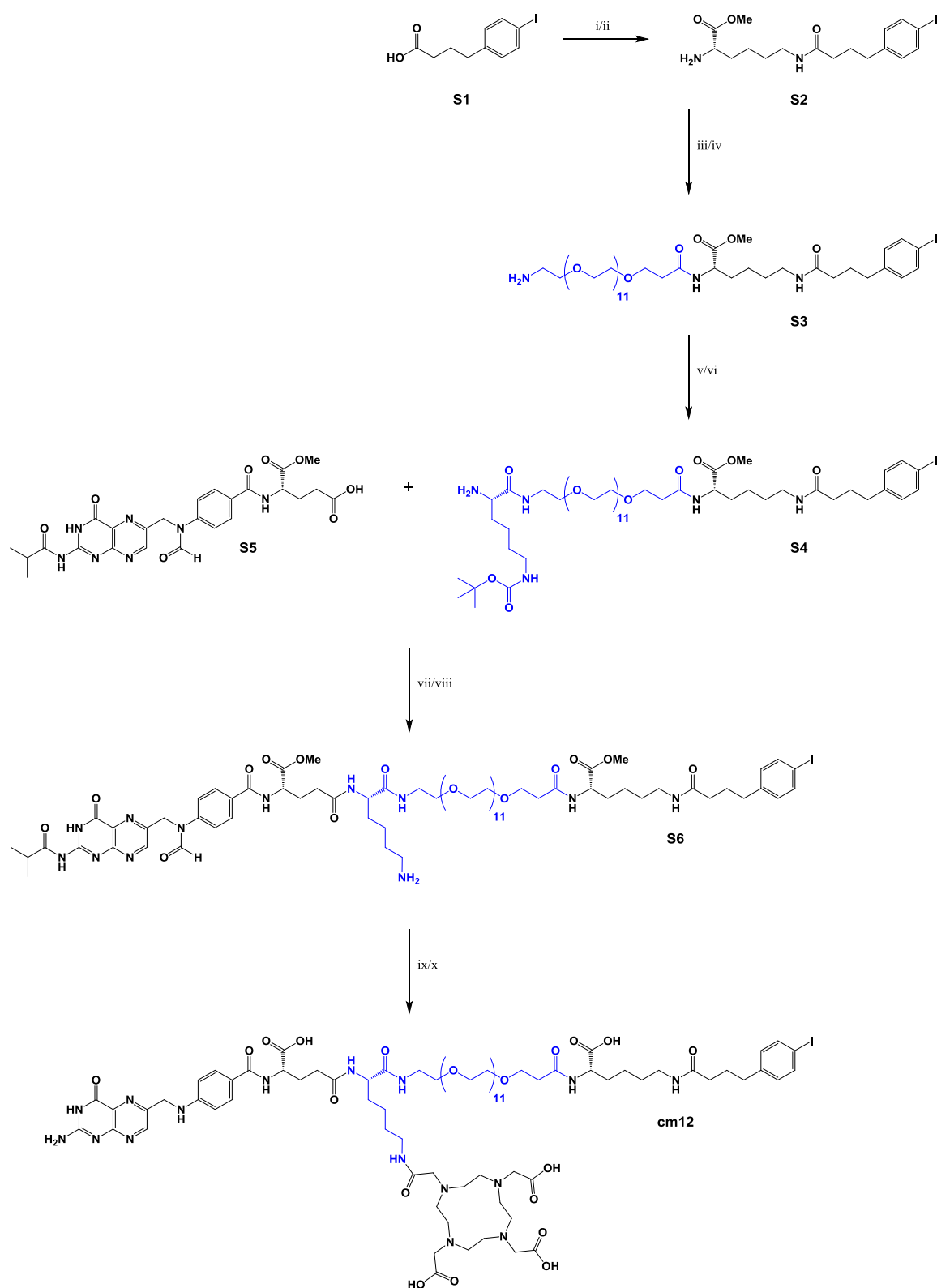


FIGURE S2. i) Boc-L-Lys-OMe x CH₃COOH (1.0 eq.), HBTU (1.1 eq.), Et₃N (4.0 eq.), DMF, 83% yield ii) excess TFA/CH₂Cl₂ (1:1), 1%TIPS, 70% yield iii) Fmoc-NH-(PEG)₁₁-COOH (1.0 eq.), HBTU (1.0 eq.), Et₃N (2.0 eq.), CH₂Cl₂, 74% yield iv) excess Et₂NH/CH₂Cl₂ (1:1), 68% yield v) Fmoc-L-Lys(Boc)-OH (1.1 eq.), HBTU (1.1eq.), Et₃N (2.0 eq.), CH₂Cl₂, 76% yield vi) excess Et₂NH/CH₂Cl₂ (1:1) , 71% yield vii) **S5** (1.0 eq.), HBTU (1.0 eq.), Et₃N (2.0 eq.), DMF, 79% yield viiii) excess 10% TFA/ CH₂Cl₂, 1%TIPS ix) DOTA-tris(*t*-Bu ester) (1.0 eq.), HBTU (1.0 eq.), Et₃N (11.0

eq.), DMF, 40% yield over step viii) and ix) x) excess TFA/ CH₂Cl₂ (1:1), 1%TIPS, then aq. LiOH (20 eq.) / THF (1:1), 19% yield.

Organic Synthesis of the Folate Conjugate **cm13**

Compound **S2** was prepared as described for **cm12** (Supplemental Figure S2). Boc-7-aminoheptanoic acid (Boc-NH(CH₂)₆-COOH; purchased from Bachem AG) was activated with HBTU in methylenechloride (CH₂Cl₂) and reacted with compound **S2**. Then, the Boc group was cleaved with TFA in methylenechloride in the presence of TIPS. The resulting amine **S7** was coupled to Fmoc-Lys(Boc)-OH which was activated with HBTU. After cleavage of the Fmoc group with diethylamine (Et₂NH) in methylenechloride the resulting amine **S8** was coupled to the γ -carboxyl group of *N*²-pivaloyl-*N*¹⁰-formylfolic acid α -methylester **S5** which was activated with HBTU. Cleavage of the Boc-protective group with TFA in methylenechloride in the presence of TIPS resulted in compound **S9**. The amine of the lysine side chain of **S9** was reacted with DOTA-tris(*t*-Bu ester) in the presence of HBTU and Et₃N resulting in the protected DOTA-folate conjugate **cm13**. Deprotection of **cm13** was achieved by treatment with TFA in methylenechloride and - after removal of solvents - with aqueous lithiumhydroxide solution (aq. LiOH) in the presence of THF. The product **cm13** was precipitated at pH 3.0 by addition of aqueous 1 M HCl. It was then isolated by centrifugation, washed with water and dried at 40 °C in vacuum (Supplemental Fig. S3). The reactions were monitored by HPLC and LC-MS. The final product was characterized by LC-MS. The synthesis of compound **cm13** resulted in an overall yield of 2%. The compound was obtained in >75% purity as confirmed by HPLC. Additional purification of this compound was performed using semipreparative HPLC yielding the compound with >95% purity. Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF MS): [M+H]⁺ = calculated for C₆₄H₉₂IN₁₆O₁₇ 1484.44; found 1484.51.

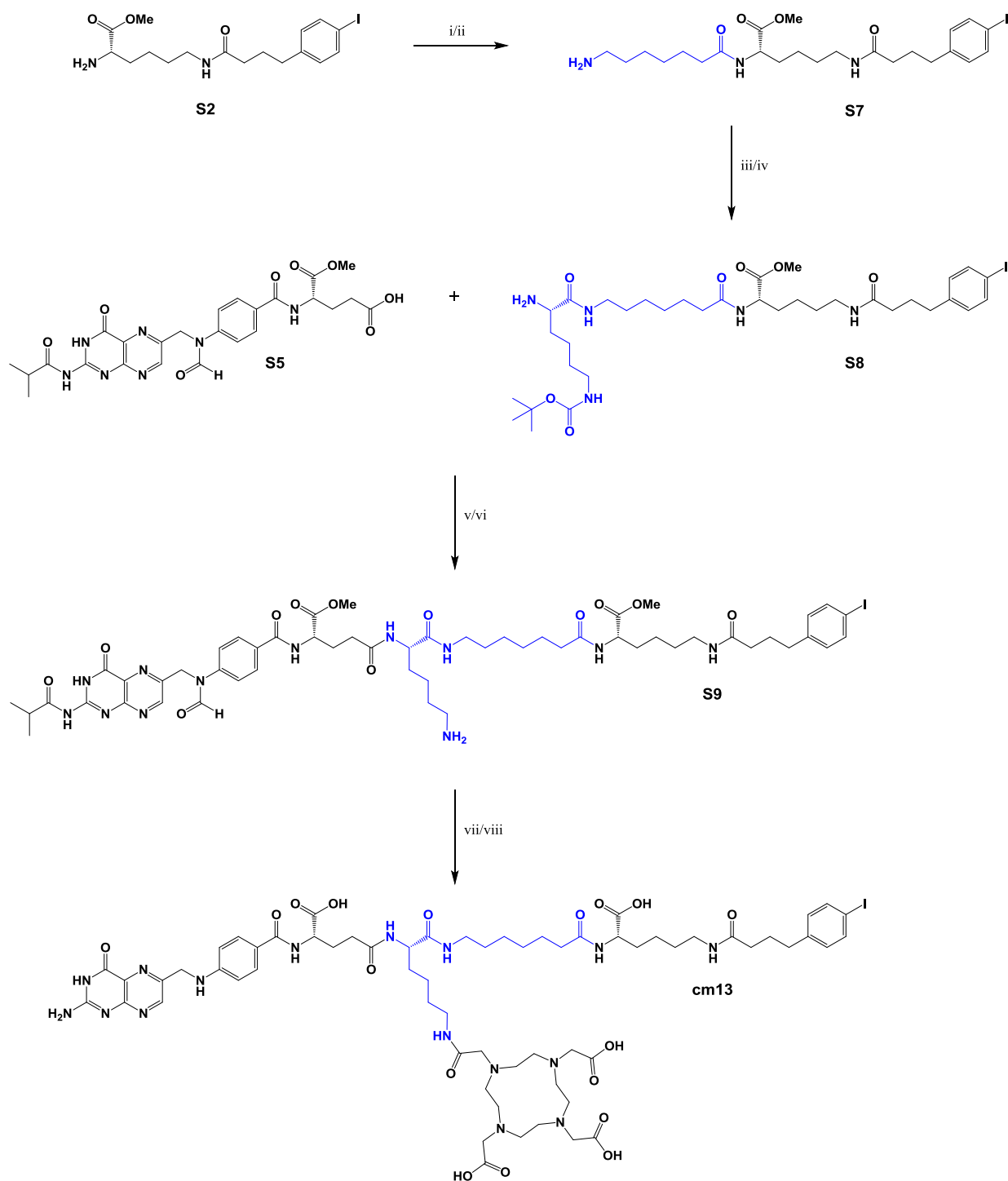


FIGURE S3. i) Boc-NH(CH₂)₆-COOH (1.0 eq.), HBTU (1.0 eq.), Et₃N (4.0 eq.), CH₂Cl₂ ii) excess 10%TFA/CH₂Cl₂, 1%TIPS iii) Fmoc-L-Lys(Boc)-OH (1.0 eq.), HBTU (1.0 eq.), Et₃N (4.0 eq.), DMF iv) excess Et₂NH/ CH₂Cl₂ (1:1), 55% yield over steps i) to iv) v) **S5** (1.0 eq.), HBTU (1.0 eq.), Et₃N (2.0 eq.), DMF, 22% yield vi) excess 10%TFA/ CH₂Cl₂, 1%TIPS vii) DOTA-tris(*t*-Bu ester) (1.0 eq.), HBTU (1.0 eq.), Et₃N (11.0 eq.), DMF, 28% yield over step vi) and vii) viii) excess TFA/CH₂Cl₂ (1:1), then aq. LiOH (20.0 eq.)/THF (1:1), 59% yield.

Organic Synthesis of the Folate Conjugate **cm14**

*N*²-pivaloyl-*N*¹⁰-trifluoroacetylfolate α -methylene ester **S10** was activated with HBTU in DMF and reacted with H-L-Lys(Boc)-OMe·HCl (purchased from Bachem AG) in the presence of Et₃N. Then, the Boc group was cleaved with TFA in methylenechloride (CH₂Cl₂) in the presence of TIPS. The resulting amine **S11** was reacted in DMF with DOTA-tris(*t*-Bu ester) in the presence of HBTU and Et₃N resulting in the protected DOTA-folate conjugate **cm14**. Deprotection of **cm14** was achieved by treatment with TFA in methylenechloride and - after removal of solvents - with aqueous lithiumhydroxide solution (aq. LiOH). The product **cm14** was precipitated at pH 1-2 by addition of 2 M HCl. After addition of acetone it was isolated by centrifugation, washed with water and dried at 40 °C in vacuum (Supplemental Fig. S4). The reactions were monitored by HPLC and LC-MS. The final product was characterized by LC-MS. The synthesis of compound **cm14** resulted in an overall yield of 38%. The compound was obtained in >93% purity as confirmed by HPLC. Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF MS): [M+H]⁺ = calculated for C₄₁H₅₈N₁₃O₁₄ 956.4; found 956.4.

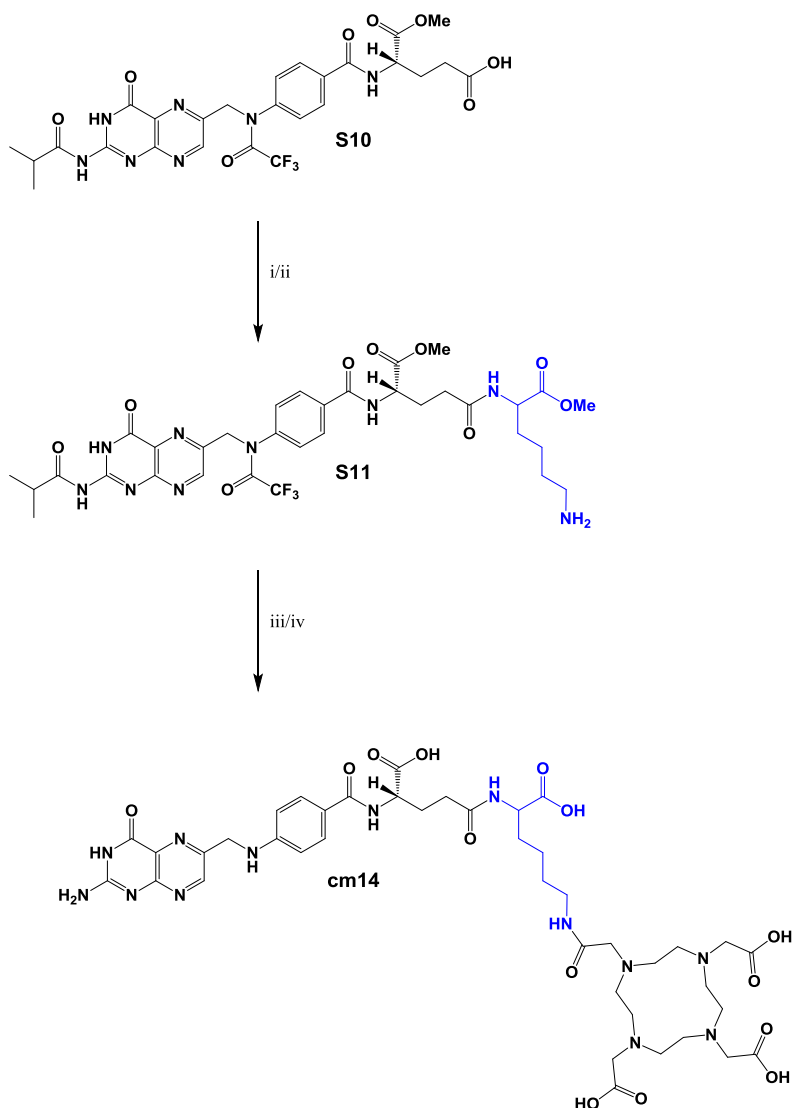


FIGURE S4. i) H-L-Lys(Boc)-OMe (1.0 eq.), HBTU (1.0 eq.), Et₃N (2.0 eq.), DMF, yield 91% ii) excess 10% TFA/CH₂Cl₂, 1%TIPS, yield 100% iii) DOTA-tris(*t*-Bu ester) (1.0 eq.), HBTU (1.0 eq.), Et₃N (2.0 eq.), DMF, yield 54% iv) excess TFA/CH₂Cl₂ (1:1), then aq. LiOH (28.0 eq.), 77% yield.

Final Remark

The reason for the very low overall yield of compound cm12, as compared to compound cm13 and compound cm14, was the last reaction step in which the final product was precipitated from an aqueous solution by addition of HCl. Due to the higher solubility of cm12 in water as compared to cm13 and cm14, the isolated yield of the last step was only ~19% for cm12 while a large amount of product remained in the mother liquor and could not be isolated with an acceptable purity.

2. Determination of FR-Binding Affinity

Experimental

The experimental procedure of how the in vitro assays were performed to determine K_D values as a measure of FR-binding affinities of the radiofolates is described in the main manuscript.

Results

The results revealed K_D values in the range of ~4-7.5 nM for the tested radiofolates without any significant differences between the different radiofolates as reported in the main manuscript.

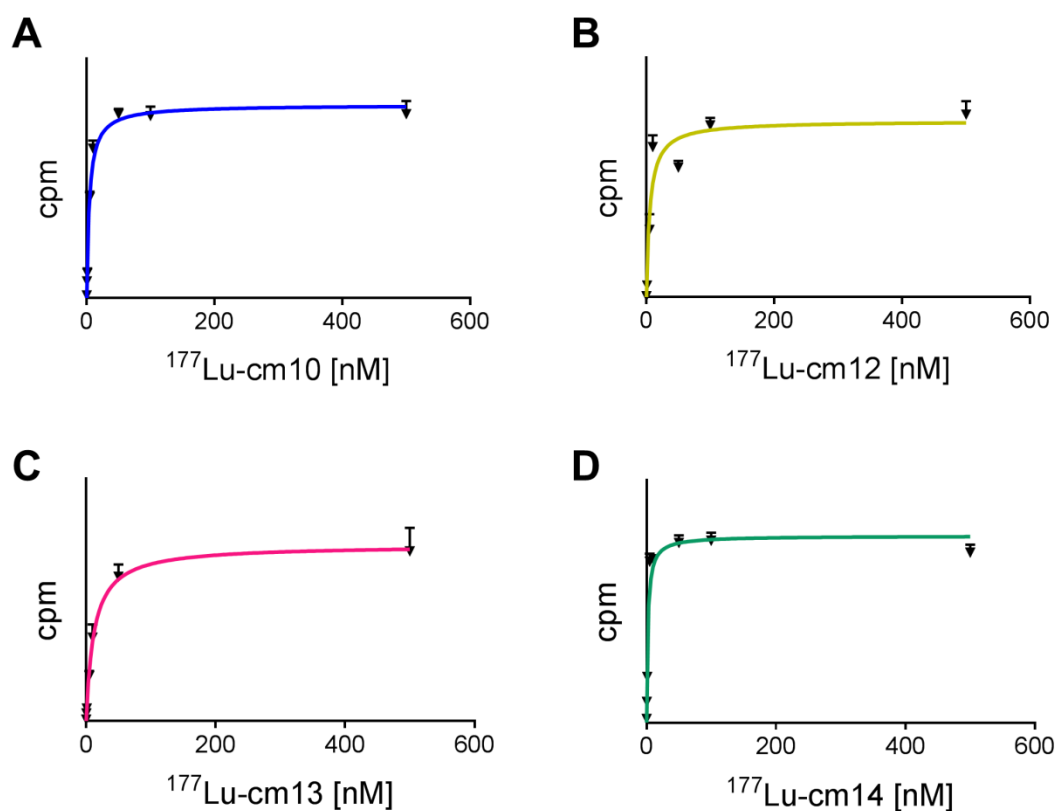


FIGURE S5. Representative saturation curves of experiments performed to determine K_D values of (A) ^{177}Lu -cm10/cm10, (B) ^{177}Lu -cm12/cm12, (C) ^{177}Lu -cm13/cm13 and (D) ^{177}Lu -cm14/cm14.

3. Biodistribution Data of the Radiofolates

Experimental

The experimental setting and preparation of the tumor-bearing mice is described in the main manuscript.

Results

The results are listed in the Supplemental Tables S1-S4. They are discussed in the main manuscript.

Table S1. Biodistribution 1 h after Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
	1 h p.i.	1 h p.i.	1 h p.i.	1 h p.i.
Blood	7.89 ± 1.36	6.53 ± 0.84	10.2 ± 2.52	0.20 ± 0.02
Lung	4.20 ± 0.75	3.69 ± 0.53	4.22 ± 0.18	1.40 ± 0.27
Spleen	1.61 ± 0.19	1.35 ± 0.12	1.76 ± 0.10	0.50 ± 0.10
Kidneys	22.6 ± 3.87	22.4 ± 0.38	18.2 ± 0.70	58.7 ± 5.17
Stomach	1.85 ± 0.42	1.41 ± 0.31	1.54 ± 0.15	1.78 ± 0.40
Intestines	1.37 ± 0.19	0.98 ± 0.25	1.18 ± 0.11	0.76 ± 0.16
Liver	5.00 ± 1.26	7.26 ± 0.47	5.76 ± 0.67	5.83 ± 1.37
Salivary glands	9.79 ± 2.42	11.5 ± 1.55	6.97 ± 0.74	7.13 ± 1.56
Muscle	1.27 ± 0.11	1.61 ± 0.30	1.22 ± 0.18	1.75 ± 0.20
Bone	1.62 ± 0.13	1.52 ± 0.19	1.53 ± 0.07	1.87 ± 0.08
KB Tumor	11.1 ± 0.49	9.51 ± 0.59	8.69 ± 0.94	6.23 ± 0.71
Tu-to-blood	1.45 ± 0.31	1.47 ± 0.13	0.88 ± 0.20	31.7 ± 6.00
Tu-to-liver	3.11 ± 1.54	3.06 ± 3.18	3.13 ± 2.78	2.13 ± 2.11
Tu-to-kidney	0.50 ± 0.09	0.43 ± 0.03	0.48 ± 0.07	0.11 ± 0.01

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

Table S2. Biodistribution 4 h After Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
	4 h p.i.	4 h p.i.	4 h p.i.	4 h p.i.
Blood	2.34 $\pm$ 0.50	2.35 $\pm$ 0.09	4.05 $\pm$ 0.64	0.09 $\pm$ 0.02
Lung	1.57 $\pm$ 0.20	1.67 $\pm$ 0.12	2.11 $\pm$ 0.47	1.21 $\pm$ 0.14
Spleen	0.67 $\pm$ 0.06	0.76 $\pm$ 0.10	0.97 $\pm$ 0.18	0.49 $\pm$ 0.07
Kidneys	21.9 $\pm$ 2.82	31.9 $\pm$ 3.21	25.8 $\pm$ 2.79	71.8 $\pm$ 22.5
Stomach	0.93 $\pm$ 0.26	0.93 $\pm$ 0.09	0.92 $\pm$ 0.08	1.90 $\pm$ 0.43
Intestines	0.63 $\pm$ 0.07	0.45 $\pm$ 0.06	0.61 $\pm$ 0.11	0.46 $\pm$ 0.19
Liver	3.14 $\pm$ 0.36	5.17 $\pm$ 0.73	4.13 $\pm$ 0.62	3.57 $\pm$ 0.62
Salivary glands	5.64 $\pm$ 0.26	6.44 $\pm$ 1.17	7.31 $\pm$ 2.01	8.47 $\pm$ 0.74
Muscle	1.12 $\pm$ 0.21	2.17 $\pm$ 0.27	1.25 $\pm$ 0.19	1.18 $\pm$ 0.14
Bone	1.27 $\pm$ 0.22	1.24 $\pm$ 0.05	1.19 $\pm$ 0.16	1.47 $\pm$ 0.26
KB Tumor	14.3 $\pm$ 1.94	19.5 $\pm$ 3.21	16.0 $\pm$ 1.58	8.56 $\pm$ 2.09
Tu-to-blood	6.39 $\pm$ 2.19	9.29 $\pm$ 1.01	3.98 $\pm$ 0.30	101 $\pm$ 26.9
Tu-to-liver	3.74 $\pm$ 0.89	3.85 $\pm$ 1.01	3.93 $\pm$ 0.38	3.11 $\pm$ 1.06
Tu-to-kidney	0.65 $\pm$ 0.02	0.61 $\pm$ 0.10	0.63 $\pm$ 0.12	0.12 $\pm$ 0.00

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

Table S3. Biodistribution 24 h After Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
	24 h p.i.	24 h p.i.	24 h p.i.	24 h p.i.
Blood	0.49 $\pm$ 0.08	0.18 $\pm$ 0.02	0.56 $\pm$ 0.10	0.03 $\pm$ 0.00
Lung	0.82 $\pm$ 0.16	0.87 $\pm$ 0.29	1.54 $\pm$ 1.15	0.98 $\pm$ 0.12
Spleen	0.55 $\pm$ 0.02	0.54 $\pm$ 0.11	0.42 $\pm$ 0.08	0.34 $\pm$ 0.05
Kidneys	28.1 $\pm$ 2.69	37.1 $\pm$ 6.32	19.5 $\pm$ 5.02	46.6 $\pm$ 9.64
Stomach	0.59 $\pm$ 0.07	0.49 $\pm$ 0.13	0.48 $\pm$ 0.18	1.28 $\pm$ 0.15
Intestines	0.20 $\pm$ 0.10	0.16 $\pm$ 0.04	0.29 $\pm$ 0.08	0.40 $\pm$ 0.14
Liver	3.10 $\pm$ 0.98	4.30 $\pm$ 0.34	2.59 $\pm$ 0.28	2.13 $\pm$ 0.80
Salivary glands	4.60 $\pm$ 1.21	4.76 $\pm$ 0.20	3.21 $\pm$ 0.30	5.38 $\pm$ 0.49
Muscle	1.00 $\pm$ 0.21	1.48 $\pm$ 0.36	0.80 $\pm$ 0.02	0.99 $\pm$ 0.12
Bone	0.74 $\pm$ 0.31	1.01 $\pm$ 0.12	0.59 $\pm$ 0.15	1.13 $\pm$ 0.36
KB Tumor	17.6 $\pm$ 2.58	16.1 $\pm$ 3.41	12.2 $\pm$ 1.95	4.80 $\pm$ 0.94
Tu-to-blood	36.2 $\pm$ 7.87	86.4 $\pm$ 11.09	21.8 $\pm$ 0.67	154. $\pm$ 26.3
Tu-to-liver	3.67 $\pm$ 1.50	3.72 $\pm$ 0.81	3.94 $\pm$ 1.10	3.13 $\pm$ 0.09
Tu-to-kidney	0.63 $\pm$ 0.10	0.43 $\pm$ 0.03	0.64 $\pm$ 0.07	0.10 $\pm$ 0.02

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

Table S4. Biodistribution 48 h After Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
	48 h p.i.	48 h p.i.	48 h p.i.	48 h p.i.
Blood	0.19 ± 0.00	0.17 ± 0.08	0.42 ± 0.10	0.02 ± 0.00
Lung	0.49 ± 0.19	1.06 ± 0.23	0.65 ± 0.16	0.54 ± 0.05
Spleen	0.33 ± 0.05	0.45 ± 0.07	0.49 ± 0.06	0.21 ± 0.03
Kidneys	18.6 ± 0.57	39.3 ± 8.52	22.5 ± 3.99	36.1 ± 2.04
Stomach	0.29 ± 0.06	0.56 ± 0.29	0.39 ± 0.07	0.64 ± 0.02
Intestines	0.23 ± 0.20	0.20 ± 0.09	0.23 ± 0.08	0.22 ± 0.01
Liver	1.86 ± 0.37	2.83 ± 0.66	2.80 ± 0.59	1.27 ± 0.36
Salivary glands	2.43 ± 0.39	9.09 ± 0.42	3.23 ± 1.28	3.52 ± 0.08
Muscle	0.58 ± 0.26	0.87 ± 0.07	0.73 ± 0.24	0.47 ± 0.04
Bone	0.50 ± 0.09	0.65 ± 0.16	0.57 ± 0.08	0.52 ± 0.06
KB Tumor	10.12 ± 1.20	10.8 ± 0.67	13.3 ± 2.94	3.34 ± 0.27
Tu-to-blood	54.1 ± 5.86	78.6 ± 46.9	31.6 ± 3.18	175 ± 17.3
Tu-to-liver	4.83 ± 2.34	3.97 ± 1.32	4.12 ± 1.12	1.86 ± 0.86
Tu-to-kidney	0.55 ± 0.07	0.28 ± 0.07	0.59 ± 0.03	0.09 ± 0.01

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

Table S5. Biodistribution 72 h After Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13
	72 h p.i.	72 h p.i.	72 h p.i.
Blood	0.10 $\pm$ 0.02	0.05 $\pm$ 0.00	0.26 $\pm$ 0.02
Lung	0.34 $\pm$ 0.04	0.55 $\pm$ 0.19	0.48 $\pm$ 0.15
Spleen	0.35 $\pm$ 0.03	0.34 $\pm$ 0.03	0.41 $\pm$ 0.04
Kidneys	17.2 $\pm$ 1.99	27.9 $\pm$ 6.18	13.7 $\pm$ 2.01
Stomach	0.29 $\pm$ 0.10	0.31 $\pm$ 0.03	0.40 $\pm$ 0.10
Intestines	0.28 $\pm$ 0.31	0.13 $\pm$ 0.05	0.15 $\pm$ 0.03
Liver	1.83 $\pm$ 0.59	2.72 $\pm$ 0.56	1.95 $\pm$ 0.18
Salivary glands	1.08 $\pm$ 1.53	4.97 $\pm$ 1.69	2.48 $\pm$ 0.14
Muscle	0.50 $\pm$ 0.21	0.59 $\pm$ 0.05	0.46 $\pm$ 0.08
Bone	0.29 $\pm$ 0.09	0.61 $\pm$ 0.03	0.41 $\pm$ 0.09
KB Tumor	8.59 $\pm$ 0.56	8.34 $\pm$ 1.11	8.81 $\pm$ 0.81
Tu-to-blood	89.4 $\pm$ 17.3	157 $\pm$ 13.6	33.5 $\pm$ 0.95
Tu-to-liver	3.88 $\pm$ 2.59	3.13 $\pm$ 0.23	3.58 $\pm$ 1.59
Tu-to-kidney	0.50 $\pm$ 0.07	0.30 $\pm$ 0.03	0.65 $\pm$ 0.05

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

Table S6. Biodistribution 120 h After Injection of ^{177}Lu -Folate Conjugates in KB Tumor-Bearing Female Nude Mice

	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13
	120 h p.i.	120 h p.i.	120 h p.i.
Blood	0.02 ± 0.01	0.02 ± 0.01	0.10 ± 0.02
Lung	0.22 ± 0.02	0.48 ± 0.20	0.39 ± 0.16
Spleen	0.25 ± 0.05	0.36 ± 0.13	0.29 ± 0.05
Kidneys	9.46 ± 0.38	21.9 ± 8.11	9.98 ± 1.11
Stomach	0.19 ± 0.01	0.31 ± 0.04	0.18 ± 0.05
Intestines	0.12 ± 0.03	0.20 ± 0.04	0.08 ± 0.02
Liver	0.93 ± 0.12	1.75 ± 0.56	1.32 ± 0.35
Salivary glands	1.60 ± 0.21	2.73 ± 0.37	1.62 ± 0.33
Muscle	0.19 ± 0.06	0.44 ± 0.15	0.28 ± 0.08
Bone	0.20 ± 0.04	0.36 ± 0.08	0.21 ± 0.06
KB Tumor	6.02 ± 1.17	6.66 ± 2.43	6.04 ± 0.98
Tu-to-blood	320 ± 136	270 ± 11.3	62.6 ± 12.0
Tu-to-liver	4.84 ± 3.85	3.34 ± 1.50	3.37 ± 2.27
Tu-to-kidney	0.63 ± 0.11	0.30 ± 0.03	0.61 ± 0.12

values shown represent the mean $\pm$ S.D. of data from three animals (n=3)

4. In Vivo Stability of Albumin-Binding Radiofolates

Experimental

Additional animal studies were performed with the same mouse strain (female CD-1 Foxn-1/nu, Charles River) in order to determine the in vivo stability of the radiolabeled folates. Mice without tumors were injected with the radiofolates (50 MBq, 2 nmol per mouse). Blood was taken under anesthesia from the retrobulbar vein after 4 h or after 24 h in the case of ^{177}Lu -cm10, ^{177}Lu -cm12 and ^{177}Lu -cm13 followed by euthanasia of the mouse. In the case of ^{177}Lu -cm14 which was rapidly cleared from the blood circulation, it was necessary to perform the blood sampling already 25 min after injection. Blood samples were centrifuged (4°C, 20 min, 1600 rpm) to allow collecting blood plasma. A volume of 200 μL methanol was added to ~ 130 μL plasma for precipitation of the proteins. After centrifugation (3 min, 8000 rpm), the supernatant was collected and centrifuged for a second time before filtration of the supernatant through a polytetrafluorethylene filter (0.2 μm) prior to injection into HPLC using the same gradient as described in the main manuscript but performed on a different HPLC system.

Results

The HPLC analysis revealed over 98% intact radiofolate up to 24 h after injection of all albumin-binding radiofolates (^{177}Lu -cm10, ^{177}Lu -cm12 and ^{177}Lu -cm13). In the case of ^{177}Lu -cm14 >87% of the compound was intact at 25 min p.i. (Supplemental Figure S6). It appears that the albumin-binding radiofolates were all stable over an extended time period in the blood circulation. Hence, the high renal accumulation of activity in the kidneys in the case of ^{177}Lu -cm12 was not due to metabolite formation but a result of different pharmacokinetics of this radiofolate.

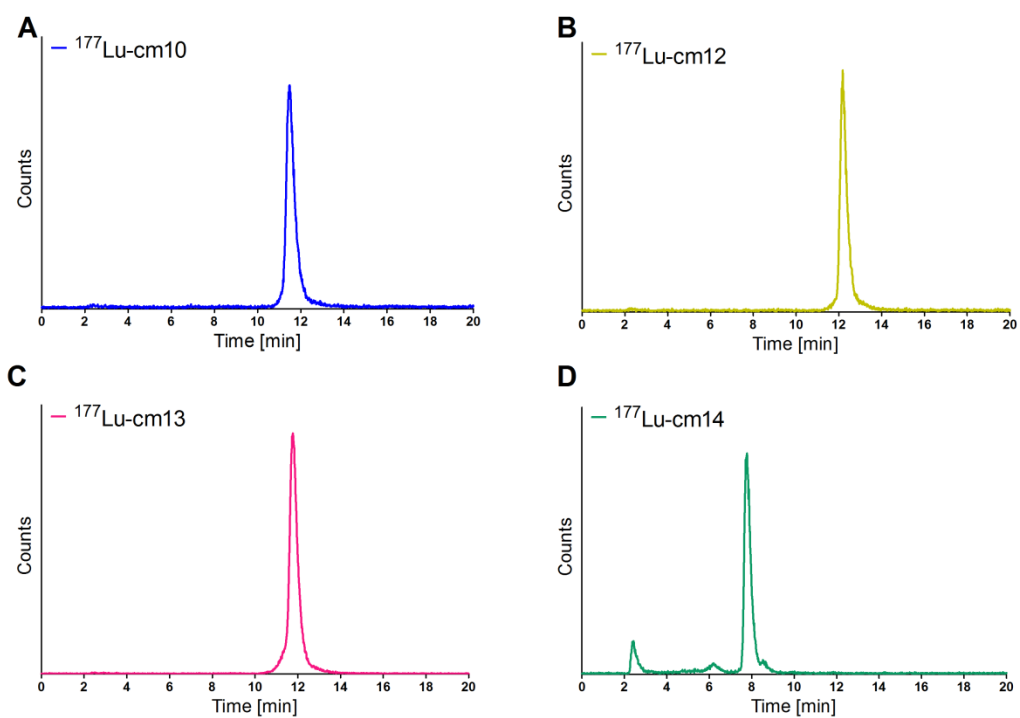


FIGURE S6. HPLC chromatograms obtained with processed blood samples taken 24 h after injection of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12 and (C) ^{177}Lu -cm13. HPLC chromatogram obtained with processed blood sample taken 25 min after injection of (D) ^{177}Lu -cm14.

5. Sections of SPECT/CT Imaging Studies in KB Tumor-Bearing Mice

In vivo SPECT/CT images shown in the main manuscript are maximal intensity projections. The sections through the center of the right KB tumor xenografts are shown in a separate figure (Supplemental Figure S7).

Experimental

The experimental procedure is reported in the main manuscript.

Results

The sections of these SPECT/CT imaging studies show an inhomogeneous distribution of accumulated radioactivity with higher accumulation in the outer rim of the xenograft and clearly less activity in the center of the tumor. The reason for these observations could be a poor vascularization of the tumor center, the presence of necrotic cells in the center of the tumor xenograft or acidification of the tumor which could influence the binding of the radiofolates to the FR (known to be reduced at a low pH).

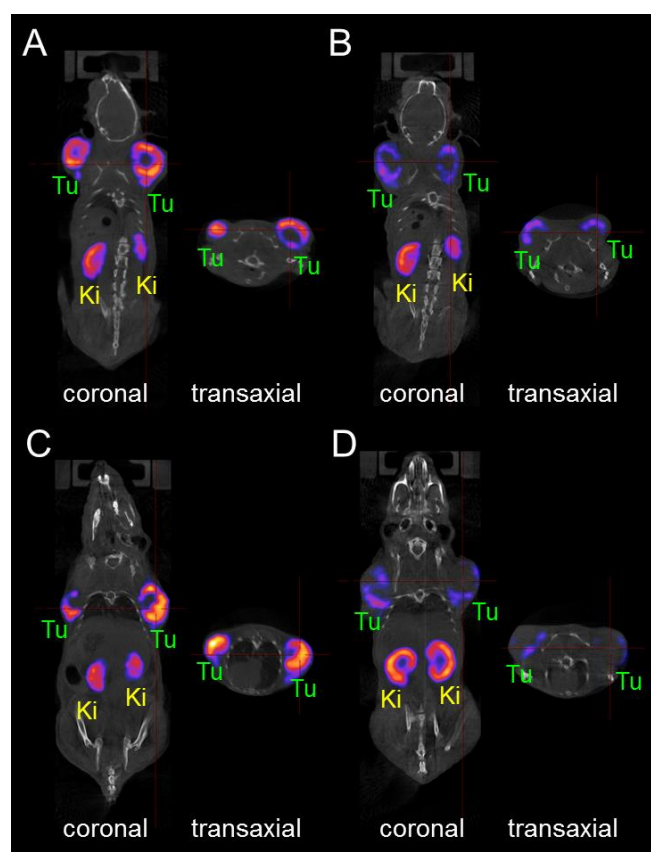


FIGURE S7. SPECT/CT image of KB tumor-bearing mice 24 h after injection of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12, (C) ^{177}Lu -cm13 and (D) ^{177}Lu -cm14 (50 MBq, 2 nmol per mouse). The images are presented as coronal and transaxial sections. The windowing has been adjusted so that the accumulation of activity in kidneys was comparable for all mice. (Tu = IGROV-1 tumor xenograft, Ki = kidney).

6. Biodistribution and SPECT/CT Imaging Studies in IGROV-1 Tumor-Bearing Mice

In additional in vivo experiments, the tissue distribution of the radiofolates was investigated in IGROV-1 tumor-bearing mice in order to confirm the tumor-to-kidney ratios in a mouse model other than the standard model with KB tumor xenografts.

Experimental

IGROV-1, a FR-positive, human tumor cell line was a kind gift from Dr. Gerrit Janssen. (Free University Medical Center Amsterdam, the Netherlands).² The cells were cultured under the same conditions (folate-deficient culture medium, FFRPMI) as were used for the KB tumor cells. Mice of the same strain (female CD-1 Foxn-1/nu, Charles River) were inoculated with a suspension of IGROV-1 tumor cells in PBS pH 7.4 (5×10^6 cells per mouse). After 2-3 weeks when the tumor xenografts reached a size of $\sim 200 \text{ mm}^3$, mice were injected with radiofolates (3 MBq, 0.5 nmol) for biodistribution studies at 24 h p.i. performed in triplicate. The experimental procedure was the same as for KB tumor-bearing mice described in the main manuscript.

SPECT/CT experiments were performed 24 h after intravenous injection of the radiofolates (50 MBq, 2 nmol per mouse). SPECT/CT scans of 45 min were performed under anesthesia of the mice as described in the main manuscript. Images were prepared using the *VivoQuant* post-processing software (version 2.5, inviCRO Imaging Services and Software, Boston, U.S.). A Gauss post-reconstruction filter was applied for the presentation of the SPECT images and the scale was adjusted to allow the best visualization of tumors and kidneys in which radioactivity accumulated.

Results

The biodistribution data obtained in IGROV-1 tumor-bearing mice revealed similar distribution profiles of the radiofolates as shown for KB tumor-bearing mice. As compared to the accumulation of the radiofolates in KB tumor xenografts, the tumor uptake in IGROV-1 xenografts was increased at 24 h after injection of all radiofolates (^{177}Lu -cm10: $25.6 \pm 8.85\% \text{ IA/g}$; ^{177}Lu -cm12: $13.8 \pm 1.86\% \text{ IA/g}$; ^{177}Lu -cm13: $24.6 \pm 2.16\% \text{ IA}$ and ^{177}Lu -cm14: $12.8 \pm 2.64\% \text{ IA/g}$). This may be a result of an increased vascularization of IGROV-1 tumors as compared to KB tumors. In addition, there may be a difference in the recycling rate of the FRs among KB and IGROV-1 tumors which – according to in vivo studies reported in the literature³ - may be more relevant with regard to the accumulation of folates in FR-positive tumor cells than the FR expression level. As a result, the tumor-to-kidney ratios of IGROV-1 tumor-bearing mice were slightly improved as compared to the tumor-to-kidney ratios obtained in KB tumor-bearing mice (Supplemental Table S7). The best tumor-to-kidney ratios were obtained with ^{177}Lu -cm10 and ^{177}Lu -cm13 followed by ^{177}Lu -cm12. ^{177}Lu -cm14 showed an inferior value of the tumor-to-kidney ratio which was, however, in the same range in both tumor-mouse models (~ 0.10 - 0.15).

Table S7. Tumor-to-Kidney Ratios of Radiofolates in KB and IGROV-1 Tumor-Bearing Mice 24 h after Injection.

Tumor type	^{177}Lu -cm10	^{177}Lu -cm12	^{177}Lu -cm13	^{177}Lu -cm14
IGROV-1	1.03 ± 0.37	0.55 ± 0.10	0.97 ± 0.07	0.15 ± 0.03
KB	0.63 ± 0.10	0.43 ± 0.03	0.63 ± 0.07	0.10 ± 0.02

SPECT/CT images were obtained 24 h after injection of the radiofolates using IGROV-1 tumor-bearing mice (Supplementary Figures S8 and S9). At scan start the activity in the respective mice was 56% of the injected activity 24 h after injection of ^{177}Lu -cm10, 33% of the injected activity 24 h after injection of ^{177}Lu -cm12, 67% of the injected activity 24 h after injection of ^{177}Lu -cm13 and 23% of the injected activity 24 h after injection of ^{177}Lu -cm14. The scale has been adjusted so that the uptake in kidneys was comparable for all mice. This allowed better visualization of the different tumor-to-kidney ratios although it does not reflect the absolute renal retention of activity which was different among radiofolates. The tumor-to-kidney ratios calculated based on these SPECT acquisitions confirmed that the tumor-to-kidney ratios were best for ^{177}Lu -cm13 (1.34), followed by ^{177}Lu -cm10 (1.23) whereas ^{177}Lu -cm12 and ^{177}Lu -cm14 showed clearly reduced tumor-to-kidney ratios (0.93 and 0.81, respectively).

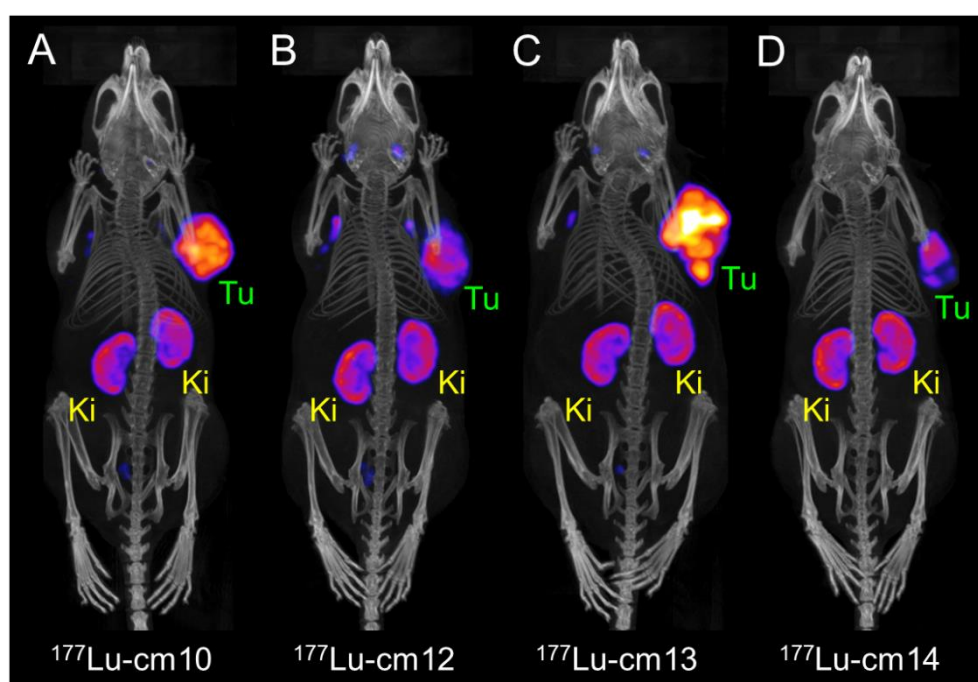


FIGURE S8. SPECT/CT image of IGROV-1 tumor-bearing mice 24 h after injection of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12, (C) ^{177}Lu -cm13 and (D) ^{177}Lu -cm14 (50 MBq, 2 nmol per mouse). The images are presented as maximal intensity projections. The windowing has been adjusted so that the accumulation of activity in kidneys was comparable for all mice. (Tu = IGROV-1 tumor xenograft, Ki = kidney)

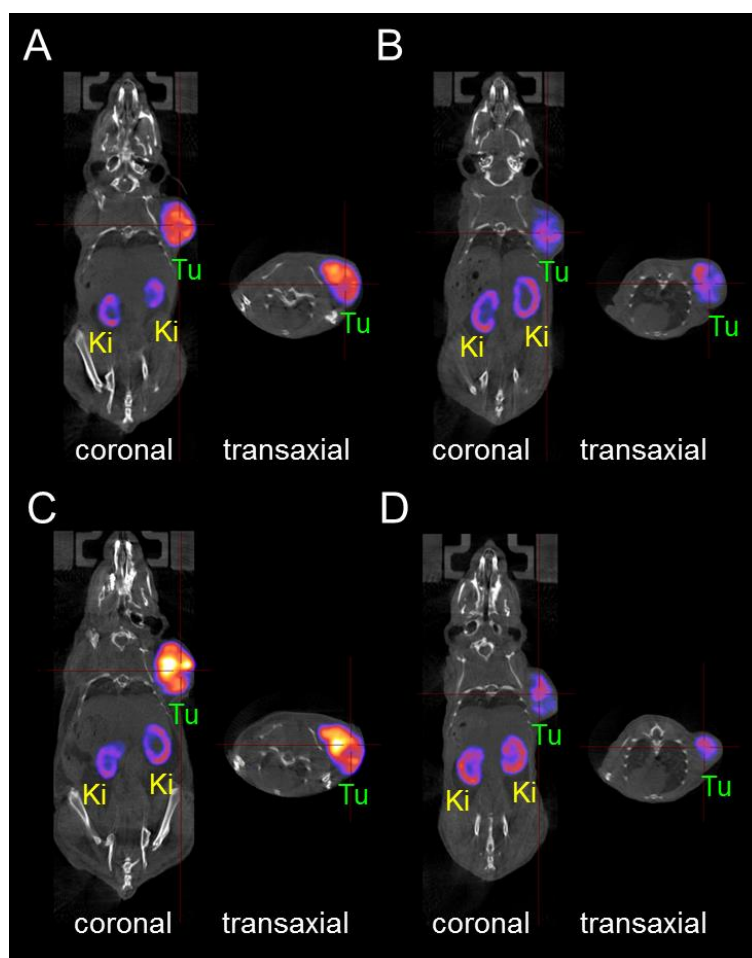


FIGURE S9. SPECT/CT image of IGROV-1 tumor-bearing mice 24 h after injection of (A) ^{177}Lu -cm10, (B) ^{177}Lu -cm12, (C) ^{177}Lu -cm13 and (D) ^{177}Lu -cm14 (50 MBq, 2 nmol per mouse). The images are presented as coronal and transaxial sections. The windowing has been adjusted so that the accumulation of activity in kidneys was comparable for all mice. (Tu = IGROV-1 tumor xenograft, Ki = kidney)

References

1. Müller, C.; Struthers, H.; Winiger, C.; Zhernosekov, K.; Schibli, R., DOTA conjugate with an albumin-binding entity enables the first folic acid-targeted ^{177}Lu -radionuclide tumor therapy in mice. *J Nucl Med* **2013**, *54* (1), 124-31.
2. Müller, C.; Schibli, R.; Krenning, E. P.; de Jong, M., Pemetrexed improves tumor selectivity of ^{111}In -DTPA-folate in mice with folate receptor-positive ovarian cancer. *J Nucl Med* **2008**, *49* (4), 623-9.
3. Paulos, C. M.; Reddy, J. A.; Leamon, C. P.; Turk, M. J.; Low, P. S., Ligand binding and kinetics of folate receptor recycling in vivo: impact on receptor-mediated drug delivery. *Molecular Pharmacology* **2004**, *66* (6), 1406-1414.