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PEGylation enables the specific tumor accumulation of a peptide identified by phage display

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Walter Mier,*[a] Susanne Krämer,[a] Sabine Zitzmann,[a,b,c] Annette Altmann,[a,b] Karin Leotta,[b] Ursula Schierbaum,[b] Martina Schnölzer,[d] Michael Eisenhut[e] and Uwe Haberkorn[a,b]

Peptides are excellent alternatives to small molecules and proteinaceous drugs. Their high medicinal potential for diagnostic and therapeutic applications has prompted the development of tumor targeting peptides. Despite its excellent tumor binding capacity, FROP–DOTA (H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-NH_2), a peptide that we had identified in phage display libraries, revealed slow binding kinetics. Consequently, biodistribution studies showed that its excretion forestalled a significant tumor accumulation. The aim of this study was to investigate whether the conjugation of PEG to FROP–DOTA resulted in a derivative with a prolonged residence time in the blood. A synthetic method for the PEGylation of the tumor specific peptide FROP–DOTA was developed. Thereafter, binding studies were done *in vitro* and a biodistribution was performed in tumor bearing animals. These were compared to the data obtained with FROP–DOTA. The binding kinetics of the PEGylated FROP–DOTA was even slower than that of FROP–DOTA. Biodistribution studies of the labeled conjugate in mice bearing human FRO82-2 tumors showed a time dependent increased uptake of the PEGylated peptide with a high retention (at 24 h p.i. 76% of the maximal activity concentration persisted in the tumor). The highest uptake values were determined at 120 min p.i. reaching 2.3%ID/g tumor as compared to 0.06%ID/g observed for the non-PEGylated derivative at 135 min p.i. Apparently, PEGylation provides a substantially improved stabilization in the circulation which allowed a stable tumor accumulation.

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Introduction

Systemic toxicity is one of the main limitations of chemotherapy. While the traditional chemotherapeutic drugs affect all proliferating cells, targeted drugs preferentially pinpoint tumor cells and allow us to go beyond the possibilities of current drug therapy. However, also these novel agents sometimes lead to severe side effects. The success of new therapy modalities depends on the development of new vector systems to target tumors. The study of numerous naturally occurring peptides, in particular small regulatory peptides, has demonstrated that peptides provide excellent targeting properties. DOTATOC, a peptide derivative successfully used for the diagnosis and therapy of neuroendocrine tumors,[1,2] is the prime example for this class of therapeutics. Unfortunately, the hope to discover peptides that meet this performance was lost because the number of similar natural peptides is limited and problems with unwanted accumulation in critical organs may occur.[3] Alternatively, attempts have been made to exploit antibodies that bind epitopes specifically expressed on tumors. Again, the promises for many therapeutic antibodies were disillusioned because of their unfavorable pharmacokinetics observed as a consequence of their high molecular weight.

The best results with therapeutic antibodies were obtained with Zevalin,[4] (^{90}Y-ibritumomab tiuxetan) a radiolabeled derivative of the anti-CD20 antibody rituximab – the first radiopharmaceutical approved for the radioimmunotherapy of patients with B-cell non-Hodgkin lymphomas. These successes and failures clearly define the ideal properties of tumor binding molecules. The identification and characterization of novel peptides that bind to cell surface receptors overexpressed on tumors can be achieved by the screening of phage display libraries.[5] However, the high expectations for this technology have not been met for *in vivo* applications. We have shown that the coupling of DOTA to FROP-1, a peptide that enables the

[a]*Department of Nuclear Medicine, University Hospital Heidelberg, INF 400, 69120 Heidelberg, Germany. E-mail: walter.mier@med.uni-heidelberg.de; Tel: +49-6221-567720*
[b]*Clinical Cooperation Unit Nuclear Medicine, German Cancer Research Center (DKFZ), INF 280, 69120 Heidelberg, Germany. Tel: +49-6221-420*
[c]*Bayer Pharma AG, Research Laboratories, Müllerstr. 178, 13353 Berlin, Germany. Tel: +49-30 468 1111*
[d]*Research Group Functional Proteome Analysis, German Cancer Research Center (DKFZ), INF 280, 69120 Heidelberg, Germany. Tel: +49-6221-420*
[e]*Department of Radiopharmaceutical Chemistry, German Cancer Research Center (DKFZ), INF 280, 69120 Heidelberg, Germany. Tel: +49-6221-420*

selective targeting of thyroid carcinoma and a series of other tumors,[6] resulted in a dramatically improved *in vitro* binding capacity. Unfortunately, the biodistribution study of this peptide, originally identified using a phage display library, revealed that the binding kinetics of FROP–DOTA (H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-NH_2) was slow and consequently excretion impeded a significant tumor accumulation.[7] It is known that peptides can be retained in the circulation by either albumin binding upon acylation[8,9] or by suppressing the renal filtration by increasing their molecular weight.[10] PEGylation, a technique first described in 1977 by Davies and Abuchowsky,[11,12] is most commonly applied for the modification of proteins.[13] However, it has been shown to be advantageous for small molecules such as MRI contrast agents.[14] More importantly several of the shortcomings of peptides as pharmaceuticals[15] such as short duration of action, their lack of oral bioavailability and their susceptibility to proteolysis might be solved by PEGylation.

Among the different techniques of post-production modification (*i.e.* amino acid manipulation, HESylation polysialylation and PEGylation) PEGylation is still the most commonly applied technique. It allows us to increase the circulation half-life of peptide drugs and consequently the reduction of the doses required. Conjugation of PEG causes a significant change in the physicochemical characteristics, as a result the pharmacokinetic and pharmacodynamic properties are altered – in general at a reduced immunogenic and toxicological potential.[16–18]

The circulation half-life of PEG-conjugates is generally increased by longer PEG chains. Even at extremely high concentrations the toxicological effects of PEG–protein conjugates have been shown to be limited to a transient renal tubular vacuolization, a side effect that does not cause functional effects.[19] Therefore, PEG–protein conjugates can be considered immunologically safe and non-toxic.

The aim of this study was to investigate whether the conjugation of PEG to FROP–DOTA results in a derivative with a prolonged residence time in the blood.

Results and discussion

In order to improve the retention time of FROP-1 in the circulation, a PEGylated derivative of this peptide as shown in Fig. 1 was required.

A synthetic method for the PEGylation of the tumor specific peptide FROP–DOTA had to be developed. Unfortunately, all attempts to couple different PEG derivatives on the solid support did not yield satisfactory results. A possible explanation is the relatively low molar concentrations achievable and the slow reaction kinetics to be expected with the 20 kDa PEG building block which shows a slow diffusion rate in the polymer. The bulky PEG building block hardly reaches its reaction partner buried at the C-terminal end of the resin bound peptide. Consequently, the PEGylation was performed in solution. For this purpose H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-Cys-NH_2 (a FROP–DOTA derivative with a C-terminal cysteine) was obtained by solid phase synthesis. The product was characterized by HPLC and mass spectrometry. This cysteine derivative was reacted in solution with a 20 000 Da maleimido-modified PEG-oligomer. A first attempt to analyze the reaction was made by using RP-HPLC as shown in Fig. 2. Unfortunately, the chromatographic behavior of the conjugate did not differ from the pure PEG synthesis precursor.

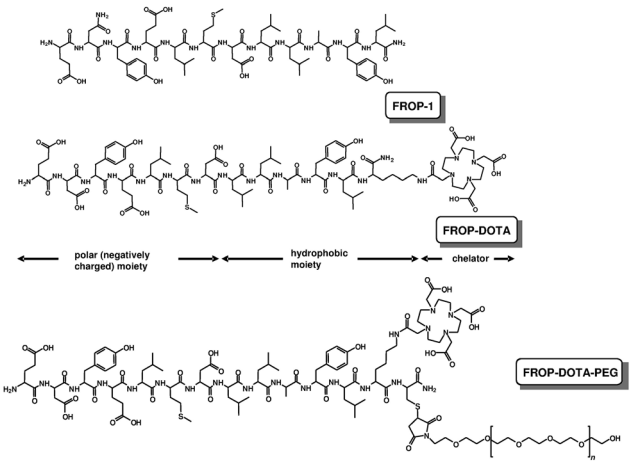

Fig. 1 The chemical structure of the PEGylated peptide and its precursor peptide generations.

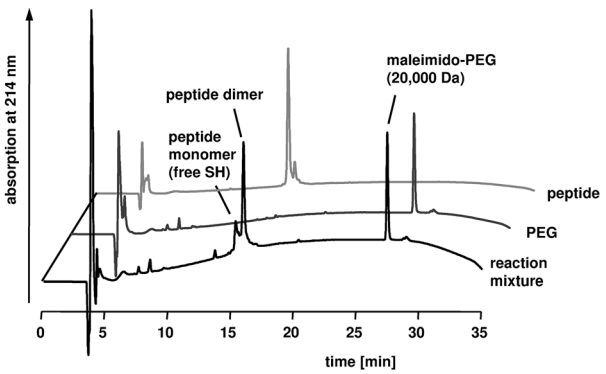

Fig. 2 Chromatographic survey of the PEGylation reaction. HPLC of PEG and the maleimido-peptide *versus* the PEGylation reaction mixture.

Upon conjugation of the peptide the average molecular weight of the PEG-polymer shifted from 22 401 g mol^{-1} to 24 239 g mol^{-1} as determined by MALDI-TOF mass spectrometry. This clear shift revealed that the excess of peptide (5 equivalents) allowed a complete conversion of the PEG derivative into its peptide conjugate as judged by MALDI-TOF mass spectrometry as revealed by a shift of approximately 1.8 kDa corresponding to the DOTA-peptide moiety of the conjugate.

Due to the relatively slight change of the molecular weight, size exclusion chromatography did not reveal a visible change of the retention time of the polymer as shown in Fig. 3. As the mass spectrometry had revealed a complete reaction of the PEG polymer, purification could be accomplished by

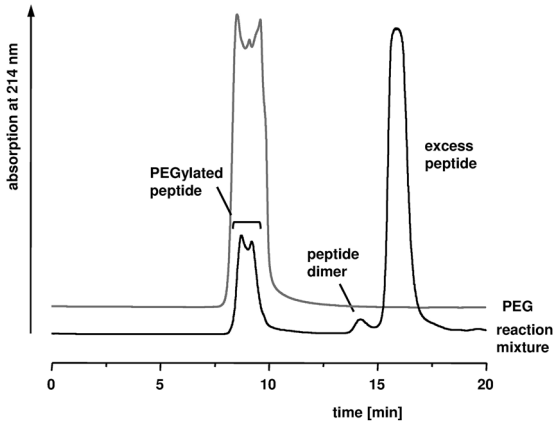

Fig. 3 Chromatographic survey of the PEGylation reaction. Size exclusion chromatography of the 20 kDa PEG-maleimide *versus* the PEGylation reaction mixture obtained by the reaction of 5 equivalents of CH_3O-PEG(20 000)C_2H_4-maleimide with the peptide H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-Cys-NH_2.

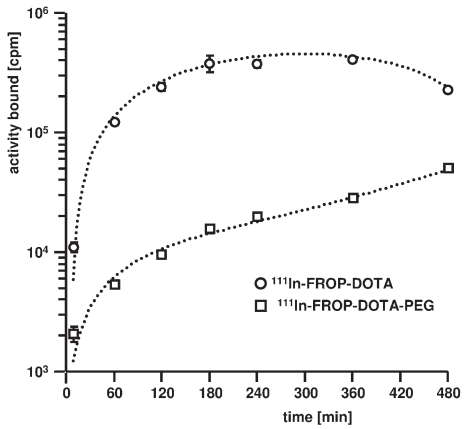

Fig. 4 *In vitro* binding kinetics of the ^{111}In-labeled peptide FROP–DOTA and its PEGylated conjugate FROP–DOTA–PEG.

preparative size exclusion chromatography. The final product was desalted on a Sephadex column equilibrated and eluted with water.

The *in vitro* binding kinetics of the PEGylated conjugate FROP–DOTA–PEG was compared to the kinetics of the original peptide FROP–DOTA. Fig. 4 shows these data obtained in the cell line FRO82-2. Apparently, the PEGylation caused a substantial change in the binding kinetics of the ^{111}In-labeled compound. ^{111}In-FROP–DOTA showed a slow accumulation to reach a relatively high value of almost 400 000 cpm per 10^6 cells and a plateau which was reached at 2 to 3 h exposure, corresponding to 42 percent of the total activity applied. The binding of the PEGylated conjugate ^{111}In-FROP–DOTA–PEG was even slower. However, the accumulation of this compound showed a continuous increase within the period of time examined.

The biodistribution studies of the labeled conjugate in mice bearing human FRO82-2 showed a time dependent increasing uptake of the PEGylated peptide over the first two hours after injection, while activity in the blood and most of the other organs decreased (Table 1). The accumulation in the tumor showed a high retention, *e.g.* at 24 h p.i. 76% of the maximal activity concentration persisted in the tumor. The highest uptake values were determined at 120 min p.i. reaching 2.3%ID/g tumor as compared to 0.06%ID/g observed for the non-PEGylated derivative at 135 min p.i. None of the other organs showed an increase of activity over time. In contrast to FROP–DOTA–PEG, FROP–DOTA showed very fast elimination with most of the activity already out of the circulation at 15 min p.i. Accumulation in the tumor is initially higher for FROP–DOTA than for FROP–DOTA–PEG but decreases rapidly while FROP–DOTA–PEG showed an increase in tumor activity.

In summary, the data reveal the positive effect of PEGylation including a substantially improved stabilization in the circulation which allows stable tumor accumulation. Compilation of these data as tumor to organ ratios allows a clear exposition of this effect. The values presented in Table 2 show that the changes of the tumor to organ ratios over time dramatically increase in all organs in the case of the PEGylated peptide. In the case of the tumor–blood ratio a 33-fold increase is observed when comparing the value determined at 5 min after injection and the value at 24 h post injection. In contrast the relative values observed for the unconjugated peptide are rather stable or even decreasing – apparently the result of the lack of a specific uptake into the tumor. In order to discover novel targeting molecules combinatorial phage display has been extensively used for the identification of protein-ligands.[20,21] Using this technique we had identified the thyroid carcinoma binding peptide FROP-1.[6] Conjugation of the chelator DOTA induced a secondary structure resulting in significantly improved *in vitro* properties; however the peptide retained an unfavorable pharmacokinetics. The discrepancy between the high *in vitro* binding capacity and the lack of tumor uptake was explained by the slow binding kinetics that allowed excretion to forestall an efficient tumor uptake.[7]

Here, we were able to show that PEGylation, an often successfully used method to modulate the pharmacokinetics and immunogenicity of proteins,[22,23] efficiently prolonged the residence time of the peptide in the circulation which reversed its tumor accumulation profile as shown in Fig. 5. The long circulation time of the PEGylated peptide was in contrast to its moderately high molecular weight. It is not easy to extrapolate the kidney excretion limit of PEG by looking only at the kidney clearance threshold of macromolecules which is assumed to be in the range of 50 kDa.[24] However, this default value cannot be assumed for PEG conjugates as the high water coordination causes strongly increased hydrodynamic volume of PEG when compared to that of a globular protein having the same molecular weight.[25] It is unlikely that the increased and persistent accumulation of FROP–DOTA–PEG in the tumor is due to the blood unspecifically found in the highly vascularized tumor.

A comparison between tumor and blood values showed that tumor accumulation increased whereas at the same time point radioactivity in the blood decreased. Also the other organs

Table 1 %ID/g values obtained for the biodistribution in female Balb/c nu/nu mice carrying FRO82-2 tumors after i.v. injection (n = 3)

Time [min]	Blood	Heart	Lung	Spleen	Liver	Kidney	Muscle	Colon	Brain	Tumor
^{111}In-labeled FROP–DOTA–PEG										
5	23.8 ± 8.3	6.5 ± 1.8	13.6 ± 3.4	4.0 ± 1.7	8.0 ± 3.5	23.4 ± 10.3	1.2 ± 0.3	3.0 ± 1.4	0.7 ± 0.2	1.0 ± 0.4
15	23.5 ± 3.1	6.3 ± 0.7	14.5 ± 3.1	4.2 ± 1.1	7.5 ± 2.2	32.5 ± 6.9	1.2 ± 0.2	3.9 ± 0.5	0.7 ± 0.2	1.7 ± 0.2
60	11.8 ± 3.0	3.3 ± 0.7	7.5 ± 2.8	2.0 ± 0.8	3.4 ± 0.8	27.6 ± 10.0	0.9 ± 0.3	3.0 ± 1.1	0.3 ± 0.1	2.1 ± 0.9
120	11.0 ± 1.7	3.7 ± 1.2	5.8 ± 1.4	1.8 ± 0.3	3.0 ± 0.4	27.9 ± 5.5	0.9 ± 0.1	2.2 ± 0.5	0.3 ± 0.0	2.3 ± 0.3
240	8.0 ± 1.1	2.6 ± 0.7	5.3 ± 1.7	1.7 ± 0.4	2.8 ± 0.6	31.1 ± 1.8	0.9 ± 0.1	1.5 ± 0.2	0.3 ± 0.1	2.0 ± 0.5
1440	1.2 ± 0.2	0.6 ± 0.1	1.0 ± 0.4	1.1 ± 0.2	1.6 ± 0.3	23.6 ± 6.4	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	1.7 ± 0.3
^{111}In-labeled FROP–DOTA										
5	3.5 ± 1.8	1.1 ± 0.5	2.4 ± 1.3	0.7 ± 0.4	4.9 ± 2.4	11.4 ± 3.6	0.6 ± 0.3	nd	0.1 ± 0.0	1.6 ± 0.8
15	1.5 ± 0.1	0.5 ± 0.1	1.4 ± 0.4	0.4 ± 0.0	1.7 ± 0.1	5.5 ± 0.7	0.3 ± 0.0	nd	0.1 ± 0.0	1.1 ± 0.2
45	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	3.6 ± 1.1	0.1 ± 0.0	nd	0.0 ± 0.0	0.2 ± 0.0
135	0.2 ± 0.4	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	3.2 ± 1.0	0.0 ± 0.0	nd	0.0 ± 0.0	0.1 ± 0.0

Table 2 Tumor to organ ratios of the organ uptake values: the comparison of the biodistribution values of ^{111}In labeled FROP–DOTA and FROP–DOTA–PEG with respect to their tumor to organ ratios reveals the highly specific tumor accumulation of FROP–DOTA–PEG in the FRO82-2 tumors

Time [min]	tu/blood	tu/heart	tu/lung	tu/spleen	tu/liver	tu/kidney	tu/muscle	tu/brain
^{111}In-labeled FROP–DOTA								
5 min	0.5	1.4	0.7	2.4	0.3	0.1	2.7	15.2
135 min	0.3	1.0	1.1	1.4	0.6	0	2.4	4.7
Factor	0.6	0.7	1.7	0.6	1.7	0.1	0.9	0.3
^{111}In-labeled FROP–DOTA–PEG								
5 min	0.0	0.2	0.1	0.3	0.1	0.0	0.8	1.6
24 h	1.4	3.1	1.8	1.5	1.1	0.1	4.1	21.6
Factor	33.3	19.8	23.5	6	8.4	1.7	4.9	13.9

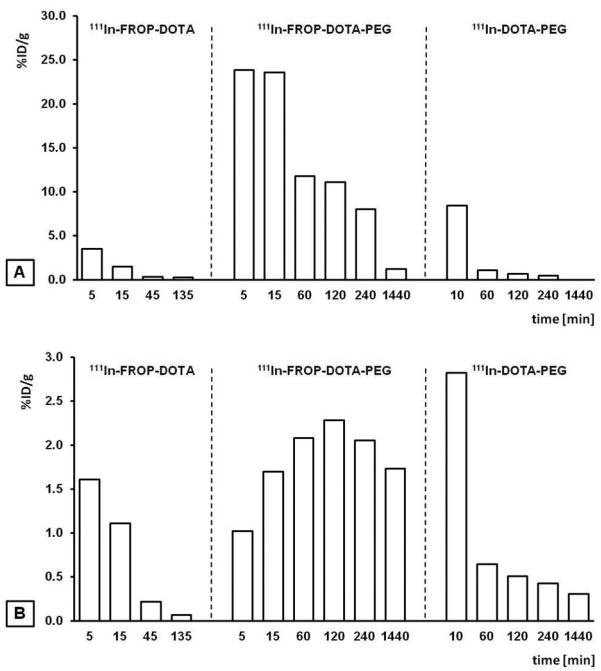

Fig. 5 Kinetics of the blood (A) and the tumor accumulation (B) of ^{111}In-labeled FROP–DOTA–PEG, ^{111}In-labeled FROP–DOTA and ^{111}In-labeled DOTA–PEG.

showed no signs of increased accumulation of FROP–DOTA–PEG over time which rules out unspecific peptide–organ interaction which could lead to increased tumor uptake. Therefore it seems that the slower elimination from the circulation allowing for a longer exposition of the peptide to the tumor cells is the major factor for the increased tumor accumulation. FROP–DOTA–PEG attains a stable accumulation in the tumor tissue, as shown in Fig. 5b. In contrast, ^{111}In-labeled DOTA–PEG shows only a transient uptake. Consequently, the EPR (enhanced permeability and retention) effect may be excluded as the cause for the tumor accumulation. As one of the motivations for the clinical application of peptides is to benefit from their small size, the upsizing of a peptide to achieve improved properties has to be carefully scrutinized. It might be worthwhile to compare this situation with the development process of antibodies, where efforts were made to achieve improved properties. The example of antibodies shows that the relationship between protein size and pharmacological properties should not be overrated. Several antibody fragments, a large variety of miniaturized antibody forms, Fab fragments, scFv, F(ab′)2, diabodies, minibodies, tandem scFv and nanobodies have been developed. Interestingly, despite the numerous efforts of antibody miniaturization the engineered smaller formats with improved biodistribution and blood clearance properties often do not achieve sufficient efficacy and stability *in vivo*. Even formats such as ALX-0081 – a bivalent nanobody suppressing thrombus formation,[26] are far from overrating the natural intact IgG format. Consequently, properly engineered small protein scaffolds might represent the basis for the development of efficient therapeutics.[27] These peptides show the advantages of being accessible by chemical synthesis and to variable modifications.[28] As shown here, these modifications can be essential to achieve the pharmacokinetic profile required.

Conclusion

Phage-display is the most widely practiced display technology for the *de novo* selection of peptides with selective affinity for a specific target. Due to several limitations of the peptides identified such as their relatively low affinity, low stability and inability to cross biological membranes several screening methods such as CIS display,[29] ribosome display[30] and mRNA display[31] and maturation strategies[32] and other alternatives have been developed. While these techniques offer advantages with respect to their screening speed, library size and the display of unnatural amino acids the applications of the resulting peptides are prone to unfavorable pharmacokinetic properties which can only be ruled out by manual processing. Here we were able to show that PEGylation can be applied to successfully modify peptides – an eventual loss of biological activity caused by PEGylation is compensated for by the prolonged body-residence time, as a result of the increased stability and higher hydrodynamic volume. Thanks to these favorable properties, PEGylation might enhance the potential of peptides and proteins identified by screening technologies.

Experimental procedures

Peptide synthesis

H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-Cys-NH_2, (FROP-1 bearing a C-terminal DOTA residue to allow labeling with ^{111}In and a cysteine residue to attach a maleimido-modified PEG oligomer) was obtained by solid phase synthesis using Fmoc-chemistry on a Rink amide resin (Rapp Polymere, Tübingen, Germany). Nα-Fmoc amino acids with the following side chain protecting groups were employed: Asp(tBu), Glu(tBu) and Tyr(tBu). The peptide chain was constructed in an automated peptide synthesizer (ABI 433A) using reaction cycles consisting of deprotection (10 min) with 20% piperidine in NMP and 30 min coupling with 10 equivalents of the HBTU-activated Fmoc-protected amino acid. The Mtt protected lysine residue was deprotected with 1.5% TFA in DCM and conjugated to 4 equivalents of tris-tBuDOTA (CheMatech, Dijon, France) using HATU activation. The subsequent cleavage from the resin was performed with 95 : 2.5 : 2.5 TFA/H_2O/triisopropylsilane for 1 h at room temperature and subsequent precipitation with cold diethyl ether. The precipitate was dried and left to stand for 12 h in neat TFA. Purification was accomplished by reversed-phase HPLC on a Chromolith® SemiPrep 100 × 10 mm column (Merck, Darmstadt, Germany) using a linear gradient from 20% to 70% of acetonitrile in water (both containing 0.1% TFA) over 10 min, the product eluted at 4.3 min. The purified peptide showed a single peak with a retention time of 2.79 min in an analytical HPLC using a linear gradient from 0% to 100% of acetonitrile in water (both containing 0.1% TFA) over 5 min. The mass signal at 1052.5237 *m*/*z* for the $[M + 2H]^{2+}$ peak (calculated: 1052.5025) confirmed the identity on an Exactive® mass spectrometer (Thermo Fisher Scientific), linked to an Agilent 1200 HPLC system.

PEGylation of the peptide

The PEGylation was performed in solution. For this purpose CH_3O-PEG(20 000)C_2H_4-maleimide (Rapp Polymere, Tübingen, Germany) was incubated with 5 equivalents of the peptide H-Glu-Asn-Tyr-Glu-Leu-Met-Asp-Leu-Leu-Ala-Tyr-Leu-Lys(DOTA)-Cys-NH_2. The peptide (6.7 mg = 3.2×10^{-3} mmol, 5 eq.) and 13 mg CH_3O-PEG(20 000)C_2H_4-maleimide (Rapp Polymere, Tübingen, Germany) (0.65×10^{-3} mmol) each dissolved in 200 µl PBS were incubated in 0.1 M phosphate buffer/0.15 M NaCl at pH 7.2 for 20 h at room temperature. The conjugate was purified by size exclusion chromatography on an Agilent 1100 HPLC system equipped with a Superdex 75 column (GE Healthcare, München, Germany) using 0.05 M phosphate buffer/0.15 M NaCl at pH 7 as the eluent. The fractions containing the product were desalted on a Hi Trap Sephadex G-25 superfine desalting column (GE Healthcare, München, Germany) equilibrated with neat H_2O. A total amount of 7.61 mg, corresponding to a yield of 48%, was obtained. The characterization of the conjugate FROP–DOTA–PEG was performed by MALDI mass spectrometry.

Peptide labeling

The radiolabeling was performed by complexation with ^{111}In chloride. In brief 5 µl of a 10^{-3} M solution of FROP–DOTA–PEG or FROP–DOTA was mixed with 50 µl of 0.4 M sodium acetate buffer (pH 5) and 6 to 18 MBq $^{111}InCl_3$ (Tyco Healthcare Neustadt, Germany) were added. The solution was heated at 80 °C for 30 min. A quality control of the radiolabeled peptide performed by HPLC on a monolithic RP HPLC 100 × 4.6 mm column (Merck, Darmstadt, Germany) using 0.1% TFA in water and 0.1% TFA in acetonitrile as eluents (in the case of ^{111}In-FROP–DOTA) or thin layer chromatography on ITLC SG showed yields >95%.

Cell line

The human thyroid follicular carcinoma cell line FRO82-2 (University of California, Los Angeles, Ca, USA) was cultured in RPMI 1640 with Glutamax containing 10% FCS, 0.15% sodium bicarbonate, 1.5 mM sodium pyruvate, 1.5 × non-essential amino acids (all Invitrogen Karlsruhe, Germany) and 25 mM HEPES. The cells were cultivated at 37 °C in a 5% CO_2-incubator.

In vitro binding experiments

400 000 cells (FRO82-2) were seeded into 6-well plates and cultivated for 24 hours. The medium was replaced by 1 ml fresh medium (without FCS). 10 µl ^{111}In-FROP–DOTA or ^{111}In-FROP–DOTA–PEG was added to three wells each and incubated for 10 min, 60 min, 2 h, 3 h, 4 h, 6 and 8 h. The cells were washed three times with 1 ml PBS and subsequently lysed with 0.5 ml of 0.3 M NaOH. Radioactivity was determined with a gamma counter and calculated as percent applied dose per 10^6 cells.

Animals and tumor growth

5×10^6 cells of the thyroid carcinoma cell line FRO 82-2 were suspended in a Matrigel matrix (BD Biosciences, Heidelberg, Germany) and subcutaneously inoculated into the anterior region of the mouse trunk of female 7–8-week-old BALB/c nu/nu mice (Charles River WIGA, Sulzfeld, Germany) and the tumors were allowed to grow for about 10 to 14 days until approximately 1 cm^3 in size. All animals were cared for according to the national animal guidelines.

Organ distribution of ^{111}In-labeled FROP–DOTA and FROP–DOTA–PEG

The radiolabeled peptides (0.5–1 MBq per mouse) were injected *via* the tail vein of the Balb/c nu/nu mice, transplanted with FRO82-2 thyroid tumors. The animals were sacrificed at the indicated times (5, 15, 60, 120, 240 and 1440 min) and selected organs (blood, heart, lung, spleen, liver, kidney, muscle, colon, brain, and tumor) were removed, blotted dry and weighed. The radioactivity was measured with a γ-counter (Cobra II, Canberra Packard, Meriden, USA) along with a sample of the injection solution to calculate the percentage of injected dose per gram of the tissue (%ID/g).

Acknowledgements

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