Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 7594

www.rsc.org/obc

PAPER

Dual-targeting conjugates designed to improve the efficacy of radiolabeled peptides†

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Received 12th June 2012, Accepted 1st August 2012
DOI: 10.1039/c2ob26127h

Radiolabeled regulatory peptides are useful tools in nuclear medicine for the diagnosis (imaging) and therapy of cancer. The specificity of the peptides towards GPC receptors, which are overexpressed by cancer cells, and their favorable pharmacokinetic profile make them ideal vectors to transport conjugated radionuclides to tumors and metastases. However, after internalization of the radiopeptide into cancer cells and tumors, a rapid washout of a substantial fraction of the delivered radioactivity is often observed. This phenomenon may represent a limitation of radiopeptides for clinical applications. Here, we report the synthesis, radiolabeling, stability, and *in vitro* evaluation of a novel, dual-targeting peptide radioconjugate designed to enhance the cellular retention of radioactivity. The described trifunctional conjugate is comprised of a Tc-99m SPECT reporter probe, a cell membrane receptor-specific peptide, and a second targeting entity directed towards mitochondria. While the specificity of the first generation of dual-targeting conjugates towards its extracellular target was demonstrated, intracellular targeting could not be confirmed probably due to non-specific binding or hindered passage through the membrane of the organelle. The work presented describes a novel approach with potential to improve the efficacy of radiopharmaceuticals by enhancing the intracellular retention of radioactivity.

Introduction

Conjugation of radionuclides to tumor-targeting peptides enables their application for the diagnosis (using gamma- or positron-emitting nuclides) and therapy (employing beta- or alpha-particle emitters) of cancer. In particular, radiometal-labeled regulatory peptides, which target G-protein-coupled receptors (GPCRs) overexpressed in different cancers, have been a focus of radiopharmaceutical and nuclear medicinal research over the past decade. As a result, some radiometal-labeled peptides have found routine application in the clinic (*e.g.*, somatostatin derivatives for the management of neuroendocrine tumors), while other promising candidates are currently the subject of preclinical evaluation or have recently advanced to clinical trials.[1–4]

Radiolabeled regulatory peptides internalize into tumor cells by GPCR-mediated endocytosis. A potential drawback of a number of reported radiopeptides is represented by a rapid washout of a substantial fraction of the intracellular radioactivity from cancer cells (*in vitro*) and tumors (*in vivo*). In some instances, the *in vitro* externalization of radioactivity from cancer cells occurs at a rate comparable to the internalization.[5–8] Also, a significant washout of radioactivity from tumors over time is often observed *in vivo*.[6,9–11] This phenomenon can render the initial efforts of delivering specifically radioactive nuclides to cancer cells and tumors in part futile. The mechanism by which the radioactivity is externalized from cancer cells and tumors is yet not fully understood and can vary depending on, *e.g.*, the peptide carrier or the composition of the radioconjugate employed. For example, cellular washout of radioactivity in the form of metabolic degradation products has been reported for radiometal-labeled derivatives of bombesin and minigastrins.[12,13] On the other hand, the externalization of intact radiometal conjugates has been observed for somatostatin and exendin-4 derivatives.[7,9,11] Regardless of the metabolic fate of the tumor-targeting peptide vector, it is generally accepted that the efficacy of radioconjugates correlates with a high rate of internalization into, and a low rate of externalization from targeted tissue.

We herein wish to report our efforts to combine tumor-targeting radiolabeled peptides with a second moiety which targets intracellular components. We hypothesized that such an additional moiety (*e.g.*, directed against intracellular proteins, enzymes, or cell compartments) of the resulting dual-targeting radioconjugates will decrease the externalization rate of radioactivity from cancer cells and tumors independent of the vector used. An increased retention time of the delivered radioactivity within tumors holds the promise to improve the signal-to-background ratio important for diagnostic (imaging) applications and

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c2ob26127h

to enhance the efficacy of therapeutic radionuclides as a result of the increased energy deposited in cancerous tissue.

In recent years, new approaches for the development of radiopharmaceuticals have emerged, which aim at the combination of the tumor-targeting properties of radiolabeled peptides with other entities of interest. Reported examples include the combination of radiopeptides with pharmacological modifiers to improve their pharmacokinetic and/or –dynamic characteristics[14–16] or therapeutic efficacy,[7,17–19] optical dyes for the design of dual-modal imaging probes for high sensitivity and high resolution imaging,[20,21] and therapeutic agents[22] for the development of theranostics.[23] The potential of multifunctional radioconjugates, which exploit the individual properties of components or synergistic effects thereof, respectively, for applications in nuclear medicine is intriguing, however, their synthesis can be challenging.

We have previously reported a modular synthetic approach which employs the Cu(I)-catalyzed alkyne–azide cycloaddition (CuAAC; click chemistry)[24,25] for the efficient preparation of multifunctional radioconjugates.[14,26] In this approach, an *N*(α)-propargyl derivative of the amino acid lysine functions as the center piece of the final conjugate by providing the means for conjugation of two different chemical/biological moieties by selective amide bond formation and CuAAC (Fig. 1). In addition, the *N*(α)-propargyl lysine derivative is a prochelator which forms, after formation of the 1,2,3-triazole by CuAAC, a tridentate ligand system for the stable complexation of the ^{99m}Tc-tricarbonyl core suitable for imaging by single photon emission computed tomography (SPECT).[27,28] The diagnostic isotope technetium-99m forms a "matched pair" with chemically related therapeutic β$^-$-particle emitters rhenium-186/188 and, therefore, the chemistry developed has the potential to be used for the development of radioconjugates for both diagnostic and therapeutic applications in nuclear medicine.[29]

We set out to investigate the use of the "click chemistry" strategy for the assembly of trifunctional conjugates made up of a ^{99m}Tc-tricarbonyl reporter probe for SPECT, a tumor-targeting peptide, and an additional entity specific for an intracellular target, *e.g.*, a cellular compartment. For the first generation of the proposed dual-targeting radioconjugates, we selected a reported, stabilized variation of the minimal binding sequence of bombesin (BBS), [Nle^{14}]BBS(7–14),[12] as an extracellular tumor-targeting component. BBS is a regulatory peptide that targets the gastrin-releasing peptide (GRP) receptor, which is overexpressed by various cancer cell types including prostate, breast, and small-cell lung cancer.[30] The potential of derivatives of [Nle^{14}]-BBS(7–14) radiolabeled with the ^{99m}Tc(CO)$_3$ core for cancer imaging has been demonstrated *in vitro* and *in vivo*.[12,15,28] Based on literature precedence, we introduced a spacer made of multiple β-alanine units to separate the tumor-targeting peptide from other parts of the conjugate in order to minimize potentially unfavorable interferences.[10,12] As an intracellular target, we chose the organelle mitochondria. It has been shown that lipophilic, cationic species such as triphenylphosphonium (TPP) moieties can be driven electrophoretically through negatively charged transmembranes, resulting in an accumulation of the compounds inside the energized mitochondria of cells. TPPs have been used as a drug delivery system for the transport of therapeutic agents to the mitochondria.[31] Also, radioactive labeled TPP derivatives have been studied for myocardial imaging and tumor imaging.[32]

The combination of receptor-targeting radiopeptides with moieties specific for the cell nucleus (*e.g.*, DNA intercalator or cell penetrating peptides) has been reported to exploit potentially the therapeutic effect of short-range Auger electron-emitting radionuclides, however with varying degrees of success.[7,19] The use of mitochondria-specific entities (*e.g.*, TPPs) for improving the intracellular retention of radioactivity after selective delivery by tumor-avid peptides has yet not been described in the context of dual-targeting radioconjugates.

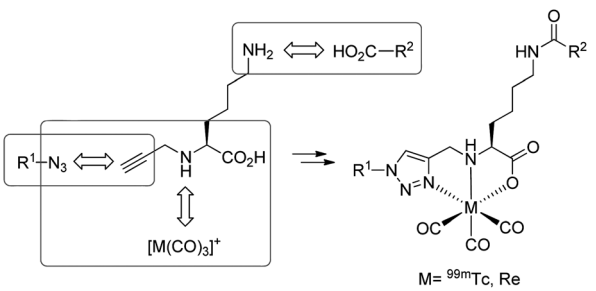
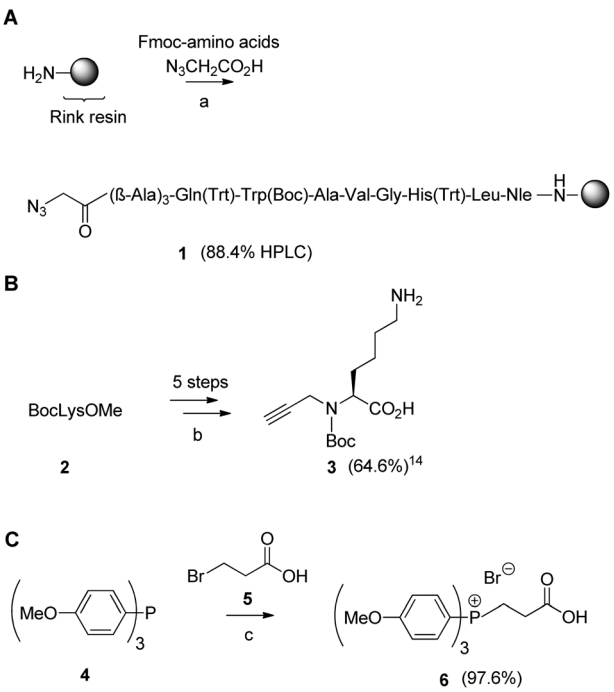


Fig. 1 Assembly of trifunctional conjugates by selective amide bond formation, CuAAC (click chemistry), and (radio)metal complexation; residues R^1 and R^2 represent two different moieties of biological function to be combined in the final (radio)metal conjugate.

Results and discussion

Scheme 1 outlines the synthesis of building blocks required for the assembly of trifunctional conjugates. The preparation of the



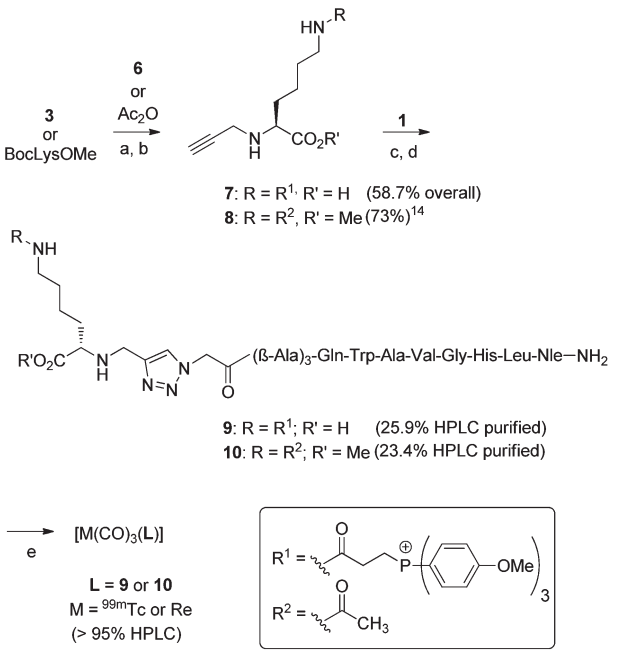
Scheme 1 Synthesis of building blocks. (a) SPPS: (i) piperidine (20%) in DMF; TBTU–HOBt (4 equiv.), (ii) Fmoc-amino acids or azidoacetic acid (3 equiv.), HATU (3 equiv.); (b) see ref. 14; (c) toluene, reflux, 5 h.

tumor targeting peptide [Nle[14]]BBS(7–14), its N-terminal elongation with three β-Ala units, and azidoacetic acid was accomplished by standard solid phase peptide synthesis (SPPS) yielding protected azide-functionalized peptide **1** on the resin (Scheme 1A; see the Experimental section for details). The central piece of the trifunctional conjugate, *N*(α)Boc-*N*(α)propargyl lysine **3**, was prepared from commercial BocLysOMe (**2**) in five steps as previously described (Scheme 1B).[14] An appropriately functionalized mitochondria-targeting entity, 3-tris(*p*-methoxyphenyl)-phosphonium propionic acid **6**, was synthesized by the reaction of tris(*p*-methoxyphenyl)phosphine (**4**) with 3-bromo-propionic acid (**5**; Scheme 1C).[33]

The assembly of the trifunctional conjugate started with the coupling of TPP derivative **6** to the *N*(ε)-amine of the side chain of Lys-derivative **3** followed by TFA-mediated removal of the *N*(α)Boc protective group (Scheme 2). The resulting intermediate **7** was then reacted with protected, azide-functionalized peptide **1** by CuAAC on a solid support. After cleavage from the resin and deprotection, conjugate **9** was purified by preparative HPLC and its structure was confirmed by mass spectrometric analysis (Table 2). To study the effect of the mitochondria-targeting TPP moiety, we also synthesized reference compound **10** from intermediate **8**[14] by the same synthetic pathway (see the Experimental part for details). Compound **10** is identical to conjugate **9** in all respects but lacks the TPP moiety at the *N*(ε)-amine of the Lys residue (replaced by an acetate group).

Both peptide conjugates **9** and **10** were readily radiolabeled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ within 30 min at 100 °C, providing the desired radiopeptides $[^{99m}Tc(CO)_3(L)]$ (L = **9**, **10**) in >95% radiochemical yield and purity. The radioconjugates were obtained with a specific activity of up to 17.4 GBq μmol^{-1} (not optimized). In the case of methyl ester **10**, ester hydrolysis and labeling with ^{99m}Tc-tricarbonyl occurred simultaneously under the reaction conditions applied.[14] For characterization purposes, the corresponding non-radioactive rhenium tricarbonyl complexes $[Re(CO)_3(L)]$ (L = **9**, **10**) were prepared by the reaction of conjugates **9** and **10** with $[Re(CO)_3Br_3][Et_4N]_2$ [34] for 60 min at 100 °C and analyzed by mass spectrometry. The identity of $[^{99m}Tc(CO)_3(L)]$ (L = **9**, **10**) was confirmed in each case by comparison of the γ-HPLC trace with the UV trace of the corresponding rhenium complexes $[Re(CO)_3(L)]$ (L = **9**, **10**), a procedure which is common practice with ^{99m}Tc complexes on an n.c.a. (no carrier added) level (Fig. 2).

The stability of the radiolabeled peptides was evaluated in different media at 37 °C. Both $[^{99m}Tc(CO)_3(L)]$ (L = **9**, **10**) were stable (>95% as determined by HPLC) in PBS (pH 7.4), NaCl (0.9%), and in a cell culture medium (1% FBS) for >4 h, the period of time required for the *in vitro* experiments described below (see the Experimental part for details). We also verified the stability of the Tc-99m complexes in a ligand challenge experiment.[35] Thus, $[^{99m}Tc(CO)_3(L)]$ (L = **9**, **10**) were incubated at 37 °C in PBS containing a 4500-fold excess of histidine, an excellent tridentate chelator for complexation of the Tc-99m tricarbonyl core. No transmetallation was observed over a period of 24 h, therefore demonstrating the suitability of the employed Lys-based chelator unit of the conjugates.



Scheme 2 Assembly of dual targeting radiopeptide conjugates. (a) For **7**: **6**, $SOCl_2$, CH_2Cl_2, rt overnight; then **3** and Et_3N, −78 °C, 4 h; for **8**: see ref. 14; (b) CH_2Cl_2–TFA (3 : 1), rt overnight; (c) DIPEA, $[Cu(CH_3CN)_4]PF_6$, TBTA, DMF, rt overnight; (d) trifluoroacetic acid, phenol, water, triisopropylsilane (87.5/5/5/2.5%), 2 h, rt; (e) for M = Tc-99m: 0.1 mM of **9** or **10** in water, $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (100–250 MBq), 100 °C, 30 min; for Re complexes: 0.4 mM of **9** or **10** in water, $[Re(CO)_3Br_3][Et_4N]_2$ (1.5 equiv.), 100 °C, 60 min.

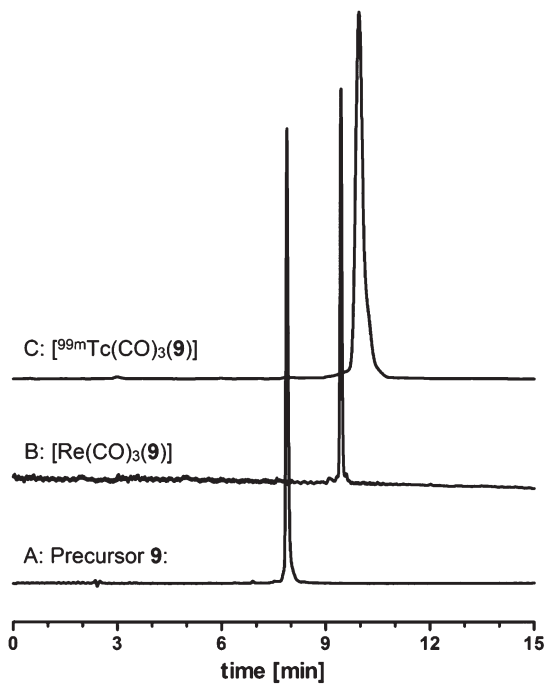


Fig. 2 HPLC chromatographs of peptide precursor **9** (UV-trace A), the corresponding metal conjugates $[Re(CO)_3(\mathbf{9})]$ (UV-trace B), and $[^{99m}Tc(CO)_3(\mathbf{9})]$ (γ-trace C). The small difference of retention times between $[Re(CO)_3(\mathbf{9})]$ and $[^{99m}Tc(CO)_3(\mathbf{9})]$ (traces B and C) is due to the serial arrangement of the UV- and γ-detectors.

We next investigated *in vitro* the receptor affinity as well as internalization and externalization characteristics of the conjugates [^{99m}Tc(CO)$_3$(L)] (L = **9**, **10**) using PC-3 cells (n = 3–4 in triplicate). The binding affinity of the bombesin derivatives towards GRP-receptors was determined by receptor saturation binding experiments employing increasing amounts of the radioconjugates (Fig. 3). Non-specific receptor binding was assessed by using an excess of natural bombesin BBS(1–14). Reference peptide [^{99m}Tc(CO)$_3$(**10**)] displayed a dissociation constant (K_d) of 5.6 ± 0.8 nM. In comparison, dual-targeting [^{99m}Tc(CO)$_3$(**9**)] exhibited a decreased receptor binding affinity with a K_d of 41.6 ± 5.8 nM. Values of dissociation constants in the single- to lower double-digit nanomolar range are usually reported for radiolabeled regulatory peptides.

Despite the differences in receptor binding affinities, we observed that both radioconjugates internalized into PC-3 cells to the same extent and at a comparable rate (approx. 25% of the applied radioactivity within 30–90 min; Fig. 4). These results are comparable with those reported for related BBS derivatives labeled with the ^{99m}Tc-tricarbonyl core.[15] In comparison to reference compound [^{99m}Tc(CO)$_3$(**10**)], [^{99m}Tc(CO)$_3$(**9**)] exhibited some degree of non-specific internalization as determined by blocking experiments using a 1000-fold excess of natural bombesin BBS(1–14). Albeit not ideal, low levels of non-specific binding and internalization have been described for a number of receptor-specific radiotracers, some of which have advanced to the stage of preclinical or clinical investigations nevertheless.[11,36] In the case reported herein, however, the lipophilicity introduced by the TPP moiety and/or the ^{99m}Tc-(CO)$_3$ core could also be in part responsible for the observed non-specific binding and internalization of the trifunctional conjugate.

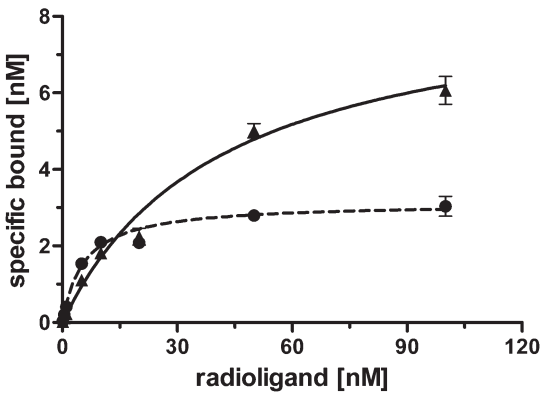


Fig. 3 Receptor saturation binding experiments of radiometal conjugates using GRP-receptor expressing PC-3 cells; normalized to 10^6 cells per well; specific binding of [^{99m}Tc(CO)$_3$(**10**)] (●, dotted line) and [^{99m}Tc(CO)$_3$(**9**)] (▲, continuous line); K_d values were determined by nonlinear regression.

Determination of the log D of peptide precursors **9** and **10**, and the corresponding metal conjugates by HPLC indeed revealed increased lipophilicities for TPP-containing peptide **9** and, more pronounced, its metal-labeled analogues [M(CO)$_3$(L)] (M = ^{99m}Tc, Re; L = **9**) (Table 1, n = 3). While some non-specific binding and internalization of a peptidic radiotracer is not necessarily a criterion for exclusion from further developments, one has to critically acknowledge that an increased lipophilicity can result in an unfavorable biodistribution. On the other hand, it is known that the lipophilicity of a radiopeptide can be adjusted by the attachment of polar groups (*e.g.*, by pegylation or glycosylation), or the use of charged and/or polar linkers.[16,37]

Experiments performed with radiopeptides [^{99m}Tc(CO)$_3$(L)] (L = **9**, **10**) showed, undesirably, a very similar externalization pattern for both conjugates, resulting in a washout of *ca.* 50% of intracellular radioactivity after 90 min, approximately the same time required for specific internalization of the radioconjugates into GRP-receptor expressing PC-3 cells (Fig. 5).

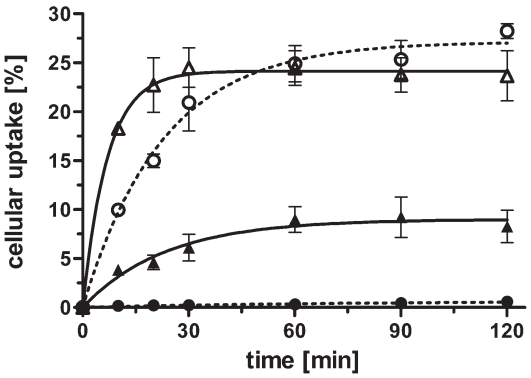


Fig. 4 Internalization of radiometal conjugates in GRP-receptor expressing PC-3 cells; normalized to 10^6 cells per well; total binding of [^{99m}Tc(CO)$_3$(**10**)] (○, dotted line) and [^{99m}Tc(CO)$_3$(**9**)] (Δ, continuous line); non-specific binding as determined by blocking experiments in the presence of excess natural BBS: [^{99m}Tc(CO)$_3$(**10**)] (●, dotted line) and [^{99m}Tc(CO)$_3$(**9**)] (▲, continuous line).

Table 1 Log D of peptides and (radio)metal-labeled analogues determined by HPLC

Compound	Log D
9	1.23 (±0.04)
[Re(CO)$_3$(**9**)]	3.01 (±0.01)
[^{99m}Tc(CO)$_3$(**9**)]	3.11 (±0.02)
10	0.51 (±0.06)
[Re(CO)$_3$(**10**)]	0.80 (±0.07)
[^{99m}Tc(CO)$_3$(**10**)]	0.78 (±0.01)

Table 2 Yields and analytical data of synthesized peptides

Compound	Molecular formula	Yield (isolated)	Calcd M_W (g mol^{-1})	MALDI-MS m/z	Purity (HPLC)
9	$C_{88}H_{123}N_{21}O_{19}P$	25.9%	1808.90	$[M]^+$: 1808.86	98.6%
10	$C_{67}H_{103}N_{21}O_{16}$	23.4%	1457.79	$[M + H]^+$: 1459.2, $[M + 2H]^{2+}$: 730.2	97.6%

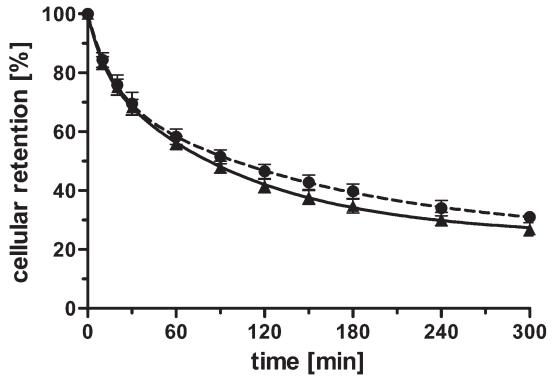


Fig. 5 Externalization of radioactivity from GRP-receptor expressing PC-3 cells. Externalization of the total internalized fraction at $t = 0$ (100%) of radiotracers: [^{99m}Tc(CO)$_3$(**10**)] (●, dotted line) and [^{99m}Tc(CO)$_3$(**9**)] (▲, continuous line).

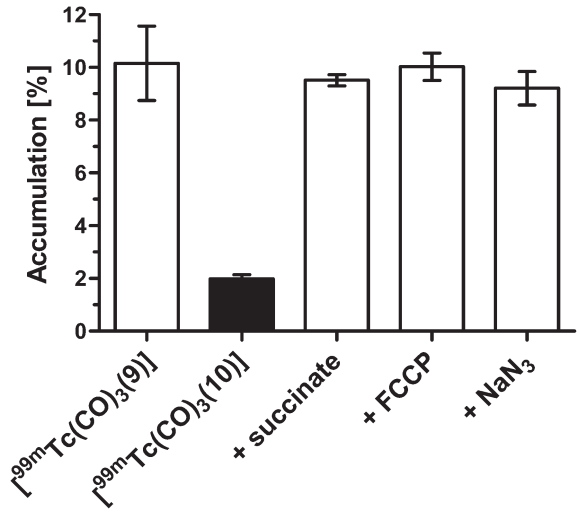


Fig. 6 Accumulation of radiometal conjugates in mitochondria. [^{99m}Tc(CO)$_3$(**9**)] (white bars) and [^{99m}Tc(CO)$_3$(**10**)] (black bar) without additives, and [^{99m}Tc(CO)$_3$(**9**)] in the presence of succinate (10 mM), FCCP (0.5 μM), or sodium azide (20 mM).

Puzzled by the results obtained from these experiments we resorted to investigating in more detail the binding of the radioconjugates to mitochondria. Subcellular fractionation following internalization experiments did not reveal a statistically significant accumulation of the dual-targeting conjugate [^{99m}Tc(CO)$_3$(**9**)] in the target organelle (data not shown). To simplify the experimental set-up, additional tests were conducted with mitochondria isolated from PC-3 cells (see the Experimental part). In brief, [^{99m}Tc(CO)$_3$(L)] (L = **9**, **10**; 11.5 kBq and 15.3 kBq, respectively; 5 pmol of peptide) were incubated with mitochondria (1 μg protein per mL) for 30–120 min (Fig. 6; $n = 4$ for all experiments described henceforth). In these experiments, the multifunctional conjugate [^{99m}Tc(CO)$_3$(**9**)] exhibited a steady and statistically significantly enhanced binding to mitochondria (10.15 ± 1.41%) in comparison to reference compound [^{99m}Tc(CO)$_3$(**10**)] (1.98 ± 0.16%; unpaired t-test; $p < 0.0001$). To the best of our knowledge these data represent the first report of quantification of the binding of a radiotracer to isolated mitochondria.

In a further effort to verify the specificity of [^{99m}Tc(CO)$_3$(**9**)] towards mitochondria, we performed experiments following procedures described by Murphy *et al.* and others,[38–40] in which isolated mitochondria are pretreated with established reagents that either disrupt the transmembrane potential of the organelle (sodium azide (NaN_3) or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)) or enhance it (succinate), respectively. In all cases, the binding of [^{99m}Tc(CO)$_3$(**9**)] to mitochondria was not influenced by the presence of the additive (Fig. 6). It is therefore possible that the binding of [^{99m}Tc(CO)$_3$(**9**)] to mitochondria described above is not driven by the transmembrane potential of the organelle but may be the result of non-specific binding. Alternatively, the ability of TPPs to shuttle conjugated cargo into mitochondria can vary depending on the composition of the conjugate. For instance, examples for successful and unsuccessful mitochondria-targeting of TPP moieties conjugated to (not receptor specific) peptides have both been reported.[38,41] Thus, it is also feasible that the passage of [^{99m}Tc(CO)$_3$(**9**)] through the membrane of mitochondria is hindered and therefore interfered with the intracellular targeting. Distinction of the two possible processes, which could impede the specific accumulation of the dual-targeting radioconjugate in mitochondria, may be challenging and requires further investigations. In the meantime, we are examining a second generation of dual-targeting radioconjugates, which target cytosolic proteins instead of organelles and therefore do not require trafficking through intracellular membranes. By simplifying the intracellular targeting system we expect to gain more insights into the structural requirements of a dual-targeting radioconjugate leading to an improved retention of radioactivity in cancer cells and tumors.

Experimental

CAUTION: ^{99m}Tc is a γ-emitter (140 keV) with a half-life of 6.01 h. All reactions involving ^{99m}Tc were performed in a laboratory approved for the handling of radionuclides and appropriate safety procedures were followed at all times to prevent contamination.

General procedures

Fmoc-amino acids, Rink Amide MBHA LL resin (100–200 mesh), HOBt, HATU, and TBTU were purchased from Merck Biosciences (Nottingham, UK). Bombesin (1–14) trifluoroacetate and BocLysOMe were purchased from Bachem (Bubendorf, Switzerland). Solvents and all other chemicals were purchased from Acros Organics (Geel, Belgium), Merck (Darmstadt, Germany), or Sigma Aldrich (Buchs, Switzerland) and used as supplied unless stated otherwise. Polypropylene syringes for manual peptide couplings, fitted with polypropylene frits and a polypropylene plunger, were obtained from MultiSyntech (Witten, Germany) and Teflon taps from Biotage (Uppsala, Sweden). Na[^{99m}TcO$_4$] was eluted from a Mallinckrodt ^{99}Mo/^{99m}Tc generator (Tyco Healthcare, Petten, The Netherlands) using 0.9% saline. The precursor [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ was prepared by using the IsoLink™ kit (Mallinckrodt-Tyco,

Petten, The Netherlands). Column chromatography was carried out using silica gel C60 (particle size 0.04–0.063 mm) (Sigma) and TLC was performed on precoated silica gel plates (0.25 mm, 60F^{254}, Merck). ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were recorded on a Bruker DPX 400 instrument. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (0.00 ppm) and coupling constants (*J*) in hertz (Hz). Standard abbreviations indicating multiplicity are singlet (s), doublet (d), doublet of doublets (dd), triplet (t), and multiplet (m). Analytical and preparative HPLC were carried out with systems from Bischoff Chromatography, equipped with a λ-1010 UV/Vis and an LB509 radioflow detector (Berthold Technologies), using C18 reversed-phase columns (Macherey Nagel Nucleodur C18 ISIS, 5 µm, 250 × 4.6 mm (column A) or Phenomenex Jupiter 4u Proteo 90 Å, 4 µm, 250 × 4.6 mm (column B) for analysis and Macherey Nagel Nucleodur C18 ISIS, 5 µm, 250 × 16 mm (column C) or Nucleosil 100-5 C18, 5 µm, 250 × 21.0 mm (column D) for purifications). HPLC solvents were 0.1% TFA in H_2O (A) and MeCN (B). Quality control of (radio)metal labeled peptides was performed using column B and a linear gradient from 80% to 30% A in 15 min (flow: 1.5 mL min^{-1}); peptide purification was performed using column C and a linear gradient from 70% to 50% A in 15 min (flow: 8 mL min^{-1}); purification of compound **7** was done using column D and a linear gradient from 55% to 35% A in 20 min (flow: 10 mL min^{-1}). Log *D* measurements were performed using column A and an isocratic gradient 25% phosphate buffer (pH 7.4) and 75% methanol (run time: 15–40 min; flow: 1 mL min^{-1}). LRMS analyses were performed on a 4800 MALDI TOF/TOF™ analyzer (Applied Biosystems) or on an ESI Bruker Esquire 3000 plus. HRMS analyses were performed by LC-ESI on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Quantitative γ-counting was performed on a COBRA II auto-gamma system (Model 5003; Packard Instruments).

Solid phase synthesis

Automated solid-phase peptide synthesis (SPPS; scale: 0.1–0.25 mmol) was run on a Pioneer synthesizer (Applied Biosystems) using standard Fmoc chemistry with TBTU–HOBt as coupling reagents and 20% piperidine in DMF as the deprotection reagent. The elongation was carried out using a 4-fold excess of protected amino acids and coupling reagents. For manual solid phase peptide synthesis, Fmoc-protected amino acids or azidoacetic acid (3 equiv.) were coupled onto the resin (scale: 0.03–0.1 mmol) in a syringe fitted with a polypropylene frit and a Teflon tap in the presence of HATU (3 equiv.) and DIPEA (5 equiv.) in DMF for 2 h. The completion of the reaction was verified by the Kaiser test and repeated if necessary. Elongation yields were determined by the UV-absorption of the fluorenylmethylpiperidine adduct after treatment of the resin with 5 mL of a 20% piperidine–DMF solution (3 × 3 min).

CuAAC on a solid support was carried out under argon in oxygen-free solvents by a modified procedure reported for CuAAC in solution.[42] In brief, the resin (loaded with 0.03 mmol peptide) was swollen several times in degassed DMF (5 mL). The solvent was drained off thoroughly and a solution of lysine derivatives **7** or **8** (0.06 mmol) and DIPEA (0.03 mmol) in degassed DMF (2–3 mL) were added to the resin. To the suspension was added tetrakis(acetonitrile)copper(I) hexafluorophosphate ($[Cu(CH_3CN)_4]PF_6$) (0.015 mmol), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (0.015 mmol) and the mixture was vigorously shaken at rt overnight. The resin was washed with DMF and a solution of diethyldithiocarbamate (0.5% in DMF) to remove the remaining copper species. The completion of the reaction was verified by a colorimetric test for solid-supported azides.[43] The peptides were cleaved from the resin and deprotected using a solution of trifluoroacetic acid, phenol, water and triisopropylsilane (0.5–1 mL; 87.5/5/5/2.5%) as a cleavage cocktail at rt for 2 h. After precipitation in ice-cold diethyl ether (approx. 40 mL), the crude mixture of peptides was recovered by centrifugation and washed twice with cold diethyl ether. The precipitate was dissolved in water (5–10 mg mL^{-1}), purified by preparative HPLC, and lyophilized (Table 2).

In solution synthesis of building blocks and intermediates

Compounds **3** and **8** were prepared as previously described.[14]

Compound 6. The synthesis was accomplished according to a modified procedure reported by Leavens *et al.*[33] In brief, tris(*p*-methoxy)phenylphosphine (501 mg, 1.42 mmol) and 3-bromopropionic acid (363 mg, 2.37 mmol) in toluene (15 mL) were stirred at reflux for 5 h and then cooled to rt. The mixture was centrifuged, the precipitate was washed with toluene (4 × 10 mL), and dried under reduced pressure. The crude product was dissolved in acetonitrile (2 mL) and precipitated at 4 °C by addition of diethyl ether (12 mL). After drying under reduced pressure, product **6** was isolated as a white solid (bromide salt, 702 mg, 98%); purity according to HPLC > 95%; ^{1}H NMR (MeOH-d$_4$): δ = 7.68 (dd, 6H, J_{H-H} = 9.0 Hz, J_{H-P} = 12.1 Hz), 7.27 (dd, 6H, J_{H-H} = 9.0 Hz, J_{H-P} = 2.6 Hz), 3.93 (s, 9H), 3.55–3.46 (m, 2H), 2.74–2.63 (m, 2H) ppm; ^{13}C NMR (MeOH-d$_4$): δ = 173.7, 166.6 (d, J_{C-P} = 3.0 Hz), 136.9 (d, J_{C-P} = 11.6 Hz), 117.3 (d, J_{C-P} = 13.8), 110.3 (d, J_{C-P} = 95.3 Hz), 56.6, 27.6 (d, J_{C-P} = 52.5 Hz), 20.1 (d, J_{C-P} = 57.8 Hz) ppm; ^{31}P NMR (MeOH-d$_4$): δ = 21.81 ppm (s); HRMS (ESI) *m*/*z*: 425.15119 $[M]^+$ (calcd for $C_{24}H_{26}O_5P$: 425.15124).

Compound 7. Compound **6** (bromide salt: 86.6 mg, 0.17 mmol) was dissolved under argon in CH_2Cl_2 (1.5 mL) and $SOCl_2$ (0.4 mL, 5.51 mmol) was added dropwise. The resulting solution was stirred at rt overnight. The excess of $SOCl_2$ was removed with a nitrogen stream and then evaporated to dryness under reduced pressure. The acid chloride of compound **6** was dissolved under argon in CH_2Cl_2 (2 mL) at −78 °C, Et_3N (0.4 mL, 2.87 mmol) and compound **3** (34 mg, 0.12 mmol) were added. The mixture was stirred for 4 h at −78 °C before quenching the reaction by the addition of water (2 mL) followed by warming to rt. The reaction mixture was washed with 0.1 M citric acid and water. The aqueous phases were extracted twice with CH_2Cl_2. The organic phases were combined, dried over Na_2SO_4, and concentrated under reduced pressure. The Boc-protected intermediate was directly deprotected by stirring it in a mixture of CH_2Cl_2–TFA (2 : 1; 6 mL) at rt for 5 h followed by drying under reduced pressure. Purification by preparative HPLC afforded compound **7** as a yellow oil (TFA salt; 50 mg; yield for

3 steps: 59%); purity according to HPLC >95%; ^{1}H-NMR (400 MHz, MeOH-d_4; recorded after completed H/D exchange): δ = 7.62 (dd, 6H, J_{H-H} = 9.0 Hz, J_{H-P} = 12.1 Hz), 7.21 (dd, 6H, J_{H-H} = 9.0 Hz, J_{H-P} = 2.5 Hz), 4.04 (dd, 1H, J_{H-H} = 6.6 Hz, J_{H-H} = 5.1 Hz), 3.99 (dd, 1H, J_{H-H} = 16.7 Hz, J_{H-H} = 2.6 Hz), 3.93 (dd, 1H, J_{H-H} = 16.7 Hz, J_{H-H} = 2.6 Hz), 3.88 (s, 9H), 3.49–3.38 (m, 2H), 3.16 (t, 1H, J_{H-H} = 2.6 Hz), 3.06 (t, 2H, J_{H-H} = 6.6 Hz), 2.60–2.50 (m, 2H), 2.00–1.83 (m, 2H), 1.50–1.23 (m, 4H) ppm; ^{13}C NMR (MeOH-d_4): δ = 171.7 (d, J_{C-P} = 13.6 Hz), 171.0, 166.6 (d, J_{C-P} = 3.0 Hz), 136.9 (d, J_{C-P} = 11.6 Hz), 117.3 (d, J_{C-P} = 13.8 Hz), 110.3 (d, J_{C-P} = 95.0 Hz), 79.7, 74.4, 60.1, 56.6, 55.0, 40.3, 36.5, 30.0 (d, J_{C-P} = 7.2 Hz), 29.2 (d, J_{C-P} = 2.8 Hz), 23.2, 20.2 (d, J_{C-P} = 57.8 Hz) ppm; ^{31}P NMR (MeOH-d_4): δ = 21.92 ppm (s); HRMS (ESI) m/z: 591.26208 $[M]^+$ (calcd for $C_{33}H_{40}O_6N_2P$: 591.26185).

(Radio)metal labeling of peptides

Na[$^{99m}TcO_4$] was eluted from a Mallinckrodt ^{99}Mo/^{99m}Tc generator (Tyco Healthcare, Petten, The Netherlands) using 0.9% NaCl. The precursor $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared according to published procedures.[44] In brief, $[^{99m}TcO_4]^-$ (1 mL, 1.5–2 GBq) in 0.9% NaCl was added to the IsoLink™ kit (Mallinckrodt-Tyco, Petten, The Netherlands) and heated for 20 min at 100 °C. After the solution was cooled to rt, the pH was adjusted to pH 7 with a 1 : 2 mixture of 1 M phosphate buffer (pH 7.2) and 1 M HCl (final pH 1.4). Aliquots of 1 mM stock solutions of the peptides **9** or **10** in water (10–20 μL; 10–20 nmol) were added to a solution of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (90–180 μL; 100–250 MBq) to reach a final peptide concentration of 0.1 mM. The reaction mixture was heated at 100 °C for 30 min and the radiochemical yield and purity of [^{99m}Tc-$(CO)_3$(L)] (L = **9**, **10**) was determined by HPLC.

Rhenium complexes were prepared by mixing aqueous stock solutions of peptides **9** or **10** (100 μL, 1 mM) with $[Re(CO)_3Br_3]$-$[N(Et)_4]_2$ [34] (150 μL, 1 mM) and heating at 100 °C for 60 min. The products were purified by HPLC and analyzed by MS. [Re$(CO)_3$(**9**)]: quantitative conversion of peptide **9** (HPLC); purity >99%; MS (MALDI TOF) m/z: 2078.8 $[M]^+$ (calcd for $C_{91}H_{122}N_{21}O_{22}PRe$: 2078.84). [Re$(CO)_3$(**10**)]: quantitative conversion of peptide **10** (HPLC); purity >99%; MS (MALDI TOF) m/z: 1714.7 $[M + H]^+$ (calcd for $C_{69}H_{100}N_{21}O_{19}Re$: 1713.71).

Stability studies

The radiolabeled peptides [^{99m}Tc$(CO)_3$(L)] (L = **9**, **10**) (100 μL; 100 pmol, 0.6 MBq) were incubated at 37 °C with 900 μL PBS (pH 7.4), NaCl (0.9%), or a cell culture medium (1% FBS) respectively. At different time points (1, 2, and 4 h), aliquots were analyzed by HPLC (n = 3). In the case of the cell culture medium, serum proteins were precipitated prior to HPLC analysis by the addition of aliquots to ethanol followed by centrifugation (2500g, 5 min).

Histidine challenge. Compounds [^{99m}Tc$(CO)_3$(L)] (L = **9**, **10**) (100 μL; 200 pmol, 1.1 MBq) were incubated at 37 °C with 900 μL of a solution of histidine (1 mM in PBS; 4500-fold excess). Aliquots were analyzed by HPLC after 1, 4, and 24 h (n = 3).

Log *D* determination

Determination of log D values of radiotracers is usually accomplished by partitioning between n-octanol and PBS (pH 7.4) using the "shake flask method". However, the OECD guidelines for testing of chemicals (Nr. 117) recommend an HPLC method for the determination of log D values in the range of log D 0–6.[45] Applying this method also allows the evaluation of radioactive and non-radioactive species, *e.g.*, peptide precursors, and their radioactive and non-radioactive metal-labeled analogues. In brief, log D values of analytes were determined by correlating their retention time with those of standard compounds of known log D values using a calibration curve with at least six reference compounds. All measurements were done with a Bischoff HPLC system, column A and an isocratic mobile phase composed of methanol (75%) and phosphate buffer (pH 7.4; 25%) at a flow rate of 1 mL min^{-1}. Standard compounds (L-sodium ascorbate (t_0), aniline (log D = 0.9), benzaldehyde (log D = 1.5), anisole (log D = 2.1), bromobenzene (log D = 3.0), naphthalene (log D = 3.6), and dibenzyl (log D = 4.8); 0.1% in methanol)[45] and testing compounds were injected three times and analyzed either by UV or gamma detection. The differences in retention times (approx. 0.3 min) of radioactive analytes (^{99m}Tc-complexes) and the corresponding non-radioactive Re-compounds as a result of the serial arrangement of the UV/Vis and γ-detectors were corrected by analysis of co-injections. The calibration curve was calculated by linear regression analysis using GraphPad Prism 5.0 (see ESI†).

In vitro experiments

Cell culturing. Human Caucasian prostate adenocarcinoma (PC-3) cells were obtained from HPA Culture Collections (Salisbury, UK) and cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, high glucose) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS superior, OXOID, Pratteln, Switzerland), L-glutamine (200 mM), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All culture reagents, except FBS, were purchased from BioConcept (Allschwil, Switzerland). The cells were subcultured weekly after detaching them with a commercial solution of trypsin–EDTA (1 : 250) in PBS.

Saturation binding assay. On the day prior to the experiment PC-3 cells (10^6 cells per well) were plated in a six-well plate and incubated at 37 °C and 5% CO_2 overnight, allowing them to attach. The next day, the cell-culture medium (1% FBS) was changed (0.8 mL) and the cells were kept in the fridge for 30 min to stop cell activity prior to the start of the experiment. In order to reach receptor saturation, the cells were incubated with increasing concentrations (0.1, 0.5, 1, 5, 10, 20, 50, and 100 nM per well; each 100 μL) of the radiolabeled peptides [^{99m}Tc-$(CO)_3$(L)] (L = **9**, **10**). Non-specific receptor binding was determined using an excess of natural bombesin (2.5 μM per well for concentrations of the radiopeptide <10 nM, and 10 μM per well for higher concentrations). After incubation at 4 °C for 2 h, the supernatant was removed and the cells were washed twice with 1 mL of ice-cold PBS (pH 7.4). The combined supernatants represent the free, unbound radiopeptide fraction. To determine the

receptor bound fraction, the cells were lysed with 1 M NaOH (1 mL) for 10 min at 37 °C and the wells were washed twice with 1 M NaOH (1 mL). The free and receptor bound fractions were measured in a gamma counter for quantification. Dissociation constants (K_d) were calculated from the data for specific binding with nonlinear regression using GraphPad Prism5, normalized to 10^6 cells ($n = 3$ in triplicate).

Internalization studies. On the day prior to the experiment, PC-3 cells (10^6 cells per well) were placed in six-well plates with a cell culture medium (1% FBS) and incubated at 37 °C, 5% CO_2 overnight to let them attach. On the day of the experiment, the medium was removed, and cells were incubated with fresh medium (1.3 mL) for 1 h. Radiolabeled peptides [^{99m}Tc-(CO)$_3$(L)] (L = **9**, **10**; per well: 100 μL; 0.25 pmol; 1.5 kBq) were added and the cells were incubated for different time points (10, 20, 30, 60, 90, and 120 min) in triplicate to allow binding and internalization. Non-specific receptor binding and internalization was determined in the presence of a 1000-fold excess of natural bombesin (250 pmol; 100 μL per well) as the receptor blocking agent. After each time point, the supernatant was collected, and the cells were washed twice with PBS (1 mL; pH 7.4). The combined supernatants represent the free, unbound radiopeptide fraction. Receptor-bound radioactivity was isolated by incubating the cells twice for 5 min with an acidic glycine solution (100 mM NaCl, 50 mM glycine, pH 2.8; 1 mL) on ice followed by removal of the supernatant. The internalized fraction was determined by cell lysis with 1 M NaOH (1 mL; 10 min; 37 °C, 5% CO_2) and the wells containing the cell lysate were washed twice with NaOH (1 M, 1 mL). All fractions were measured radiometrically in a gamma counter and calculated as percentage of applied dose normalized to 10^6 cells ($n = 3$ in triplicate).

Externalization studies. PC-3 cells (10^6 cells per well) were placed in six-well plates with a cell culture medium (1% FBS) and incubated at 37 °C and 5% CO_2 overnight to let them attach. On the day of the experiment, the medium was removed and cells were incubated with fresh medium (1.3 mL) for 1 h. Radiolabeled peptides [^{99m}Tc(CO)$_3$(L)] (L = **9**, **10**; per well: 100 μL; 0.25 pmol; 1.3 kBq or 2.5 pmol; 13.7 kBq) were added and the cells incubated for 1 h in triplicate to allow internalization into the cells. Non-specific receptor binding and internalization was determined in the presence of a 1000-fold excess of natural bombesin (250 pmol or 2500 pmol; 100 μL per well) as the receptor blocking agent. After 1 h, the supernatant was collected (free fraction) and receptor-bound radiopeptides were removed by washing the cells two times for 5 min with an acidic glycine buffer on ice (bound fraction). A fresh cell culture medium (1 mL) was added and the cells were incubated for 10, 20, 30, 60, 90, 120, 150, 180, 240, and 300 min. At each time-point, the supernatant was collected and cells were washed twice (1 mL PBS; pH 7.4). These fractions represent the externalized amount of radioactivity. The remaining internalized amount of radioactivity was recovered by cell lysis as described above. All fractions were measured in a gamma counter for quantification calculated as percentage of the total internalized fraction at $t = 0$ min ($n = 3$ in triplicate).

Mitochondria-binding experiments. Mitochondria were isolated from cultured PC-3 cells using the Thermo SCIENTIFIC mitochondria isolation kit for cultured cells according to the procedure described in the operating manual. Isolated mitochondria obtained by this procedure maintain their integrity.[46,47] Protein concentration was measured by the Bradford method using the Bio-Rad protein assay and BSA as a standard. Mitochondria binding experiments were performed following procedures published by Murphy *et al.*[38,39] In brief, isolated mitochondria were resuspended in KCl medium (120 mM KCl, 10 mM Hepes (pH 7.2), and 1 mM EGTA). The mitochondria (0.9 mL, 1 μg protein per mL) were incubated with 100 μL of each radiolabeled peptide [^{99m}Tc(CO)$_3$(L)] (L = **9**, **10**; 5 pmol; 11.5 and 15.3 kBq, respectively) at rt on a shaking plate for 30 min. To investigate the specificity of [^{99m}Tc(CO)$_3$(**9**)], mitochondria were preincubated with sodium succinate (10 mM), FCCP (0.5 μM) or sodium azide (20 mM)[40] respectively for 10–30 min prior to the addition of the radiolabeled conjugate. After the experiments, the mitochondria were pelleted (centrifugation at 12 000*g* for 5 min) and the supernatant was removed. The pellet was washed with KCl medium (1 mL), the supernatants combined and measured in a gamma counter; this fraction represents unbound radioactivity. The mitochondria precipitate was resuspended in 1 mL of triton X-100 (10% v/v) and measured in a gamma counter to quantify the amount of the radioactive peptide associated with the mitochondria relative to total added activity ($n = 4$ for all experiments).

Conclusion

In summary, we report the synthesis and *in vitro* evaluation of novel dual-targeting radiopeptide conjugates with the goal of improving the cellular retention of radioactivity in cancer cells after specific delivery. Starting from readily accessible building blocks, a tumor-targeting peptide ([Nle^{14}]BBS(7–14)), a ^{99m}Tc-based SPECT reporter probe ([^{99m}Tc(CO)$_3$]$^+$), and a mitochondria-seeking triphenylphosphonium (TPP) moiety were selectively and efficiently assembled into a trifunctional conjugate. With the exception of the radiometal labeling step, the assembly of the described multifunctional conjugates was accomplished conveniently on a solid support. While the first generation conjugate bound and internalized specifically into GRP receptor expressing PC-3 cells, it did not display specific accumulation in its intracellular target, the mitochondria, resulting in an overall unaltered externalization rate of radioactivity from PC-3 cells. Nevertheless, the combination of two different entities specific for an extra- and an intracellular target in the same radioconjugate is an attractive approach to improve the efficacy of radiotracers. We are currently employing the same modular synthetic strategy for the development of a simplified, second generation of dual-targeting radiopeptide conjugates in an effort to identify the features required for improving the retention time of radioactivity in cancer cells and tumors.

Acknowledgements

We thank Covidien for providing Isolink™ kits, and Kayhan Akyel, Dieter Staab, Christian Guenat and Ingo Muckenschnabel

(Novartis, Basel, Switzerland) for assistance with NMR and MS analysis.

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