Radiofolates and Radiopeptides: Exploration of Radionuclide Therapies towards Reduced Renal Side Effects

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Radiofolates and Radiopeptides: 
Exploration of Radionuclide Therapies towards Reduced Renal Side Effects

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. Sc. ETH Zurich)

presented by

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2015
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A safe and effective radionuclide tumor therapy requires a high tumor uptake of the targeting agent and at the same time the dose burden to non-targeted tissues should be low. The research activities of our laboratory focus on the development of radiolabeled small molecules. Challenging for a therapy with these radioconjugates is their renal excretion as this comprises a risk for radionephrotoxicity. The first goal of the thesis was to study the renal side effects in mice after therapy with $^{177}$Lu-labeled folic acid conjugates. For this purpose, the two radiofolates, $^{177}$Lu-cm09 and $^{177}$Lu-EC0800, were used. Both compounds target the folate receptor (FR), which is overexpressed in various tumor types but present in only few healthy tissues, including the kidneys. The albumin-binding radiofolate $^{177}$Lu-cm09 was recently shown to have a 10-fold improved tumor-to-kidney ratio and an enhanced anti-tumor effect compared to $^{177}$Lu-EC0800 which does not bind to albumin. We investigated whether $^{177}$Lu-cm09 is also favorable to reduce renal toxicity. For this purpose, the kidney function of mice applied with various doses of $^{177}$Lu-cm09 and $^{177}$Lu-EC0800 was monitored over eight months. The kidney function was checked by measuring the blood plasma parameters blood urea nitrogen and creatinine. Moreover, the renal function was investigated by determining the renal uptake of $^{99m}$Tc-DMSA non-invasively by single photon emission computed tomography (SPECT). First signs of renal impairment were observed 3-4 months after radiofolate injection as indicated by reduced renal uptake of $^{99m}$Tc-DMSA. Blood urea nitrogen and creatinine levels in the blood plasma increased time- and dose-dependently. Kidney damage was confirmed by reduced kidney mass and morphological changes. Microscopic evaluation of stained kidney sections revealed, amongst others, the presence of collapsed glomeruli and tubular atrophy. Our data showed a threshold dose of $\sim$23 Gy (10 MBq of $^{177}$Lu-cm09) to prevent renal toxicity after therapy with $^{177}$Lu-radiofolates. Comparison of $^{177}$Lu-cm09 and $^{177}$Lu-EC0800 showed that binding of the radiofolate to albumin improved the anti-tumor effect and reduced the renal dose burden. However, tumor doses of $\sim$28 Gy (injection of 20 MBq of $^{177}$Lu-cm09) which resulted in complete tumor remission were nephrotoxic. To further improve this therapy strategy, the following approaches were studied: i) co-administration of potential radioprotectors, ii) application of short-ranging high energetic particle emitters and iii) increase of radiofolate’s tumor uptake by up-regulating FR expression.

Amifostine and captopril are known radioprotectors used during external beam irradiation. Herein, we investigated whether these two compounds protect the kidneys from radiation damage.
damage during radiofolate therapy. The renoprotective effects of amifostine were investigated in mice over eight months. This study demonstrated that the co-treatment of $^{177}$Lu-cm09 (20 MBq, renal dose of ~46 Gy) with amifostine (2.5 mg; 4 h, 24 h and 48 h p.i. of $^{177}$Lu-cm09) did not protect mouse kidneys from radiation damage. We unexpectedly found in SPECT studies that co-application of captopril improved the tumor-to-kidney ratio of albumin-binding radiofolates by a factor of ~2 in some mice while it had no effect in others. Because of the observed high interindividual variability this effect was not further investigated in long-term studies.

In a next study, it was investigated whether the renal toxicity is different after applying $\alpha$-therapy ($^{149}$Tb-cm09) than after performing $\beta$-therapy ($^{161}$Tb-cm09). In previous studies it was demonstrated that the application of 3 MBq of $^{149}$Tb-cm09 (tumor dose of ~1.6 Gy) and 10 MBq of $^{161}$Tb-cm09 (tumor dose of ~22 Gy) resulted in comparable tumor growth inhibition. Hence, we compared in this work the kidney function of mice applied with 2.5 MBq or 5 MBq of $^{149}$Tb-cm09 or 10 MBq of $^{161}$Tb-cm09 over eight months. Mice treated with $^{149}$Tb-cm09 (kidney dose of ~1.7 Gy and ~3.3 Gy, respectively) did not show increased blood urea nitrogen and creatinine levels. Moreover, renal $^{99m}$Tc-DMSA uptake was unchanged compared to control mice at the end of the study. In mice injected with 10 MBq of $^{161}$Tb-cm09 (kidney dose of ~30 Gy) $^{99m}$Tc-DMSA-based investigations revealed slightly impaired kidney function. However, morphological studies showed pathological changes of the kidneys after therapy with both, $^{149}$Tb-cm09 and $^{161}$Tb-cm09. Based on these preliminary data, final conclusions about the severity of renal side effects caused by $\alpha$-therapy in comparison to $\beta$-therapy are not yet possible. More extended studies with larger cohorts might reveal the differences between the damage caused by these radiation types.

The third approach aimed to increase the tumor uptake of radiofolates by up-regulating FR expression at the tumor site. The idea was to reduce the injected amount of radioactivity without changing the anti-tumor effect and, hence, to reduce the renal dose burden. In the literature it is known that glucocorticoids (GCs) stimulate FR expression in tumor cells. Therefore, we studied the impact of GCs on the cellular uptake of radiofolates and their inhibitory effects on tumor cell viability. In vitro studies performed with FR-positive KB and IGROV-1 cells (human cervical and ovarian cancer cells, respectively) showed that the increased FR expression levels did not enhance cellular uptake of radiofolates and, hence, the anti-cancer effect was not improved. In contrast, it was found that KB cells co-exposed to GCs were ~5-fold less sensitive towards ionizing radiation than KB cells incubated with the radiofolate only. Such radioresistant effects were, however, not observed in IGROV-1 cells. Further studies revealed that radioresistance development was also evident in AR42J cells (rat pancreatic cancer cells) co-exposed to radiolabeled somatostatin analogs and GCs. In PC-3 cells (human prostate cancer cells) GCs did not influence the effect of radiolabeled
bombesin analogs. Hence, it was demonstrated that development of radioresistance upon GC exposure was cell type specific but independent on the targeting agent.

In conclusion, kidneys are the dose-limiting organs during tumor therapy with radiolabeled small molecules. Our studies showed time- and dose-dependent nephrotoxicity in mice which received a renal dose of more than \(~23\) Gy during radiofolate therapy. In this work, it was proved that enhancing the blood circulation time of radiofolates by binding to albumin was favorable regarding the risk-benefit profile of a therapeutic application. Improvement of radiofolate therapy might be the co-application of antihypertensive drugs such as captopril, as this would potentially improve the tumor-to-kidney ratio of radiofolates.

\(^{161}\)Tb is a promising radionuclide because of the emission of Auer/conversion electrons in addition to the \(\beta^+\)-particle emission. It was previously demonstrated by our group that the application of 10 MBq of \(^ {161}\)Tb-radiofolate resulted in a slightly improved anti-tumor effect compared to the treatment with 10 MBq of \(^ {177}\)Lu-radiofolate. The renal functional impairment after treatment with 10 MBq of \(^ {161}\)Tb-radiofolate and 10 MBq of \(^ {177}\)Lu-radiofolate was, however, comparable. In a side project, we therefore investigated potential benefits of Auger/conversion electron emission during radionuclide therapy. For this purpose, DOTA-[Tyr\(^3\)]-octreotide (DOTATOC) targeting the somatostatin receptors (SSTRs) and the model compound MG136 were used. MG136 is a DOTATOC analog with a nuclear localization signal for deposition of the radioconjugate in the cell nucleus. In vitro studies with AR42J cells (SSTR-positive) demonstrated that the cell viability was \(~7.5\)-fold more effectively reduced when \(^ {161}\)Tb-MG136 was applied compared to \(^ {161}\)Tb-DOTATOC at the same activity concentration. The same trend but a less significant difference in cell viability inhibition was observed for \(^ {177}\)Lu-MG136 and \(^ {177}\)Lu-DOTATOC. This data imply that nuclear deposition of radioactivity might be generally favorable, but in particular if Auger/conversion electron emitters are being used for radionuclide therapy.


In einer weiteren Studie wurde untersucht, ob die renale Toxizität nach α-Therapie ($^{149}$Tb-cm09) und β-Therapie ($^{161}$Tb-cm09) unterschiedlich ausgeprägt ist. In früheren Studien wurde gezeigt, dass die Applikation von 3 MBq $^{149}$Tb-cm09 (Tumordosis von ~1.6 Gy) und 10 MBq $^{161}$Tb-cm09 (Tumordosis von ~22 Gy) zu einer vergleichbaren Hemmung des Tumorwachstums führen. Deshalb injizierten wir Mäuse mit 2.5 MBq oder 5 MBq $^{149}$Tb-cm09 oder 10 MBq $^{161}$Tb-cm09 und verglichen die Nierenfunktion während acht Monaten. Mäuse, welche mit $^{149}$Tb-cm09 (renale Strahlendosis von ~1.7 Gy und ~3.3 Gy) behandelt wurden, wiesen keine erhöhten Werte von Blut-Harnstoff-Stickstoff und Kreatinin auf. Ausserdem war die renale Aufnahme von $^{99m}$Tc-DMSA bis zum Studiende unverändert im Vergleich zu Kontrollmäusen. In Mäusen, in welche 10 MBq $^{161}$Tb-cm09 (renale Strahlendosis von ~30 Gy) injiziert wurde, ergaben die $^{99m}$Tc-DMSA-basierten Funktionsstudien eine leicht eingeschränkte Nierenfunktion. Morphologische Untersuchungen deckten jedoch pathologische Veränderungen der Nieren nach Therapie mit $^{149}$Tb-cm09 und $^{161}$Tb-cm09 auf. Aufgrund dieser Vorstudie können noch keine finalen Aussagen über den Vergleich des Schweregrads der Nierenschädigung nach α- und β-Therapie gemacht werden. Studien mit größeren Kohorten könnten jedoch mögliche Unterschiede der Nierenschädigung nach Anwendung dieser beiden Strahlungsarten zeigen.

In einer weiteren Studie war das Ziel, die Tumoraufnahme von Radiofolaten zu steigern, indem die FR-Expression im Tumor erhöht wird. Die Idee hierbei war, die applizierte Menge an Radioaktivität zu senken und somit die Strahlenbelastung der Nieren zu minimieren, ohne


Aktivitätskonzentrationen verwendet wurden. Eine vergleichbare Tendenz, jedoch ein
geringerer Unterschied in der Effektivität der Zellwachstumshemmung, wurde auch für $^{177}$Lu-
MG136 und $^{177}$Lu-DOTATOC beobachtet. Diese Daten wiesen darauf hin, dass eine
Anreicherung von Radioaktivität im Zellkern während einer Radionuklidtherapie allgemein
von Vorteil sein könnte. Vor allem aber wird eine Anreicherung von Radioaktivität im Zellkern
bei der Anwendung von Auger-/Konversionselektronen als vorteilhaft erachtet.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>$\bar{A}(r_s)$</td>
<td>time-integrated activity in a source tissue</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BFC</td>
<td>bifunctional chelator</td>
</tr>
<tr>
<td>cm09/cm10</td>
<td>albumin-binding DOTA-folic acid conjugates</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DMSA</td>
<td>dimercaptosuccinic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DOTATATE</td>
<td>DOTA-[Tyr$^3$]-octreotate</td>
</tr>
<tr>
<td>DOTATOC</td>
<td>DOTA-[Tyr$^3$]-octreotide</td>
</tr>
<tr>
<td>D$_{(r_t)}$</td>
<td>mean absorbed dose in a target tissue</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EC0800</td>
<td>DOTA-folic acid conjugate without albumin-binding moiety</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FFRPMI</td>
<td>folate-free RPMI cell culture medium</td>
</tr>
<tr>
<td>FR</td>
<td>folate receptor</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GCR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>gastrin-releasing peptide</td>
</tr>
<tr>
<td>Gy</td>
<td>gray</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HE</td>
<td>hematoxylin-eosin</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IA</td>
<td>injected activity</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>ITG</td>
<td>Isotope Technologies Garching</td>
</tr>
<tr>
<td>LET</td>
<td>linear energy transfer</td>
</tr>
</tbody>
</table>
CHAPTER 1

General Introduction
1.1. Targeted radiopharmaceuticals for cancer diagnosis and therapy

Targeted radiopharmaceuticals are radiolabeled molecules consisting of a specific targeting ligand to which an appropriate radionuclide is linked (Fig. 1.1) [1]. The targeting ligand can be for instance a peptide, a small molecule, an antibody or an antibody fragment. For conjugation of radiometals a bifunctional chelator (BFC) has to be linked to the molecules. In contrast, non-metal radionuclides are directly integrated into the radiopharmaceutical without a chelating system. The pharmacokinetic of the radiopharmaceutical is mainly dependent on the properties of the targeting ligand. In addition, the linker and chelating system might also have an impact on the pharmacokinetic properties of the radioconjugates. Hence, to obtain a favorable tumor-to-background ratio, which is crucial for cancer diagnosis and therapy with targeted radiopharmaceuticals, these components should be accordingly modified. Important in the process of optimizing tissue distribution is to maintain binding affinity and target specificity.

![Fig. 1.1. Modular design of a targeted radiopharmaceutical consisting of a targeting ligand, a linker and a bifunctional chelator coordinating radiometals.](image-url)

Nuclear imaging can be used as a non-invasive modality for the diagnosis and monitoring of cancer [2]. The selection of radionuclides is based on the used imaging method which can be either single photon emission computed tomography (SPECT) or positron emission tomography (PET). Emitted γ-rays can be detected by SPECT cameras. Gamma-rays are photons which can penetrate the tissue deeply (Fig. 1.2). For SPECT imaging purposes γ-ray energies of 100-250 keV are ideal [2]. $^{99m}$Tc, $^{67}$Ga, $^{111}$In or $^{123}$I are radionuclides used for clinical SPECT imaging. In the case of PET imaging using $\beta^+$-emitting radionuclides such as $^{18}$F, $^{68}$Ga or $^{64}$Cu, annihilation of $\beta^+$ with an electron results in the emission of a pair of γ-rays
(each with an energy of 511 keV) which travels in opposite directions (180°) and which are simultaneously registered by the detectors of the PET camera [3, 4].

```
\begin{itemize}
  \item \textbf{Diagnosis} \\( \lambda \) (photon), \( \beta^- \) (annihilation: \( \lambda \)-ray)
  \item \textbf{Therapy} \( \beta^- \) (electron) \hspace{1cm} \text{LET} \hspace{1cm} \text{Range}
  \item \( \alpha \) (He\(^3\)) \hspace{1cm} \sim 0.2 \text{ keV/\( \mu \)m} \hspace{1cm} 0.05-12 \text{ mm}
  \item Auger/conversion electron \hspace{1cm} \sim 80 \text{ keV/\( \mu \)m} \hspace{1cm} 0.04-0.1 \text{ mm}
  \item single cell \hspace{1cm} 100 \text{ cells} \hspace{1cm} 1000-10000 \text{ cells}
\end{itemize}
```

**Fig. 1.2.** Characteristics of diagnostic and therapeutic radiation. Photons used for nuclear imaging have a tissue range up to several centimeters. In the case of therapeutic radionuclides the tissue range varies between several cell diameters (\( \beta^- \)-particles) and a single cell (Auger/conversion electrons). The linear energy transfer (LET) describing the energy deposited per path length is highest for \( \alpha \)-particles (\( \sim 80 \text{ keV/\( \mu \)m} \)) and lowest for \( \beta^- \)-particles (\( \sim 0.2 \text{ keV/\( \mu \)m} \)). Data are adapted from Kassis and Pouget et al. [5, 6].

In contrast to diagnostic radiation, therapeutic radiation has a short tissue range. This property leads to a local energy deposition and a lower whole-body irradiation (Fig. 1.2) [5]. The choice of a radionuclide for tumor therapy should be made based on the decay characteristics of the radionuclide, the biological half-life of the targeting agent, the tumor size and the availability of the radionuclide at high quality [7]. In clinics, so far \( \beta^- \)-particle-emitting radionuclides are most frequently used for therapeutic approaches [7, 8]. During \( \beta^- \)-decay negatively charged electrons are emitted. These electrons have a path-length of 0.05-12 mm in biological matter which leads to irradiation of numerous cells at the same time ("crossfire" effect, Fig. 1.2) [5]. The properties of \( \beta^- \)-particles are advantageous for the treatment of larger tumors or metastases but might comprise a potential risk of side effects to normal tissue due to the "crossfire" effect. In clinics, the radiometals \(^{177}\)Lu and \(^{90}\)Y are often used for radionuclide therapy (Table 1.1) [7, 8]. In contrast to \( \beta^- \)-particles, \( \alpha \)-particles have a shorter tissue range and the linear energy transfer (LET) is significantly higher (Fig. 1.2). Therefore, \( \alpha \)-particles might be beneficial for the treatment of cancer cell clusters or small...
metastatic lesions with limited irradiation of non-targeted tissue. The clinical experience with α-particles is limited mostly due to the restricted supply of α-emitting radionuclides. The most promising clinical application of α-particle emitters was so far the use of $^{223}$RaCl$_2$ (Xofigo®, formerly Alpharadin®) for palliation in prostate cancer patients with skeletal metastases [9]. Lately, the α-emitter $^{149}$Tb was proposed as a radiometal for future radionuclide therapy due to its suitable decay characteristics [10]. Besides α- and β+-particles, also Auger/conversion electrons have characteristics suitable for a therapeutic application. Auger/conversion electrons have even a shorter tissue range than α-particles and, hence, they deposit energy over only subcellular dimensions (Fig. 1.2) [5]. Therefore, a deposition of Auger/conversion electrons close to the cell nucleus is described to be favorable [11]. The high therapeutic effect of radionuclides (e.g $^{123/125}$I) emitting Auger/conversion electrons was demonstrated in several preclinical and clinical studies in which nuclear targeting agents have been used [11-13]. Recently, the radiolanthanide $^{161}$Tb was proposed as a promising alternative to the clinically used $^{177}$Lu due to comparable β-energies and half-lives but additional emission of Auger/conversion electrons in the case of $^{161}$Tb [10, 14].

Table 1.1. Characteristics of radionuclides suitable for therapeutic application [2, 7, 15].

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>$^{177}$Lu</td>
<td>β, γ</td>
<td>-</td>
<td>-</td>
<td>134</td>
<td>56, 113, 208</td>
<td>-</td>
<td>6.64 d</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>β</td>
<td>-</td>
<td>-</td>
<td>933</td>
<td>-</td>
<td>-</td>
<td>2.67 d</td>
</tr>
<tr>
<td>$^{161}$Tb</td>
<td>β, γ</td>
<td>yes</td>
<td>-</td>
<td>154</td>
<td>49, 75</td>
<td>-</td>
<td>6.89 d</td>
</tr>
<tr>
<td>$^{149}$Tb</td>
<td>α, γ, β</td>
<td>-</td>
<td>3.97$^2)$</td>
<td>-</td>
<td>165</td>
<td>730</td>
<td>4.12 h</td>
</tr>
<tr>
<td>$^{223}$Ra</td>
<td>α, γ</td>
<td>-</td>
<td>5.78$^3)$</td>
<td>-</td>
<td>144, 154</td>
<td>-</td>
<td>11.4 d</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>γ</td>
<td>yes</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>-</td>
<td>59.4 d</td>
</tr>
</tbody>
</table>

$^1$ Main γ-lines suitable for SPECT are given; $^2$ Intensity: 17%; $^3$ Average α-energy is given.

1.2. Dose estimations

Ionizing radiation induces damage in tumor tissue as well as in healthy tissue [16, 17]. Hence, it is crucial to quantify the applied ionizing radiation to targeted and non-targeted tissues in patients in order to predict therapeutic effects and to estimate unexpected off-target toxicity.

The quantity which describes the amount of applied ionizing radiation to a tissue is the absorbed dose given in gray (Gy). It is defined as the energy absorbed per unit tissue mass
The internal dosimetry scheme of the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine has provided a framework for performing dose estimation studies after applying internally deposited radionuclides [19]. On a regular basis they publish latest findings for refining dose estimations in so-called pamphlets. In the MIRD system source ($r_S$) and target ($r_T$) tissues are distinguished. The source organ is the organ in which the radionuclide of interest is accumulated and the target organ is the site of interest receiving absorbed radiation from elsewhere [19]. In the concept presented by MIRD the mean absorbed dose in a target tissue ($D(r_T)$) is calculated as the following:

$$D(r_T) = \sum_{r_S} A(r_S) \cdot S(r_T \leftarrow r_S)$$

$A(r_S)$ is the time-integrated activity in a source tissue (MBq·s) and therefore incorporates characteristics of uptake and retention of radioactivity and physical as well as biological half-life. This value can be determined for each source tissue by for example quantitative imaging (PET, SPECT) or by tissue and excreta sampling (biopsy, blood, urine) [20]. In preclinical studies with rodents, $A(r_S)$ is calculated as the area under the curve (AUC) from time-activity curves of biodistribution studies. The value of S is tabulated and a first compilation S-values was published by the MIRD commission in 1975 using a phantom which comprised a geometry imitating size, shape and mass of organs of an adult human body [21]. The value of S is characteristic for each radionuclide including for instance the energy per emitted particle and the fraction of energy absorbed in the target tissue [21]. Moreover, the S-factor considers the spatial relationship and tissue composition of source and target tissue including tissue mass and geometry. For dose estimations in mice it has to be considered that cross-doses are higher due to small organ size. Therefore, S-factors were also determined for mice using mathematical and voxel-based mouse models [22]. However, in preclinical studies often only the self-absorbed dose is taken into consideration for dose estimations. The fact that not for all preclinically used radionuclides S-factors are tabulated complicates dose estimation studies.

In the last decades, enormous efforts were done to refine dose estimations and as a result, the nowadays used programs are highly sophisticated (Fig. 1.3). Approximate phantom-based dose estimation models are more and more replaced by patient-specific dose calculations. Nowadays, SPECT or PET imaging after application of tracer amounts of radioactivity in parallel with anatomical imaging allows determination of patient-specific pharmacokinetic profiles of the radiopharmaceutical [23]. The combination of medical and nuclear imaging allows suborgan dose estimations. Furthermore, the option of patient-specific imaging enables re-evaluation of absorbed doses in tumor tissue over a set of time
intervals during therapy which is essential since the tumor mass varies during targeted radionuclide therapy [24]. In more sophisticated dose estimation studies the particle range, photon emission, dose fractioning and subcellular deposition are additionally taken into consideration [25]. For example, in the case of short-range high-LET $\alpha$-particles, it is fundamental to consider that the higher ionization density along a track will increase the probability for a biological damage in comparison to $\beta$-particles [26]. The concept of relative biological effectiveness (RBE) has been developed to account for this difference in energy deposition [27]. It is determined by radiation quality (LET), dose, dose rate and tissue type [28]. RBE-weighed dose has the unit gray (Gy) and is a quantity for deterministic end-points like cancer cell killing. For targeted $\alpha$-therapy a RBE value $\sim 1-8$ was recommended, depending on the reference radiation, $\alpha$-particle energy and biological end-point (e.g. cancer cell killing or induction of double strand breaks) [26, 27]. Most of the $\alpha$-particle emitters have $\alpha$- or $\beta$-emitting daughters in their decay chain. In these cases the biodistribution of both, the parent and all daughter radionuclides, has to be considered in dosimetric calculations which complicate dose estimations [26]. In the case of Auger emitters deposited in the cell nucleus the RBE is comparable to that observed for high-LET $\alpha$-particles [27, 29]. Hence, in the case of short-ranging particle emitters it is not only crucial to consider the particle range and energy deposition for dose estimation studies, but also the subcellular distribution.

Fig.1.3. Tree diagram presenting the basic formula for dosimetric calculations and several factors which are included in more sophisticated models for refining dose estimations.
1.3. Targeted radiopharmaceuticals

1.3.1. Radiolabeled somatostatin analogs

To date, the most successful targeted radiopharmaceuticals in nuclear oncology are radiolabeled somatostatin analogs targeting somatostatin receptors (SSTRs) [30-32]. SSTRs are highly expressed on malignant cells, mainly neuroendocrine tumors, but not significantly present in most healthy tissues [33]. The naturally occurring SSTR-agonist, somatostatin, is enzymatically degraded within minutes [34]. Hence, numerous analogs of the naturally occurring ligand were developed aiming to prolong the plasma half-life and to improve in vivo stability [34]. More than two decades ago, the first somatostatin analog (\(^{111}\)In-DTPA-octreotide, OctreoScan®) was approved for the diagnosis of SSTR-positive tumors [35]. Nowadays, nuclear imaging with \(^{111}\)In- or \(^{68}\)Ga-radiolabeled somatostatin analogs is a standard procedure for the diagnosis and monitoring of neuroendocrine tumors in the clinics [30]. Moreover, somatostatin analogs radiolabeled with the \(\beta\)-emitters \(^{90}\)Y or \(^{177}\)Lu are routinely applied for prolonging survival and improving quality of life of these patients [30, 31]. The currently used somatostatin analogs are all SSTR-agonists showing cell internalization. It was generally believed that receptor-binding with subsequent cell internalization is the basis for successful radionuclide diagnosis and therapy [36]. Recently published studies, however, proved the opposite [37-40]. It was shown in preclinical and clinical trials that SSTR-antagonists, which do not induce receptor internalization, show superior tumor uptake to SSTR-agonists (Fig. 1.4). This phenomenon might be explained by the fact that antagonists bind to a larger number of binding sites than agonists [37]. These findings are promising and might be revolutionary for the future development of peptide-based radioconjugates.

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**Fig. 1.4.** Somatostatin receptor (SSTR)-agonists are internalized into SSTR-positive tumor cells (A), whereas SSTR-antagonists do not induce receptor internalization (B).
1.3.2. Radiolabeled folic acid conjugates

The folate receptor (FR-α) is a promising target for targeted radionuclide therapy because of its expression in numerous human tumor types including cancers of the ovaries, colon and lung (Table 1.2) [41]. The expression in healthy tissues is restricted to only a few sites. Most importantly, the FR is expressed in the proximal tubule cells of the kidneys which leads to reabsorption of folic acid (derivatives) from the primary urine [42].

Table 1.2. Folate receptor (FR) expression in various cancer types and incidences of these tumor types in Switzerland (Statistics of Swiss Cancer League, 2014; [43]).

<table>
<thead>
<tr>
<th>Malignant tissue</th>
<th>Incidence in Switzerland</th>
<th>FR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary</td>
<td>600</td>
<td>90</td>
</tr>
<tr>
<td>Endometrium</td>
<td>850</td>
<td>90</td>
</tr>
<tr>
<td>Brain</td>
<td>600</td>
<td>90</td>
</tr>
<tr>
<td>Lung</td>
<td>3900</td>
<td>78</td>
</tr>
<tr>
<td>Kidney</td>
<td>850</td>
<td>75</td>
</tr>
<tr>
<td>Breast</td>
<td>5540</td>
<td>48</td>
</tr>
<tr>
<td>Colorectum</td>
<td>4100</td>
<td>32</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16440</strong></td>
<td><strong>58</strong></td>
</tr>
</tbody>
</table>

During the last two decades several folic acid conjugates useful for SPECT and PET imaging were developed [44, 45]. $^{111}$In-DTPA-folate and $^{99m}$Tc-etarfolatide$^\text{TM}$ (Folcepri®, $^{99m}$Tc-EC20, Endocyte Inc.) were successfully applied for imaging of FR-positive tumors in cancer patients [46, 47]. Several clinical studies demonstrated that $^{99m}$Tc-etarfolatide$^\text{TM}$ is useful for the identification of patients who can potentially benefit from FR-targeted therapeutics [47]. A number of folic acid conjugates functionalized with various chelators have been used for radiometallation using radionuclides for SPECT ($^{99m}$Tc, $^{67}$Ga, $^{111}$In, $^{155}$Tb) and PET ($^{68}$Ga, $^{152}$Tb, $^{44}$Sc) [45, 48].

The fact that the FR is not only a favorable target for cancer imaging but also for therapy was demonstrated with small molecular weight drug conjugates composed of folic acid and a chemotherapeutic agent (e.g. Vintafolide$^\text{TM}$, EC145 (clinical phase III), Endocyte Inc.). It was recently reviewed that this therapy strategy was successfully applied to cancer patients mainly suffering from FR-positive platinum-resistant ovarian cancer [49, 50].
The substantial expression of the FR in renal tissue is the limiting factor for a therapeutic application of radiofolates as it might comprise a risk of renal damage. To address this challenge, a folic acid conjugate (cm09) containing an albumin-binding moiety was recently developed in our group [51]. The resulting binding of this radiofolate \( ^{177}\text{Lu-cm09} \) to serum albumin led to an extended circulation time in the mouse. Consequently, the tumor-to-kidney ratio was \( \sim \)10-fold higher compared to the ratios obtained with conventional folic acid radioconjugates which do not bind to albumin (e.g. \( ^{177}\text{Lu-EC0800} \); Fig. 1.5) [52]. This promising biodistribution allowed using \( ^{177}\text{Lu-cm09} \) in a pilot study in tumor-bearing mice [51].

\[
\text{A} \quad \text{B}
\]

\begin{align*}
\text{M: } ^{177}\text{Lu}
\end{align*}

**Fig. 1.5.** Chemical structures of the folic acid radioconjugates \( ^{177}\text{Lu-EC0800} \) (A) and \( ^{177}\text{Lu-cm09} \) (B) and corresponding SPECT/CT images of KB (FR-positive) tumor-bearing mice 24 h p.i.. Both radiofolates comprise folic acid as a targeting ligand (blue) and DOTA (green) as a chelator for complexation of radiometals. The folic acid conjugate cm09 contains in addition an albumin-binding moiety (red). The tumor-to-kidney ratio is \( \sim \)10-fold improved in the case of \( ^{177}\text{Lu-cm09} \) compared to \( ^{177}\text{Lu-EC0800} \).
1.4. Off-target toxicity during targeted radionuclide therapy

Ionizing radiation can directly damage macromolecules in the cell (e.g. DNA) or it can lead to formation of free radicals which might damage critical targets in the cell [16]. Damaged cells are repaired and proceed through cell cycle or in the case of non-reparable damage, cells will undergo apoptosis [53]. These radiobiological effects are observed in cancer cells but also in healthy non-targeted tissues, which can lead to off-target toxicity during targeted radionuclide therapy.

The tissue radiosensitivity ranges from highly sensitive tissue (e.g. bone marrow) to barely sensitive tissue (e.g. nervous system) (Fig. 1.6). It is known that immature, undifferentiated and actively dividing cells (e.g. stem cells) are more radiosensitive compared to mature, differentiated and not actively dividing cells (e.g. neurons) [16]. Moreover, the capability of compensating non-functional units is different in every tissue [54]. Beside the radiosensitivity of the organ, the severity of tissue response to radiation depends on additional factors including i) amount of accumulated radioactivity, ii) suborgan distribution of radioactivity, iii) exposure time of the tissue to radiation, iv) decay characteristics of the radionuclide and vi) health status of the organ [54-58].

![Fig. 1.6.](image)

**Fig. 1.6.** Selected tissues in decreasing order of radiosensitivity indicated by their tolerance dose which would result in 50% probability of developing severe complications within 5 years (TD$_{50/5}$) after whole tissue irradiation in adults. Data are adapted from Hall et al. [58].
1.4.1. Bone marrow toxicity

The bone marrow is a dose-limiting organ in most types of radionuclide therapy since its rapidly dividing cells are highly sensitive towards irradiation [59]. Most problematic is bone marrow toxicity after application of long-circulating radiopharmaceuticals such as for instance radiolabeled antibodies [60]. Within weeks after therapy start acute myelosuppression can occur [61]. This can be addressed by bone marrow transplantation or application of growth factors [62]. Up to years after radiotherapy, late myelotoxic side effects such as myelodysplastic syndrome potentially leading to acute leukemia can be developed [61]. Measures to prevent bone marrow toxicity might be dose fractioning and concomitant stem cell transplantation [61, 62].

1.4.2. Radionephrotoxicity

Renal radiotoxicity is predominantly caused during targeted radionuclide therapy with small molecular weight radiopharmaceuticals due to their renal excretion (Fig. 1.7) [63, 64]. These radiopharmaceuticals predominantly accumulate in the renal cortex what should be considered for dose estimations [65].

**Fig. 1.7.** SPECT/CT images of mice illustrating the off-target accumulation of radiolabeled somatostatin analogs (A) and folic acid radioconjugates (B) in the kidneys. The transaxial sections of SPECT images demonstrate that the uptake of radioactivity is predominant in the renal cortex.
In the case of radiolabeled somatostatin analogs an unspecific uptake of radioactivity in the kidneys is observed mainly due to megalin-mediated endocytosis in the proximal tubules [66]. The renal accumulation of radiopeptides can be successfully reduced by the co-administration of positively charged amino acids such as lysine and arginine resulting in reduction of renal dose by ~50% [56, 63, 67]. However, the kidneys are still the dose-limiting organ during tumor therapy with radiolabeled somatostatin analogs. It was found in humans that the renal threshold dose for kidney toxicity after therapy with $^{177}$Lu-radiolabeled somatostatin analogs ranges between 25 Gy and 40 Gy [55, 64]. The renal tolerance against irradiation was dependent on additional risk factors of the patients such as high blood pressure or diabetes.

In the case of folic acid radioconjugates, challenging is that the FR is expressed in proximal tubule cells of the kidneys [42]. In the past, several strategies were tested in preclinical studies to reduce renal dose burden after radiofolate application, amongst others, the co-application of cationic amino acids or diuretics [68]. None of those strategies allowed decreasing the renal accumulation of radiofolates effectively. So far, the most favorable methods to reduce renal uptake of radiofolates was the co-administration of the anti-folate pemetrexed and the enhancement of blood circulation time by binding of the radiofolate to serum albumin [51, 69, 70]. In the case of the albumin-binding radiofolate ($^{177}$Lu-cm09) the renal uptake was ~60% (4 h p.i.) lower compared to the non-albumin binding radiofolate $^{177}$Lu-EC0800 [51]. For future application in the clinics using the most promising radiofolate, $^{177}$Lu-cm09, it will be crucial to perform dosimetric calculations based on tissue distribution data of humans.

1.5. Strategies for improving targeted radionuclide therapy

For a safe radionuclide tumor therapy a high tumor-to-background ratio of the radiopharmaceutical is essential. For radiolabeled small molecules, strategies for reducing renal toxic side effects and, if possible, methods to improve the anti-tumor effect have to be evaluated (Fig. 1.8).
Fig. 1.8. Possible approaches for improving the anti-tumor effect (A) and reducing renal toxic side effects (B) during radionuclide therapy. Potential strategies for improving the anti-tumor effect might be: (a) co-applying radiosensitizers (e.g. chemotherapeutic agents) in order to make tumor tissue more sensitive towards radiation damage, (b) stimulating target expression at tumor site, (c) applying high-LET particles with high biological effectiveness (e.g. $\alpha$-particles) and (d) depositing short-ranging high energetic particles (e.g. Auger/conversion electrons) in the cell nucleus by modifying the derivative with a nuclear localization signal. Potential mechanisms for reducing renal toxic side effects might be: (e) reducing the number of free radicals by co-applying radioprotectors, (f) applying short-ranging particle emitters to minimize the "crossfire" effect, (g) enhancing cell repair mechanisms, and (h) reducing glomerular filtration or renal uptake of the radioconjugates (e.g. albumin-binding derivatives).
1.5.1. Reduction of renal toxicity

Beside the aforementioned strategies to reduce renal toxicity after targeted radionuclide therapy, the co-application of radioprotectors might reduce nephrotoxic side effects. Possible mechanisms of radiation protection might be, amongst others, reducing the number of free radicals or enhancing repair processes [17]. Amifostine (WR-2721, Ethyol®) is an approved chemo- and radioprotector which was tested in several preclinical and clinical studies as recently reviewed [71, 72]. This prodrug is activated by an enzyme, which is expressed at much higher levels in healthy blood vessels compared to tumor capillaries [72]. Therefore, it was investigated in numerous studies whether radioprotection of healthy tissue could be observed while the anti-tumor effect of radiotherapy would be maintained [71, 72]. In the case of radiolabeled somatostatin analogs it was demonstrated that the co-application of amifostine might be beneficial in terms of preventing radionephropathy [73, 74]. Beside the function of amifostine as a radical scavenger and DNA repair booster, it was shown that amifostine co-application results in decreased renal uptake of radiolabeled somatostatin analogs due to interaction with megalin/cubilin receptor complex [74]. Besides using amifostine as a radioprotector, it was demonstrated in several studies that inhibition of the renin-angiotensin system (RAS) is beneficial in the mitigation of radiation nephropathy [17, 75]. In this respect, the angiotensin-converting enzyme (ACE) inhibitor captopril was extensively studied [76-78]. Captopril contains a thiol group which allows its role as a radical scavenger [79]. Moreover, it was demonstrated that the RAS induces proliferation and hence, apoptosis of damaged renal cells after irradiation [75]. The inhibition of RAS prevents this reaction and therefore mitigates radiation nephropathy.

Important for achieving minimal renal off-target toxicity is the selection of the radionuclide. In patient studies, it was demonstrated that somatostatin analogs radiolabeled with $^{90}$Y led more frequently to impaired kidney function compared to analogs radiolabeled with $^{177}$Lu [64]. This observation can be explained by the higher energy and wider range of $\beta^-$-particles in the case of $^{90}$Y ($E_{\beta}^{\text{av}} = 933$ keV, $R_{\text{max}} = 11$ mm) compared to $^{177}$Lu ($E_{\beta}^{\text{av}} = 134$ keV, $R_{\text{max}} = 2$ mm). The fact that the tissue range of radioactive particles has a remarkable impact on the renal toxic side effects was also demonstrated after the therapeutic application of somatostatin analogs radiolabeled $^{111}$In known to emit Auger/conversion electrons [80]. It could clearly be shown that the emission of short-ranging Auger electrons accumulated in the proximal tubule cells of the kidneys was less harmful to the kidneys than $\beta^-$-particles.
1.5.2. Improvement of anti-tumor effect

Radiosensitzers increase the sensitivity of tissues towards the effects of radiation [81]. The combined application of radiation and radiosensitizing agents potentially results in an additive or even synergistic cell damage [81]. The co-treatment with radiation therapy and radiosensitizers such as chemotherapeutic agents was successfully demonstrated in preclinical and clinical trials [70, 81]. It was shown that various mechanisms (e.g. inhibited DNA repair after irradiation or cell cycle arrest in radiosensitive phases) are responsible for the observed effects [81].

Another interesting approach to improve the anti-tumor effect might be the selective up-regulation of the target in the tumor tissue without changing the expression levels in healthy tissues. It was previously demonstrated that the exposure of FR-positive tumor cells to glucocorticoids, progesterone, androgene and antiestrogens resulted in an up-regulation of the FR in tumor cells but not in renal tissue [82]. This might result in increased tumor uptake of radiofolates whilst the renal accumulation of the radioactivity would be unchanged.

As mentioned before, the selection of the radionuclide has an impact on the adverse effects of targeted radionuclide therapy. To maintain optimal anti-tumor effect the targeting system has to be chosen according to the decay properties of the radionuclide. For instance, the use of Auger/conversion electrons requires a high tumor cell uptake of the radiopharmaceutical with a preferential deposition in the cell nucleus to achieve a successful tumor therapy [11, 83]. The inclusion of a nuclear localization signal might be a possibility to reach nuclear uptake of radiopharmaceuticals and, hence, profit from radionuclides emitting Auger/conversion electrons [84]. The application of α-particle emitters might also be an option to obtain better anti-tumor effect [85].

1.6. Aim of the thesis

The aim of this thesis was to improve targeted radionuclide therapy of cancer with the focus on reduction of renal toxic side effects. At the same time, the anti-cancer effect should be maintained or increased.

In the first part of the thesis the threshold dose value for tumor treatment and nephropathy was evaluated for a radiofolate without \(^{177}\text{Lu}\)-EC0800) and with albumin-binding properties \(^{177}\text{Lu}\)-cm09) in mice (chapter 2). In the second part of the thesis several strategies for reducing renal toxicity and/or improving the therapeutic outcome were studied. The particular investigated approaches were:
i. The study of a potential benefit of the radioprotectors, amifostine and captopril, on renal protection of mice applied with a radiofolate (chapter 3).

ii. The in vivo comparison of short-ranging α- ($^{149}\text{Tb}$) and β- emitters ($^{161}\text{Tb}$) with regard to their renal toxicity during radiofolate therapy (chapter 4).

iii. The investigation of a specific up-regulation of FR in cancer cells by glucocorticoids and its impact on anti-tumor effect (chapter 5).

In a side project, the benefit of additional Auger/conversion electron emission during β-therapy was investigated in vitro (chapter 6). For this purpose, DOTA-[Tyr$^3$]-octreotide (DOTATOC) targeting SSTRs and the model compound MG136 were used. MG136 is a DOTATOC analog which is supposed to accumulate in the nucleus due to its nuclear localization signal.
CHAPTER 2

Folate Receptor-Targeted Radinuclide Therapy:
Preclinical Investigation of Anti-Tumor Effects and
Potential Radionephropathy

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Author contributions:

Stephanie Haller designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript. Josefine Reber assisted with in vivo experiments. Simone Brandt stained and analyzed tissue samples. Peter Bernhardt performed dosimetric calculations. Viola Groehn synthesized cm09. Roger Schibli reviewed the manuscript. Cristina Müller supervised the study, reviewed and revised the manuscript.
2.1. Introduction

The use of folate receptor (FR)-targeted radiopharmaceuticals is a promising strategy for imaging and therapy of cancer since the FR is expressed on a wide variety of tumor types [41, 43]. The vitamin folic acid is an almost ideal targeting agent because of its high affinity to the FR and the possibility of derivatization at the glutamate’s carboxyl groups without loss of FR-binding affinity [86]. So far, the limiting factor for a therapeutic application of radiofolates was the high renal retention of radioactivity [46, 87] as a consequence of the specific binding of radiofolates to the FR, which is also expressed in the proximal tubule cells [42, 88]. Using folic acid conjugates radiolabeled with $\beta^-$-particle-emitting radionuclides would therefore comprise a significant risk of damage to the kidneys due to the high absorbed radiation dose.

An unfavorably high absorbed kidney dose has also been experienced with other small molecular weight radiopharmaceuticals and been addressed in particular for peptide receptor radionuclide therapy (PRRT) using somatostatin analogs [63]. These radiopeptides accumulate to a high extent in the proximal tubule cells of the kidneys, mainly because of megalin/cubilin receptor-mediated uptake [66, 89]. Long-term side effects were extensively studied in preclinical and clinical studies, after PRRT with somatostatin analogs and recently summarized in comprehensive review articles [56, 64]. First signs of renal toxicity in rats and mice were observed three to nine months after injection of $^{177}$Lu-DOTATATE [90, 91]. Svensson et al. determined a threshold dose of $\sim$24 Gy which was found to be safe after application of $^{177}$Lu-DOTATATE in mice [92]. In humans a renal cumulative dose of $\sim$25 Gy was considered as safe [91]. Aspects that have to be considered for estimation of a potential renal damage include, however, also additional risk factors of the patient, such as hypertension and diabetes, as well as the radionuclide which is employed since the average energy of the $\beta^-$-particles might vary significantly (e.g. $^{90}$Y versus $^{177}$Lu) [93].

Several strategies were investigated to minimize the risk of radionephropathy after PRRT [56, 63]. The most successful approach to reduce renal uptake of radiolabeled somatostatin analogs has been the co-application of cationic amino acids as demonstrated for the first time by Hammond et al. [94]. This concept was also tested in combination with folic acid radioconjugates but, application of L-lysine or L-glutamate, did both not impact the renal retention of radioactivity [68].

Recently, we developed a novel folic acid conjugate (cm09; Fig. 2.1 [51]), which contains a small molecular weight albumin-binding entity, previously developed by Dumelin et al. [95]. This modification resulted in an enhanced serum half-life of the radiofolate as a consequence of binding to serum proteins. Compared to conventional folate radioconjugates such as $^{177}$Lu-EC0800 (Fig. 2.1), $^{177}$Lu-cm09 showed an increased tumor uptake and at the same time a reduced retention in the kidneys [51].
The goal of this study was to investigate the therapeutic anti-tumor effects and potential long-term damage to the kidneys after application of high quantities of $^{177}$Lu-cm09 and $^{177}$Lu-EC0800, respectively. For this purpose, we performed a therapy study in KB tumor-bearing mice (study 1). In a separate experiment, kidney function was investigated in nude mice without tumors over a time period of eight months (study 2). Potential damage to the renal tissue was assessed by determination of $^{99m}$Tc-DMSA uptake in the kidneys, determination of blood plasma parameters indicative for renal damage and post-mortem investigation of morphological changes on renal tissue sections.

### 2.2. Materials and methods

#### 2.2.1. Cell culture

KB cells (human cervical carcinoma cells [96], subclone of HeLa cells, ACC-136) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were cultured in folate-free RPMI medium (FFRPMI,
without folic acid, vitamin B₁₂ and phenol red, Cell Culture Technologies GmbH, Gravesano, Switzerland). The cell culture medium was supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics. Routine cell culture was performed twice a week.

2.2.2. Preparation of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09

EC0800 was kindly provided by Endocyte Inc. (Indiana, U.S.). The DOTA-folate conjugate (cm09) previously developed in our group was kindly provided by Merck & Cie (Schaffhausen, Switzerland) [51]. No-carrier added $^{177}$LuCl₃ was purchased from Isotope Technologies Garching (ITG GmbH, Garching, Germany). Radiolabeling of folic acid conjugates was performed in a mixture of hydrochloric acid (HCl, 0.05 M) and Na-acetate (0.5 M) at pH 4.5 and elevated temperature (10 min at 95°C). Quality control of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 was carried out using high-performance liquid chromatography (HPLC) as previously reported [51].

2.2.3. Animal studies

In vivo experiments were approved by the local veterinarian department and conducted in accordance with the Swiss law for animal protection. Female, athymic nude mice (CD-1 Foxn1/nu, 5-7-week-old) were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were fed ad libitum with a folate-deficient rodent diet (ssniff Spezialdiäten GmbH, Soest, Germany) starting one week prior to tumor cell inoculation. Mice without tumors of study 2, which were used for investigating potential radionephropathy, received a folate-deficient diet during the first 3 weeks after injection of the radiofolates, followed by a standard rodent diet (Kliba Nafag, Kaiseraugst, Switzerland) until end of the study.

2.2.3.1. Biodistribution studies

Biodistribution studies of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 were performed in KB tumor-bearing nude mice as previously reported [51, 70]. In brief, KB cells ($5 \times 10^6$ cells in 100 µL phosphate buffered saline (PBS) pH 7.2) were inoculated into the subcutis of each shoulder, two weeks before injection of the radiofolates. Biodistribution studies were performed in triplicates. $^{177}$Lu-EC0800 (3 MBq, 1 nmol per mouse) and $^{177}$Lu-cm09 (3 MBq, 0.5 nmol per mouse) were administered intravenously (i.v.) into a tail vein. Mice were euthanized at predetermined points of time between 1 h p.i. and 10 d p.i.. Selected tissues and organs were collected, weighed and counted for radioactivity using a $\gamma$-counter (Packard Canberra Cobra II, Meriden, U.S.). The uptake of radioactivity was expressed as non-decay corrected
accumulated radioactivity per tumor (40-50 mg), and per kidney (~125 mg), respectively, calculated per MBq injected radiofolate.

2.2.3.2. Dosimetry

To estimate the equivalent absorbed radiation dose for $^{177}$Lu-cm09 and $^{177}$Lu-EC0800 in tumor xenografts and in the kidneys, the following calculations were made: (i) the cumulative radioactivity was calculated by fitting a bi-exponential curve to the non-decay corrected biodistribution data (% injected activity (IA)/g). The areas under the curve (AUCs, MBq·s) were determined by integrating the bi-exponential function to infinity. (ii) The mean absorbed dose to the tumor xenografts was assessed for the approximate tumor mass (40-50 mg) at the time of injection and a kidney mass of ~125 mg. The absorbed fraction to tumors and kidneys, with the radioactivity uniformly distributed was simulated by PENELOPE [97]. (iii) The specific mean absorbed dose (mGy/MBq) was calculated by multiplying the AUC (normalized to 1 MBq injected activity) with the absorbed fraction and the emitted energy per decay for $^{177}$Lu (ENSDF decay data in the MIRD format, www.nndc.bnl.gov) and multiplied with a conversion factor. (v) The mean absorbed dose (mGy) to tumors and kidneys was calculated by multiplying the specific mean absorbed dose (mGy/MBq) with the amount of injected radioactivity.

2.2.3.3. Tumor therapy study

For testing the therapeutic effects in study 1, mice were subcutaneously inoculated with $4.5 \times 10^6$ KB tumor cells (in 100 µL PBS pH 7.2) 4 d before start of the therapy using the radiofolates at a specific activity of 20 MBq/nmol. Control animals were i.v. injected with only saline (group A1). Groups of 5 mice each (groups B1-F1) were i.v. administered with different quantities of $^{177}$Lu-EC0800 (B1: 10 MBq, C1: 20 MBq) and $^{177}$Lu-cm09 (D1: 10 MBq, E1: 20 MBq, F1: 30 MBq), respectively. Body weights and tumor volumes were determined at day 0 followed by measurements three times per week (Table 2.1). The body weight was indicated as the average of the individual relative body weight (RBW = $W_x/W_0$; $W_x$: weight at day $x$; $W_0$: weight at day 0). The tumor volume (V) was determined according to the equation $[V = 0.5 \times (L \times W^2)]$, where L is the longest axis and W is the perpendicular axis to L [98]. The values were expressed as the average of the individual relative tumor volumes (RTV = $V_x/V_0$; $V_x$: volume at day $x$; $V_0$: volume at day 0). The anti-tumor efficacy of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 was expressed as percentages tumor growth inhibition (% TGI), using the equation [100-((T/C) x 100)], where T is the mean RTV of treated mice and C is the mean RTV of control mice at the time of euthanasia of the first mouse of the control group [99]. The tumor
growth delay (TGDₜ) was calculated for the time required until the RTVs reached a 5-fold (TGD₅) and 10-fold (TGD₁₀) increased volume compared to day 0 [99]. The tumor growth delay index (TGDIₓ) was determined by dividing TGDₜ of treated mice by TGDₜ of control mice (TGDIₓ = TGDₜ(T)/TGDₜ(C)). Endpoint criteria were defined as (i) body weight loss of >15% of the initial body weight, (ii) a tumor volume of >1000 mm³, (iii) active ulceration of the tumor or (iv) signs of unease.

### 2.2.3.4. Long-term side effects after application of ¹⁷⁷Lu-EC0800 and ¹⁷⁷Lu-cm09

For investigating potential long-term side effects to the kidneys in study 2, groups consisting of 6-9 mice without tumors were i.v. injected with the radiofolates at a specific activity of 20 MBq/nmol. Mice received 10 MBq or 20 MBq of ¹⁷⁷Lu-EC0800 (groups B2 and C2) and 10 MBq, 20 MBq or 30 MBq of ¹⁷⁷Lu-cm09 (groups D2, E2 and F2). End-point criteria were predefined as (i) body weight loss >15% of the initial weight and (ii) signs of unease. The body weight was measured once a week (Table 2.1). The study was performed over eight months.

### Table 2.1. Parameters monitored in the tumor therapy (study 1) and long-term kidney toxicity study (study 2).

<table>
<thead>
<tr>
<th>Tumor therapy (study 1)</th>
<th>Kidney toxicity (study 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Determination</td>
</tr>
<tr>
<td>Body weight</td>
<td>Three times a week</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>Three times a week</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
</tr>
<tr>
<td>WBC count</td>
<td></td>
</tr>
<tr>
<td>Kidney size/mass</td>
<td></td>
</tr>
<tr>
<td>Kidney morphology</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DMSA, dimercaptosuccinic acid; SPECT, single photon emission computed tomography; WBC, white blood cells.

¹) Terminal means “before euthanasia” when an endpoint criterion was reached or at the end of the study.
2.2.3.4.1. Quantification of renal uptake of $^{99m}$Tc-DMSA using SPECT

Kidney function was monitored by quantification of the renal uptake of $^{99m}$Tc-dimercaptosuccinic acid ($^{99m}$Tc-DMSA) using single photon emission computed tomography (SPECT) [100]. For this purpose DMSA (TechneScan® DMSA, Mallinckrodt Pharmaceuticals, Petten, The Netherlands) was radiolabeled according to the instructions of the manufacturer with $[^{99m}\text{Tc}]$NaTcO$_4$ which was eluted from a $^{99}$Mo/$^{99m}$Tc-generator (Mallinckrodt Pharmaceuticals, Petten, The Netherlands). The animals were i.v. injected with 30-40 MBq of $^{99m}$Tc-DMSA at weeks 3, 15 and 30 after injection of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09, respectively. SPECT scans were performed 2 h after the injection of $^{99m}$Tc-DMSA with 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 collimator of nine 1.4 mm-diameter pinholes and a thickness of 10 mm. The energy peak for the camera was set at 140.5 keV ± 10%. The SPECT scans were acquired with Nucline Software (version 1.02, Bioscan Inc., Poway, U.S.). The scan range was 3 cm which resulted in a scan time of ~7 min. After the performance of the SPECT acquisitions, the data were reconstructed with HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany). The NanoSPECT was calibrated in advance with a phantom filled with a known radioactive amount of $^{99m}$Tc allowing the determination of the accumulated radioactivity in a defined volume of tissue. The uptake of $^{99m}$Tc-DMSA in the kidneys was determined in a defined volume covering the size of a kidney using VivoQuant post-processing program (version 1.23 inviCRO Imaging Services and Software, Boston, U.S.). The determined activities were decay-corrected and expressed as percentage of injected radioactivity (% IA) per kidney.

2.2.3.4.2. Determination of blood plasma parameters

Blood urea nitrogen, creatinine, total bilirubin and creatinine were determined in blood plasma samples in week 8, 19, 26 and before euthanasia using a Fuji Dri-Chem 4000i analyzer (Polymed Medical Center AG, Glattbrugg, Switzerland). For this purpose, plasma samples were prepared from blood samples taken from the sublingual vein of each mouse.

2.2.3.4.3. Kidney size and morphology

Terminal collected kidneys were weighed, fixed in formalin (Formafix buffered 4%, Formafix Switzerland AG, Hittnau, Switzerland) and embedded in paraffin. In a blinded study the morphological changes of the renal tissue, which was cut into sections (4 µm), were evaluated by an independent pathologist after staining with hematoxylin-eosin (HE), periodic
acid-Schiff (PAS), silver methenamine or acid-fuchsin orange G (SFOG). Each compartment (glomeruli, tubules, interstitium and blood vessels) was evaluated separately for signs of renal damage. Several parameters (thrombi, fibrinoid necrosis, interstitial fibrosis, tubular atrophy etc.) were taken into account for the scoring. A semi-quantitative scoring of 0-4 indicating no (0), mild (1), moderate (2), moderately severe (3) and severe (4) damage was employed.

2.2.3.4.4. Determination of white blood cell death by FACS

Fluorescence activated cell sorting (FACS) was used for white blood cell counting in week 8 and 19. Blood samples were 1:5 diluted in PBS (pH 7.4) and mixed 1:10 with Guava ViaCount Reagent (Merck Millipore, Darmstadt, Germany). The samples were incubated at room temperature for 10 min before analysis by flow cytometry (Guava EasyCyte Plus Flow Cytometer System, Merck Millipore, Darmstadt, Germany). All samples were measured in duplicates.

2.2.4. Statistical analysis

Data are presented as mean ± standard. Statistics was conducted by using one-way ANOVA with Bonferroni’s multiple comparison post-test (GraphPad Prism, version 5.01).

2.3. Results

2.3.1. Biodistribution of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 and dose estimations

Biodistribution data of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 have been previously published by our group [51, 70]. Herein, further time points after injection of the radiofolates were investigated in order to refine the dosimetric calculations (Table 2.2). For both derivatives, the highest uptake of radioactivity into FR-positive tumors was observed 4 h p.i. (Fig. 2.2, A). At that time, $3 \pm 0.5$ kBq per MBq injected $^{177}$Lu-EC0800 and $8 \pm 0.8$ kBq per MBq injected $^{177}$Lu-cm09 were found in tumor xenografts. The uptake per MBq applied $^{177}$Lu-EC0800 into one kidney (~125 mg) was about $91 \pm 14$ kBq, 1 h p.i. and declined to $9 \pm 2$ kBq 7 d p.i. (Fig. 2.2, B). In the case of $^{177}$Lu-cm09, the maximal renal accumulation per MBq injected activity was $34 \pm 2$ kBq 4 h p.i. (Fig. 2.2, B). Over the time of investigation, 10 d p.i. the kidney uptake decreased to about $2 \pm 0.1$ kBq per MBq applied $^{177}$Lu-cm09.
<table>
<thead>
<tr>
<th></th>
<th><strong>177Lu-EC0800</strong></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h p.i.</td>
<td>4 h p.i.</td>
<td>24 h p.i.</td>
<td>48 h p.i.</td>
<td>3 d p.i.</td>
<td>4 d p.i.</td>
<td>5 d p.i.</td>
<td>7 d p.i.</td>
</tr>
<tr>
<td>Blood</td>
<td>0.26 ± 0.05</td>
<td>0.11 ± 0.02</td>
<td>0.04 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>n.d.</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>Lung</td>
<td>1.2 ± 0.05</td>
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<td>0.33 ± 0.04</td>
<td>0.32 ± 0.02</td>
<td>0.26 ± 0.04</td>
<td>n.d.</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.76 ± 0.19</td>
<td>0.87 ± 0.77</td>
<td>0.22 ± 0.05</td>
<td>0.35 ± 0.02</td>
<td>0.49 ± 0.37</td>
<td>0.10 ± 0.01</td>
<td>n.d.</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.2 ± 0.25</td>
<td>1.7 ± 0.14</td>
<td>0.96 ± 0.06</td>
<td>0.75 ± 0.11</td>
<td>0.55 ± 0.12</td>
<td>0.30 ± 0.02</td>
<td>n.d.</td>
<td>0.09 ± 0.01</td>
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<tr>
<td>Kidney</td>
<td>73 ± 11</td>
<td>72 ± 3.4</td>
<td>52 ± 3.4</td>
<td>42 ± 5.6</td>
<td>32 ± 1.9</td>
<td>19 ± 4.5</td>
<td>n.d.</td>
<td>7.2 ± 1.9</td>
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<tr>
<td>Small intestine</td>
<td>0.69 ± 0.08</td>
<td>0.42 ± 0.09</td>
<td>0.27 ± 0.07</td>
<td>0.18 ± 0.03</td>
<td>0.18 ± 0.04</td>
<td>0.07 ± 0.01</td>
<td>n.d.</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>5.1 ± 2.3</td>
<td>2.5 ± 0.56</td>
<td>1.5 ± 0.45</td>
<td>1.5 ± 0.27</td>
<td>1.6 ± 0.11</td>
<td>1.2 ± 0.10</td>
<td>n.d.</td>
<td>0.35 ± 0.03</td>
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<tr>
<td>Bone</td>
<td>1.2 ± 0.24</td>
<td>1.1 ± 0.12</td>
<td>0.65 ± 0.14</td>
<td>0.36 ± 0.03</td>
<td>0.37 ± 0.04</td>
<td>0.24 ± 0.02</td>
<td>n.d.</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.2 ± 0.35</td>
<td>1.2 ± 0.15</td>
<td>0.63 ± 0.07</td>
<td>0.36 ± 0.02</td>
<td>0.34 ± 0.07</td>
<td>0.30 ± 0.06</td>
<td>n.d.</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>Tumor</td>
<td>7.0 ± 1.0</td>
<td>7.4 ± 1.1</td>
<td>6.3 ± 1.1</td>
<td>4.1 ± 0.58</td>
<td>2.2 ± 0.28</td>
<td>2.0 ± 0.39</td>
<td>n.d.</td>
<td>0.89 ± 0.20</td>
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</table>

<table>
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<tr>
<td></td>
<td>1 h p.i.</td>
<td>4 h p.i.</td>
<td>24 h p.i.</td>
<td>48 h p.i.</td>
<td>3 d p.i.</td>
<td>4 d p.i.</td>
<td>5 d p.i.</td>
<td>7 d p.i.</td>
</tr>
<tr>
<td>Blood</td>
<td>8.1 ± 1.2</td>
<td>4.3 ± 0.93</td>
<td>1.1 ± 0.17</td>
<td>0.42 ± 0.07</td>
<td>0.17 ± 0.04</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.00</td>
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<tr>
<td>Lung</td>
<td>4.6 ± 0.83</td>
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<td>0.93 ± 0.18</td>
<td>0.60 ± 0.14</td>
<td>0.39 ± 0.08</td>
<td>0.19 ± 0.06</td>
<td>0.11 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.6 ± 0.25</td>
<td>1.2 ± 0.19</td>
<td>0.57 ± 0.14</td>
<td>0.52 ± 0.13</td>
<td>0.37 ± 0.06</td>
<td>0.24 ± 0.05</td>
<td>0.15 ± 0.03</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>16 ± 2.1</td>
<td>28 ± 1.3</td>
<td>27 ± 3.6</td>
<td>24 ± 7.4</td>
<td>15 ± 3.7</td>
<td>8.4 ± 1.6</td>
<td>5.4 ± 0.46</td>
<td>4.5 ± 0.84</td>
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<tr>
<td>Stomach</td>
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<td>1.4 ± 0.25</td>
<td>0.63 ± 0.13</td>
<td>0.46 ± 0.19</td>
<td>0.29 ± 0.04</td>
<td>0.19 ± 0.12</td>
<td>0.13 ± 0.03</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.4 ± 0.31</td>
<td>0.88 ± 0.20</td>
<td>0.26 ± 0.10</td>
<td>0.14 ± 0.03</td>
<td>0.12 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>4.4 ± 0.14</td>
<td>3.8 ± 0.64</td>
<td>1.6 ± 1.4</td>
<td>1.8 ± 0.26</td>
<td>1.0 ± 0.37</td>
<td>0.77 ± 0.26</td>
<td>0.50 ± 0.11</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>Bone</td>
<td>1.5 ± 0.08</td>
<td>1.2 ± 0.14</td>
<td>0.56 ± 0.12</td>
<td>0.41 ± 0.07</td>
<td>0.27 ± 0.06</td>
<td>0.15 ± 0.03</td>
<td>0.11 ± 0.03</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.3 ± 0.06</td>
<td>1.2 ± 0.06</td>
<td>0.87 ± 0.20</td>
<td>0.52 ± 0.07</td>
<td>0.33 ± 0.07</td>
<td>0.14 ± 0.03</td>
<td>0.11 ± 0.05</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>Tumor</td>
<td>11 ± 1.3</td>
<td>18 ± 1.8</td>
<td>18 ± 2.8</td>
<td>14 ± 5.1</td>
<td>8.4 ± 2.3</td>
<td>4.7 ± 0.80</td>
<td>3.3 ± 0.66</td>
<td>3.1 ± 0.33</td>
</tr>
</tbody>
</table>

Values [% IA/g] represent the mean ± SD of data obtained from three animals per cohort.
Abbreviations: n.d., not determined.
Fig. 2.2. The graphs show the absolute quantity of accumulated radioactivity in tumor and kidney, indicated per MBq injected $^{177}$Lu-EC0800 (1 h to 7 d p.i., red) and $^{177}$Lu-cm09 (1 h to 10 d p.i., green), respectively. Areas under the curve (AUCs) for the tumor tissue (A) and for the kidney (B) were used for estimating the absorbed dose to these tissues.

In the case of $^{177}$Lu-cm09 an absorbed dose of ~1.4 Gy/MBq was estimated for tumor xenografts whereas for $^{177}$Lu-EC0800 the estimated absorbed tumor dose was ~0.44 Gy/MBq. For kidneys, an absorbed dose of ~2.3 Gy/MBq was estimated for $^{177}$Lu-cm09 and ~4.8 Gy/MBq for $^{177}$Lu-EC0800 (Table 2.3).

Table 2.3. Calculated absorbed tumor and kidney dose in nude mice after injection of different quantities of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>$^{177}$Lu-EC0800</th>
<th>$^{177}$Lu-cm09</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Injected activity [MBq]</td>
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<td></td>
</tr>
<tr>
<td>Tumor dose [Gy]</td>
<td>~4.4</td>
<td>~8.8</td>
</tr>
<tr>
<td>Kidney dose [Gy]</td>
<td>~48</td>
<td>~96</td>
</tr>
</tbody>
</table>
2.3.2. Therapy study in KB tumor-bearing mice

In study 1, the therapeutic effects of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 were compared for KB tumor-bearing nude mice over three months (Fig. 2.3, Table 2.4). Control mice showed a constant tumor growth. The first control mouse had to be euthanized after 17 d due to oversized tumors. A delayed tumor growth was observed for all groups of mice (groups B1-F1) which received radiofolate therapy. The TGI was 24% and 57% for animals treated with 10 MBq and 20 MBq of $^{177}$Lu-EC0800 (groups B1 and C1), respectively. For mice treated with 10 MBq, 20 MBq or 30 MBq of $^{177}$Lu-cm09 (groups D1, E1 and F1) the TGI was 73%, 97% and 96%, respectively.

Table 2.4. Analysis of the data obtained from KB tumor-bearing mice after injection of $^{177}$Lu-EC0800 or $^{177}$Lu-cm09, respectively. Body weight is given as mean ± SD at therapy start.

<table>
<thead>
<tr>
<th></th>
<th>Body weight [g]</th>
<th>Effective injected activity [MBq]</th>
<th>TGDI$_{5}$</th>
<th>TGDI$_{10}$</th>
<th>TGI [%]</th>
<th>Mean survival [d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A1</td>
<td>22.5 ± 0.9</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>$^{177}$Lu-EC0800</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B1</td>
<td>22.5 ± 1.6</td>
<td>10.5 ± 0.1</td>
<td>1.5</td>
<td>1.2</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Group C1</td>
<td>20.8 ± 0.8</td>
<td>21.0 ± 0.6</td>
<td>1.9</td>
<td>2.0</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>$^{177}$Lu-cm09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D1</td>
<td>24.2 ± 2.5</td>
<td>10.9 ± 0.8</td>
<td>2.3</td>
<td>1.8</td>
<td>73</td>
<td>36</td>
</tr>
<tr>
<td>Group E1</td>
<td>23.2 ± 2.5</td>
<td>21.2 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>97</td>
<td>1)</td>
</tr>
<tr>
<td>Group F1</td>
<td>21.2 ± 2.6</td>
<td>30.7 ± 1.8</td>
<td>-</td>
<td>-</td>
<td>96</td>
<td>1)</td>
</tr>
</tbody>
</table>

Abbreviations: TGDI, tumor growth delay index; TGI, tumor growth inhibition.

1) Undefined survival: ≥50% of mice were still alive at end of the study.
The application of 20 MBq and 30 MBq of $^{177}$Lu-cm09 resulted in complete tumor remission in 3 out of 5 mice and 4 out of 5 mice, respectively. Re-growth of tumors in mice that experienced complete tumor regression was not observed until the end of the study at day 90. Control mice of group A1 and mice of group B1 which received 10 MBq of $^{177}$Lu-EC0800 had comparable survival times of 20 d and 22 d, respectively (Table 2.4). For mice of group C1 which were treated with 20 MBq of $^{177}$Lu-EC0800 and mice of group D1, which received 10 MBq of $^{177}$Lu-cm09, the average survival time was 34 d (+70%) and 36 d (+80%), respectively. The average survival time remained undefined for animals of groups E1 and F1 which were injected with 20 MBq or 30 MBq of $^{177}$Lu-cm09 since more than 50% of the mice did not reach an end-point criterion until the end of the study. Based on these data the maximum tumor growth inhibition (>96%) was reached at a tumor dose of about 28 Gy (Fig. 2.4).
2.3.3. Long-term damage to the kidneys

2.3.3.1. Renal uptake of $^{99m}$Tc-DMSA

In study 2, kidney function was investigated by measuring renal uptake of $^{99m}$Tc-DMSA. Three weeks after injection of the radiofolates, $^{99m}$Tc-DMSA uptake was comparable among the treated groups and the control group (Fig. 2.5, A). At day 100 (week 15), mice of group C2, which were treated with 20 MBq of $^{177}$Lu-EC0800, and mice of group F2, which received 30 MBq of $^{177}$Lu-cm09, showed a significantly reduced renal uptake of $^{99m}$Tc-DMSA (P < 0.001) compared to untreated control mice (Fig. 2.5, B). At this stage, mice of groups B2, D2 and E2, which were injected with lower quantities of radiofolates, showed comparable renal uptake of $^{99m}$Tc-DMSA as was observed in control mice. In these animals (groups B2, D2 and E2), a significantly reduced renal uptake of $^{99m}$Tc-DMSA was, however, found 210 d p.i. (week 30; Fig. 2.5, C). At that time all mice of groups C2 and F2, which received high quantities of radiofolates, had already been euthanized due to reached endpoint criteria.
Fig. 2.5. Average $^{99m}$Tc-DMSA uptake per kidney (% injected activity (IA) per kidney) in week 3 (A), week 15 (B) and week 30 (C) after injection of various quantities of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 in study 2. * P < 0.05, *** P < 0.001.

In mice in which a significantly reduced $^{99m}$Tc-DMSA uptake was identified indicating impaired kidney function, the SPECT scans did not show a clear delineation of radioactivity accumulation in the cortex, but rather a diffuse distribution of radioactivity in the cortex as well as in the medulla and pelvis of the kidneys. As an example SPECT scans of kidneys are shown for one representative mouse of the control group (A2) as well as for groups C2 and E2 with mice receiving 20 MBq of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09, respectively (Fig. 2.6).
2.3.3.2 Renal plasma parameters

In study 2, the investigation of the levels of blood urea nitrogen and creatinine showed comparable values for control mice and mice injected with $^{177}$Lu-EC0800 or $^{177}$Lu-cm09 after 50 d (Table 2.5). After 130 d (week 19) the values for blood urea nitrogen of mice of group C2, which were treated with 20 MBq of $^{177}$Lu-EC0800, and mice of group F2, injected with 30 MBq of $^{177}$Lu-cm09, were in the same range (C2: $>35$ mM; F2: $26 \pm 13$ mM) but significantly ($P < 0.001$) different from those of control mice (A2: $9.8 \pm 0.6$ mM). At terminal state, blood urea nitrogen was significantly increased for all animals which had received radionuclide therapy, except for mice of group D2, which were treated with only 10 MBq of $^{177}$Lu-cm09 (Table 2.5). Short before euthanasia creatinine levels were also significantly higher in mice of group C2 ($114 \pm 62$ µM, $P < 0.001$) and F2 ($107 \pm 35$ µM, $P < 0.01$) in comparison to the levels determined in control mice ($<18$ µM ($n=3$), $24 \pm 5.1$ µM ($n=6$)).
Table 2.5. Values of plasma parameters from mice of study 2 after injection of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09, respectively. Blood urea nitrogen and creatinine levels were measured in week 8, 19, 26 after therapy start and before euthanasia (terminal).

<table>
<thead>
<tr>
<th>Week</th>
<th>Control Group A2</th>
<th>$^{177}$Lu-EC0800 Group B2: 10 MBq</th>
<th>$^{177}$Lu-EC0800 Group C2: 20 MBq</th>
<th>$^{177}$Lu-cm09 Group D2: 10 MBq</th>
<th>$^{177}$Lu-cm09 Group E2: 20 MBq</th>
<th>$^{177}$Lu-cm09 Group F2: 30 MBq</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood urea nitrogen [mM]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>8.8 ± 0.8 (n = 7)</td>
<td>8.4 ± 0.8 (n = 6)</td>
<td>12 ± 2.2 (n = 4)</td>
<td>11 ± 0.8 (n = 6)</td>
<td>8.3 ± 0.6 (n = 4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;50 (n = 1)</td>
<td>35 ± 9.4” (n = 5)</td>
<td>10 ± 0.7 (n = 6)</td>
<td>9.3 ± 2.5 (n = 6)</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>9.8 ± 0.6 (n = 9)</td>
<td>12 ± 3.0 (n = 6)</td>
<td>&gt;50” (n = 6)</td>
<td>9.2 ± 0.5 (n = 5)</td>
<td>21 ± 7.2” (n = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5 ± 0.8 (n = 9)</td>
<td>12 ± 1.6 (n = 5)</td>
<td>10 ± 2.6 (n = 5)</td>
<td>38 ± 4.1” (n = 4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>terminal</td>
<td>8.4 ± 1.4” (n = 9)</td>
<td>30 ± 12” (n = 5)</td>
<td>&gt;50” (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>&lt;18 (n = 3)</td>
<td>&lt;18 (n = 5)</td>
<td>&lt;18 (n = 2)</td>
<td>&lt;18 (n = 3)</td>
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<td></td>
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<td>21 ± 3.5 (n = 4)</td>
<td>20 ± 1.4 (n = 2)</td>
<td>20 ± 1.0 (n = 3)</td>
<td>18 (n = 1)</td>
<td>26 ± 6.3 (n = 6)</td>
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<tr>
<td></td>
<td></td>
<td>26 ± 9.6 (n = 8)</td>
<td>58 ± 43 (n = 6)</td>
<td>&lt;18 (n = 3)</td>
<td>22 ± 0.6 (n = 3)</td>
<td></td>
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<tr>
<td>19</td>
<td></td>
<td>26 ± 9.6 (n = 8)</td>
<td>&lt;18 (n = 1)</td>
<td>24 ± 6.1 (n = 5)</td>
<td>114 ± 62” (n = 6)</td>
<td>30 ± 19 (n = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 ± 1.7 (n = 4)</td>
<td>20.0 ± 1.6 (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>26 ± 9.6 (n = 8)</td>
<td>114 ± 62” (n = 6)</td>
<td>30 ± 19 (n = 5)</td>
<td>33 ± 7.6 (n = 5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>terminal</td>
<td>&lt;18 (n = 3)</td>
<td>25 ± 7.6 (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 ± 5.1 (n = 6)</td>
<td>114 ± 62” (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>&lt;18 (n = 2)</td>
<td>63 ± 46 (n = 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>terminal</td>
<td>&lt;18 (n = 2)</td>
<td>107 ± 35” (n = 5)</td>
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</tr>
</tbody>
</table>

Abbreviations: n.d., not determined (already euthanized). Statistics: Comparison of treated animals to control; * P < 0.05, ** P < 0.01, *** P < 0.001. Detection limits: Blood urea nitrogen: 50 mM; creatinine: 18 µM.
2.3.3.3. Morphological changes in renal tissue sections

The measurement of the kidney mass after euthanasia demonstrated a dose-dependent loss in tissue mass (Fig. 2.7). Control mice (group A2) had an average kidney mass of 234 ± 23 mg. Mice of groups B2, D2 and E2 showed comparable kidney mass (157 ± 24 mg, 178 ± 31 mg and 164 ± 47 mg, respectively). Lowest renal mass was observed in mice which were injected with high radioactive quantities of $^{177}$Lu-EC0800 (group C2, 83 ± 11 mg) or $^{177}$Lu-cm09 (group F2, 94 ± 12 mg).

![Fig. 2.7. Average kidney mass at terminal stage in control mice (group A2) and mice injected with $^{177}$Lu-EC0800 (groups B2 and C2) or $^{177}$Lu-cm09 (groups D2, E2 and F2). Significant deviation of values from treated mice compared to untreated controls; ** P < 0.01, *** P < 0.001.](image)

Investigation of renal tissue sections of radiofolate-treated mice of study 2 revealed morphological changes which indicated radiation nephropathy. The extent of damage correlated with the absorbed kidney dose. Maximum renal damage (scoring of 3.5) was reached at a kidney dose of ~69 Gy (Fig. 2.8).

![Fig. 2.8. Values of the scoring correlated with the approximate applied kidney dose in gray (Gy).](image)
Mice of group D2, which were injected with only 10 MBq of $^{177}$Lu-cm09 (~23 Gy) showed only minor signs of kidney damage (score 1). Mice of groups B2 and E2 which received 10 MBq of $^{177}$Lu-EC0800 (~48 Gy) or 20 MBq of $^{177}$Lu-cm09 (~46 Gy) were scored with 2.5, indicating moderate to moderately severe renal damage. The most pronounced damage (score 3.5) was observed in the renal tissue of mice of groups C2 and F2 which were injected with high-dosed $^{177}$Lu-EC0800 (20 MBq, ~96 Gy) and $^{177}$Lu-cm09 (30 MBq, ~69 Gy). Evaluation of HE-stained kidney sections revealed shrinkage of the parenchymal cortex in mice of groups B2, C2, E2 and F2 (Fig. 2.9). In PAS- and silver-stained tissue sections the presence of fibrin was observed in glomerular capillaries. Also fibrinoid changes of blood vessels, collapsed glomeruli and interstitial fibrosis and tubular atrophy were detected (Fig. 2.9). The presence of fibrin in glomerular blood vessels and glomeruli was confirmed by SFOG-staining of renal sections (Fig. 2.10).
Fig. 2.9. Microscopic images of transversal sections of the kidneys stained with hematoxylin-eosin (HE) and tissue samples stained with periodic acid-Schiff (PAS) or silver methenamine. Representative tissue samples are shown from control mice and from mice treated with $^{177}$Lu-EC0800 or $^{177}$Lu-cm09. Parenchymal shrinkage and morphological changes correlated with the applied renal dose. For treated animals glomeruli with collapsed capillary tufts (yellow arrows) and fibrinoid changes of glomerular capillary loops (black arrows) were observed. In addition, interstitial fibrosis and tubular atrophy (blue arrows) were detected.
2.3.4. Non-renal side effects

2.3.4.1. Determination of total bilirubin and alkaline phosphatase

Mice which received 20 MBq of $^{177}$Lu-EC0800 (group C2) or 30 MBq of $^{177}$Lu-cm09 (group F2) showed significantly increased plasma levels of total bilirubin ($P < 0.001$) short before euthanasia (Fig. 2.11, A). Moreover, mice of group C2 had also increased plasma levels of alkaline phosphatase ($P < 0.05$) at that time (Fig. 2.11, B). For mice injected with lower quantities of $^{177}$Lu-radiofolates (groups B2, D2 and E2) levels of total bilirubin and alkaline phosphatase were comparable to untreated control mice at any time of the study.

![Fig. 2.11](image-url)
2.3.4.2. Determination of viable white blood cells

Eight weeks after the treatment with $^{177}$Lu-EC0800 or $^{177}$Lu-cm09, changes in the number of white blood cells were not observed. However, compared to untreated control mice (group A2: 98 ± 1.0%) a moderate reduction in the number of viable white blood cells was noticed in mice, which were treated with 20 MBq of $^{177}$Lu-EC0800 (group C2: 92 ± 6.3%) and mice treated with 30 MBq of $^{177}$Lu-cm09 (group F2: 93 ± 4.4%) 130 d (week 19) after therapy start (Table 2.6).

Table 2.6. Determination of viable, apoptotic and dead white blood cells (WBCs) 130 d (week 19) after the injection of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 in study 2.

<table>
<thead>
<tr>
<th></th>
<th>Viable WBCs [%]</th>
<th>Apoptotic WBCs [%]</th>
<th>Dead WBCs [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A2</td>
<td>98 ± 1.0</td>
<td>1.5 ± 1.1</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>$^{177}$Lu-EC0800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B2 (10 MBq)</td>
<td>98 ± 1.6</td>
<td>1.9 ± 1.4</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>Group C2 (20 MBq)</td>
<td>92 ± 6.3**</td>
<td>3.2 ± 1.5</td>
<td>4.4 ± 5.2**</td>
</tr>
<tr>
<td>$^{177}$Lu-cm09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D2 (10 MBq)</td>
<td>98 ± 1.3</td>
<td>2.1 ± 1.3</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Group E2 (20 MBq)</td>
<td>98 ± 0.8</td>
<td>1.2 ± 0.6</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Group F2 (30 MBq)</td>
<td>93 ± 4.4´</td>
<td>3.7 ± 3.1´</td>
<td>3.2 ± 3.1</td>
</tr>
</tbody>
</table>

Statistics: Comparison of treated animals to control; * P < 0.05, ** P < 0.01, *** P < 0.001.

2.3.5. Monitoring of body weight and survival

Control mice (group A2) and mice treated with 10 MBq of $^{177}$Lu-cm09 (group D2) gained body weight up to 30% over eight months (Fig. 2.12, A). In animals of groups B2 and E2, body weight gain of about 20% was observed. In contrast, mice treated with 20 MBq of $^{177}$Lu-EC0800 (group C2) or 30 MBq of $^{177}$Lu-cm09 (group F2) lost body weight from day 120 on. The overall body weight loss until euthanasia was about 5%. At the end of the study more than 50% of the mice treated with 10 MBq or 20 MBq of $^{177}$Lu-cm09 (groups D2 and E2) were still alive (Fig. 2.12, B). Mice which received only 10 MBq of $^{177}$Lu-EC0800 (group B2) survived in average 34 weeks. Mice of groups C2 and F2 which received high doses of $^{177}$Lu-EC0800 or $^{177}$Lu-cm09 survived in average 24 weeks.
CHAPTER 2

Fig. 2.12. Average relative body weight (A) and mean survival time (B) after the injection of different quantities of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09. Relative body weight of mice of groups C2 and F2 is recorded until second to last animal was euthanized ($n \geq 2$).

2.4. Discussion

The recently developed folate radioconjugate, $^{177}$Lu-cm09, revealed enhanced blood circulation due to its albumin-binding properties [51]. As a consequence, the resulting tumor-to-kidney ratios of accumulated radioactivity were significantly increased at all points of time after injections of $^{177}$Lu-cm09, compared to those observed with $^{177}$Lu-EC0800 [51]. The increased tumor uptake of $^{177}$Lu-cm09 led to a ~3-fold higher tumor dose per MBq of injected $^{177}$Lu-cm09 (1.4 Gy/MBq) compared to the estimated absorbed dose calculated for $^{177}$Lu-EC0800 (0.44 Gy/MBq). The absorbed renal dose for $^{177}$Lu-cm09 was, however, clearly reduced (2.3 Gy/MBq) compared to the kidney dose which was estimated for $^{177}$Lu-EC0800 (4.8 Gy/MBq). In study 1, the application of $^{177}$Lu-cm09 resulted in complete tumor remission (TGI >95%) in the majority of mice of groups E1 (20 MBq) and F1 (30 MBq) which received a tumor dose of ~28 Gy and ~42 Gy, respectively. Even in mice of group D1 which received only 10 MBq of $^{177}$Lu-cm09 (~14 Gy) the tumor growth inhibition was still more than 70%. However, clearly reduced anti-tumor effects were observed in mice, treated with $^{177}$Lu-EC0800 (groups B1 and C1), where the absorbed tumor dose was below 10 Gy. A tumor dose >25 Gy resulted in maximum TGI of >96% (Fig. 2.4). Most likely, the necessary dose to treat tumors effectively would vary among different tumor types.

Potential long-term side effects to the kidneys caused by therapeutic doses of $^{177}$Lu-EC0800 and $^{177}$Lu-cm09 were investigated in study 2 using nude mice without tumors. For this purpose we used the method of quantifying renal uptake of $^{99m}$Tc-DMSA as a measure of
glomerular filtration and tubular function [100, 101]. In previous studies low inter-assay variability was demonstrated with this method which indicates its reliability for long-term investigations of kidney function in mice [102]. At day 100 (week 15) after start of the experiment, we found significantly reduced renal accumulation of $^{99m}$Tc-DMSA in mice of groups C2 and F2, which received a renal dose of $>69$ Gy, compared to the renal accumulation in untreated control mice (Fig. 2.5, B). At that time, the intrarenal distribution of $^{99m}$Tc-DMSA was diffusely distributed with a shift from the renal cortex to the medulla and pelvis of these mice (Fig. 2.6). This phenomenon has been described by Jouret et al. as an indication of dysfunctional proximal tubule cells [102]. Thus, the onset of impaired renal function was determined around 3-4 months after treatment of the mice, similar to what was previously reported for $^{177}$Lu-DOTATATE therapy in rats [90, 91]. Plasma values of blood urea nitrogen and creatinine determined in mice of groups C2 and F2 were significantly higher than the values obtained from control mice at terminal state (Table 2.5). This was again an indication of impaired kidney function. Mice injected with 10 MBq of $^{177}$Lu-cm09 did, however, not show significantly different blood urea nitrogen and creatinine levels than untreated control mice at the end of the study. These findings were in clear contrast to our observation that at that time renal uptake of $^{99m}$Tc-DMSA was significantly reduced for these mice (Fig. 2.5, C).

Lack of correlation between creatinine and blood urea nitrogen plasma levels and $^{99m}$Tc-DMSA uptake in the kidneys implies that measuring these plasma parameters is not a reliable method to determine impaired kidney function at an early stage. These results confirmed literature reports which state that serum creatinine as a reference parameter for kidney function must be regarded with caution [103]. Creatinine levels might vary with age, gender, muscle mass and metabolism, body weight and other parameters. Also the blood urea nitrogen level is not a very sensitive marker for kidney function as it might be affected by many renal and non-renal factors that are independent on renal damage [103]. It would clearly be of great benefit to determine a plasma parameter or marker in the urine, which would allow identification of renal injury at an early stage after radionuclide therapy. In this respect the retinol binding protein 4 might be a useful marker as recently demonstrated [104].

In study 2, the size and morphology of the kidneys was investigated after the mice had reached an end-point criterion or, latest after eight months, at the end of the study. Loss of kidney mass correlated with the applied renal dose (Fig. 2.7). Moreover, dose-dependent renal damage was evident based on the morphological changes of the renal tissue sections. According to the histopathological changes, it was found that a renal dose of $>69$ Gy (groups C2 and F2) led to severe renal damage (mean score 3.5), whereas the application of $\sim45-50$ Gy (groups B2 and E2) led to moderately severe renal damage (mean score 2.5, Fig. 2.8). A renal dose of $\sim23$ Gy (group D2) resulted in mild morphological alterations.
indicating only minor renal damage (mean score 1). Nevertheless, morphological changes indicating mild to severe thrombotic microangiopathy were observed in all investigated tissue sections of kidneys from treated mice. Severely damaged kidneys (groups C2 and F2) showed a majority of damaged glomeruli. In addition, extensive interstitial fibrosis and tubular atrophy as well as fibrinoid necrosis of blood vessels were determined in these tissue sections. The described time- and dose-dependent occurrence of functional and morphological changes of the kidneys was previously reported in preclinical studies performed with rodents as well as in clinical trials after whole-body irradiation or radionuclide therapy of cancer patients and is known as radiation nephropathy [75, 105]. According to the results obtained from study 2, a threshold dose limit for the kidneys, which would be considered as safe, was estimated as ~25 Gy. This value is comparable to the dose of 24 Gy which was assumed to be the threshold for renal toxicity in case of $^{177}$Lu-DOTATATE application to mice [92]. Also for humans an absorbed renal dose ~25 Gy was considered as safe during PRRT [56, 64]. Even though renal accumulation of radiofolates in mice and men is based on the same mechanism (radiofolate binding to the FR expressed in proximal tubule cells), these results which are obtained in mice will not be easily translated to humans. Importantly, there are several factors, including the kidney size and repair time of renal tissue as well as the biokinetics, which are different in mice and men as previously discussed by Svensson et al. [92].

Animals injected with 20 MBq of $^{177}$Lu-EC0800 (group C2) or 30 MBq of $^{177}$Lu-cm09 (group F2) showed increased alkaline phosphatase and/or total bilirubin plasma levels short before euthanasia (Fig. 2.11). Both parameters are used for evaluating liver function in humans [106]. Most likely, liver was injured in these mice due to high cross-organ dose from kidneys to hepatic tissue. Moreover, mice of groups C2 and F2 showed a constant decrease of body weight form day 120 on and had a reduced average survival (Fig. 2.12). This data indicated a general impaired health status of mice treated with high radioactive quantities of radiofolates.

The fraction of viable white blood cells in mice of study 2 was always above 90% and did not change significantly over the whole eight months of investigation. These findings indicated that undesired side effects of radiofolate therapy were limited to the kidneys and did not affect hematopoietic tissues, even in mice which received high quantities of $^{177}$Lu-EC0800 or $^{177}$Lu-cm09.
2.5. Conclusion

In this study, it was shown that application of a folate radioconjugates with an albumin-binding entity resulted in significantly improved anti-tumor effects and reduced renal toxicity compared to a conventional radiofolate without albumin-binding properties. In this study, we demonstrated the favorable effects of enhanced blood circulation of radiofolates regarding their risk-benefit profile. These results hold promise for future translation of the albumin binder concept to the clinics, potentially enabling FR-targeted radionuclide therapy in patients.
CHAPTER 3

Impact of Amifostine and Captopril on the Tissue Distribution and Renal Side Effects of Therapeutic Radiofolates

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Author contributions:

Stephanie Haller designed and carried out the experiments, analyzed and interpreted the data and wrote the chapter. Roger Schibli reviewed the manuscript. Cristina Müller supervised the study and revised the manuscript.
3.1. Introduction

Therapy with albumin-binding radiofolates was successfully applied in tumor xenografted mice [51]. However, it was recently shown by our group that the dose which is necessary to reach complete tumor remission (~28 Gy) lead to a renal dose burden of ~46 Gy which is not tolerated by the kidneys [107]. As this adverse effect is supposed to be the limiting factor also for a clinical application of this therapy strategy, it is essential to find methods to protect renal tissue during therapy with these radioconjugates.

The tolerability of healthy tissues towards ionizing radiation can be enhanced by the use of tissue-selective radioprotective agents allowing higher doses to be deposited at tumor site [17, 71]. Possible mechanisms of radiation protection include scavenging of free radicals, suppressing the formation of reactive oxygen species, enhancing cell repair processes and stabilization of cell compartments prone to radiation damage such as enzymes and DNA (Fig. 3.1) [17]. Since decades, efforts have been made to find tissue-selective and well-tolerated radioprotective drugs [108]. Most of the developed compounds were investigated in preclinical studies, but did not enter clinical trials. The main problem for a clinical application was the induction of acute adverse side effects and the undesired radioprotection of the tumor tissue [17, 71].

![Fig. 3.1. Possible mechanisms of action of radioprotectors including prevention of radiation damage (a-c) and enhancement of cell repair after radiation damage to the cell (d).](image-url)
One of the most promising radioprotectors developed so far is amifostine (WR-2771, Ethylol®) [17, 71, 108]. This compound was already used in numerous preclinical and clinical trials and is approved for its use as chemo- and radioprotector [71, 72]. Amifostine is a prodrug which has to be converted by alkaline phosphatase or spontaneous dephosphorylation to its active metabolite WR-1065 containing a thiol group [109]. Within the cell, WR-1065 is further metabolized to WR-33278 which is also an active metabolite of this drug (Fig. 3.2, A) [110]. The selective action of amifostine in healthy tissues is based on several mechanisms [72, 111]. Firstly, healthy tissues contain higher levels of capillary alkaline phosphatase, which is mainly responsible for the dephosphorylation of amifostine, compared to the tumor tissue. Secondly, the neutral pH in healthy tissues favors the enzymatic action of alkaline phosphatase compared to acidic conditions found at the tumor site. And thirdly, hypovascularization which is often observed in tumor tissue results in lower accumulation of amifostine and its metabolites at the tumor sites compared to healthy tissues. Besides that, the use of amifostine for renal radioprotection is favorable as alkaline phosphatase is also expressed in the proximal tubules of the kidneys and since WR-1065 is extensively taken up by the kidneys [112, 113]. Hence, the concentration of amifostine and its active metabolites was found approximately 100-fold higher in kidneys than in the tumor tissue of rats [114]. The main mechanisms of cytoprotection during radiation therapy is scavenging of free radicals by donating hydrogen atoms [115]. Moreover, oxidation reaction of WR-1065 with molecular oxygen to its disulfide compound (WR-33278) reduces oxygen levels in healthy tissues and, hence, decreases the formation of oxygen-induced radicals [116, 117]. The involvement in DNA repair and inhibition of apoptosis are also discussed in the literature and assumed to be responsible for the radioprotective effects of WR-1065 and WR-33278 [72, 118]. Furthermore, it was demonstrated that application of amifostine can induce condensation of DNA strands and, therewith it makes the DNA less vulnerable for attack by free radicals [110].

The renin-angiotensin system (RAS) plays a key role in the development of radiation nephropathy [75]. It was previously demonstrated that inhibition of the RAS by treatment with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor antagonists is not only successful in preventing, but also in treating radiation nephropathy [77, 119, 120]. The exact mechanisms responsible for these effects are not completely understood. Captopril (e.g. Captosol®) is one of the most extensively studied ACE inhibitors in the context of radioprotection. RAS inhibition by captopril leads to hypotension and, hence, the systemic pressure to organs such as lung and kidneys are decreased which was demonstrated to be a main mechanism for protecting lung from radiation pneumonitis [121]. Moreover, captopril can act as a radical scavenger as it contains a thiol functionality in its chemical structure (Fig. 3.2, B) [79]. The RAS promotes cell growth and, hence, renal cells pass more frequently
cell cycle checkpoints if the RAS is activated. In the case of activated RAS, cell damage will be recognized early and these cells will faster undergo apoptosis. RAS inhibition leads, as a consequence, to an inhibition of cell apoptosis and, therefore, mitigates radiation nephropathy [75].

Fig. 3.2. Chemical structures of amifostine and its active metabolites (A) and captopril (B). Amifostine (WR-2721) is mainly transformed by membrane-bound capillary alkaline phosphatase to its active metabolite WR-1065. Within the cell, WR-1065 is oxidized to the disulfide WR-33278, another active metabolite of amifostine. Captopril is already in its active form.

The goal of this study was to investigate the potential of amifostine and captopril to act as radioprotective agents during therapy with radiofolates. Firstly, we studied the impact of these two drugs on the tissue distribution of albumin-binding radiofolates in tumor-bearing mice by single photon emission computed tomography (SPECT). Based on these results, we decided which radioprotector will be further studied for its potential renoprotective effect. For investigating long-term side effects, mice without tumors were applied with doses of the $^{177}$Lu-radiolabeled albumin-binding radiofolate known to induce renal toxicity (~46 Gy) alone or in combination with the potential radioprotector. The kidney function was monitored by non-invasive determination of renal $^{99m}$Tc-DMSA uptake and by measuring renal function parameters in blood plasma over eight months.
3.2. Materials and methods

3.2.1. Cell culture

KB cells (human cervical carcinoma cells [96], subclone of HeLa cells, ACC-136) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were cultured in folate-free RPMI medium (FFRPMI, without folic acid, vitamin B\textsubscript{12} and phenol red, Cell Culture Technologies GmbH, Gravesano, Switzerland). The cell culture medium was supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics. Routine cell culture was performed twice a week.

3.2.2. Preparation of captopril and amifostine

Captopril (Sigma-Aldrich, St. Louis, MO, U.S.) was directly dissolved in the drinking water (0.1-0.25 mg/mL) for oral application. For intraperitoneal (i.p.) application, captopril was dissolved in sodium chloride 0.9% (NaCl 0.9%) at a concentration of 4-20 mg/mL. Amifostine (Calbiochem, Merck Millipore, Darmstadt, Germany) was dissolved in Milli-Q water at a concentration of 25 mg/mL.

3.2.3. Preparation of $^{177}$Lu-cm09 and $^{177}$Lu-cm10

The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-folate conjugates cm09 and cm10 were kindly provided by Merck & Cie (Schaffhausen, Switzerland). The two folic acid conjugates, cm09 and cm10, are composed of the same three functionalities (Fig. 3.3). The tissue distributions of $^{177}$Lu-cm09 and $^{177}$Lu-cm10 are similar and the quantity of accumulated radioactivity in the kidneys is comparable (unpublished data).
No-carrier added $^{177}$LuCl$_3$ was purchased from Isotope Technologies Garching (ITG GmbH, Garching, Germany). The radiolabeling of cm09 and cm10 was performed according to standard labeling procedures at a specific activity of 20-25 MBq/nmol. In brief, the $^{177}$Lu-radiolabeling of folic acid conjugates was performed in a mixture of hydrochloric acid (HCl, 0.05 M) and sodium acetate (Na-acetate, 0.5 M) at pH 4.5 for 10 min at 95°C. Sodium diethylenetriaminepentaacetic acid (Na-DTPA 5 mM, pH 5) was added afterwards for complexing potential traces of unreacted $^{177}$Lu(III). Quality control was performed by high performance liquid chromatography (HPLC) using a C-18 reversed phase column (XTerra™ MS C18, 5 µm, 15 cm x 4.6 cm, Waters). The mobile phase consisted of aqueous 0.1% trifluoroacetic acid (A) and acetonitrile (B) with a linear gradient from 5% B to 80% B over 15 min at a flow rate of 1 mL/min.
3.2.4. Animal studies

Animal experiments were conducted in compliance with the Swiss law of animal protection. All studies were approved by the local veterinarian department. Female athymic nude mice (CD-1 Foxn1/nu, 5-7 week-old, Charles River Laboratories, Sulzfeld, Germany) were fed ad libitum with a folate-deficient rodent diet (sنين Spezialdiäten GmbH, Soest, Germany) starting one week before tumor cell inoculation [122]. Mice without tumors of study 3, which were used for investigating potential renal toxicity, received folate-deficient diet during the first 3 weeks after injection of the radiofolate, followed by a standard rodent diet (Kliba Nafag, Kaiseraugst, Switzerland) until end of the study.

3.2.4.1. SPECT/CT

The tissue distribution of radiofolates after co-application of captopril and amifostine was investigated by SPECT imaging (pilot study 1 and 2). For this purpose, mice were inoculated with KB tumor cells (5 x 10^6 cells in 100 µL phosphate buffered saline (PBS) pH 7.2) into the subcutis of each shoulder. Two weeks after tumor cell inoculation, mice were co-applied with captopril (study 1) or amifostine (study 2) and 177Lu-radiofolate (~50 MBq, ~2 nmol) which were intravenously (i.v.) injected. The designs of pilot study 1 and 2 are described in Table 3.1. For in vivo SPECT scans the mice were anesthetized by inhalation of an isoflurane/oxygen mixture. SPECT/computed tomography (CT) images were acquired with a four-head multiplexing multipinhole small-animal SPECT/CT scanner equipped with tungsten collimators of nine 1.4-mm-diameter pinholes (NanoSPECT/CT, Mediso Medical Imaging Systems, Budapest, Hungary). SPECT images were obtained 4 h and/or 24 h after the injection of the radiofolate using Nucline Software (version 1.02, Bioscan Inc., Poway, California, U.S.). CT scans were performed with the integrated CT scanner. After acquisition, data were reconstructed with HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany) using γ-energies for 177Lu of 56.1 keV ± 10%, 112.9 keV ± 10% and 208.4 keV ± 10%. SPECT and CT data were automatically co-registered as both modalities share the same axis of rotation. Analysis of fused data sets and Gauss post-reconstruction filtering was performed using the VivoQuant post-processing software (version 1.23, inviCRO Imaging Services and Software, Boston, U.S.). The SPECT camera was calibrated in advance with a phantom filled with a known quantity of 177Lu allowing a quantification of the accumulated radioactivity in a defined volume. The average accumulation of radiofolates in tumors and kidneys was determined with VivoQuant post-processing program by using an approximate volume covering complete size of a single tumor or kidney, respectively. Tumor-to-kidney ratios were calculated for all mice.
Table 3.1. Design of the SPECT/CT studies investigating the impact of captopril (pilot study 1) and amifostine (pilot study 2) on the tissue distribution of radiofolates. In the case of drug application in the drinking water (oral), a water consumption of 3-5 mL per day was assumed [123].

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Application route</th>
<th>Dosage</th>
<th>Start before $^{177}$Lu-cm10 injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1</td>
<td>2</td>
<td>oral</td>
<td>0.1 mg/mL</td>
<td>24 h</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>oral</td>
<td>0.1 mg/mL</td>
<td>48 h</td>
</tr>
<tr>
<td>D1</td>
<td>2</td>
<td>oral</td>
<td>0.1 mg/mL</td>
<td>72 h</td>
</tr>
<tr>
<td>E1</td>
<td>2</td>
<td>oral</td>
<td>0.25 mg/mL</td>
<td>72 h</td>
</tr>
<tr>
<td>F1</td>
<td>2</td>
<td>i.p.</td>
<td>3 x 0.4 mg (every 24 h)</td>
<td>48 h</td>
</tr>
<tr>
<td>G1</td>
<td>2</td>
<td>i.p.</td>
<td>3 x 1 mg (every 12 h)</td>
<td>24 h</td>
</tr>
<tr>
<td>H1</td>
<td>2</td>
<td>i.p.</td>
<td>2 x 2 mg (every 24 h)</td>
<td>24 h</td>
</tr>
</tbody>
</table>

1) Two of the animals were injected with $^{111}$In-cm10 instead of $^{177}$Lu-cm10.

3.2.4.2. Renal side effects after co-application of $^{177}$Lu-cm09 and amifostine

Potential renoprotective effects of amifostine during therapy with albumin-binding radiofolates were investigated over eight months in non-tumor-bearing nude mice (study 3). Control mice were injected with only saline (group A3, n = 9) and mice of group B3 were applied with 20 MBq of $^{177}$Lu-cm09 (n = 6). Animals of group C3 were co-treated with 20 MBq of $^{177}$Lu-cm09 and amifostine (n = 6). The study design is presented in Table 3.2. Amifostine was i.v. injected 4 h, 24 h and 48 h after radiofolate application at a dosage of 2.5 mg per injection. End-point criteria were defined as (i) body weight loss >15% of the initial body weight or (ii) signs of unease. Determination of body weights was performed at the day of radiofolate
injection followed by a measurement once a week. The relative body weight (RBW) was calculated according to the equation \[ \text{RWB} = \frac{W_x}{W_0} \], where \( W_x \) is the weight at day \( x \) and \( W_0 \) is the weight at day 0. The average of the individual RBWs of mice was calculated for each group.

Table 3.2. Design of the long-term study investigating potential renoprotective effects of amifostine during \(^{177}\)Lu-radiofolate therapy (study 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>(^{177})Lu-cm09 (1 nmol, i.v.)</th>
<th>Application route</th>
<th>Dosage</th>
<th>Time after radiofolate injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>6</td>
<td>20 MBq</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>6</td>
<td>20 MBq</td>
<td>i.v.</td>
<td>2.5 mg</td>
<td>4 h, 24 h, 48 h</td>
</tr>
</tbody>
</table>

3.2.4.2.1. Quantification of renal uptake of \(^{99m}\)Tc-DMSA using SPECT

Kidney function was monitored by determining renal uptake of \(^{99m}\)Tc-dimercaptosuccinic acid (\(^{99m}\)Tc-DMSA) by quantitative SPECT [100]. DMSA (TechneScan® DMSA, Mallinckrodt Pharmaceuticals, Petten, The Netherlands) was radiolabeled with \(^{99m}\)TcNaTcO\(_4\), which was eluted from a \(^{99m}\)Mo/\(^{99m}\)Tc-generator (Mallinckrodt Pharmaceuticals, Petten, The Netherlands), according to manufacturer’s instructions at a specific activity of 3 GBq/1.2 mg. At day of measurement, each mouse was i.v. injected with 30-40 MBq of \(^{99m}\)Tc-DMSA 2 h before SPECT imaging. For in vivo SPECT scans, the mice were anesthetized by inhalation of an isoflurane/oxygen mixture. \(^{99m}\)Tc-DMSA uptake studies were performed in week 4, 15, 22 and 30 after radiofolate injection. SPECT imaging was performed as described above. The scan range was 3 cm which resulted in a scan time of about 7 min. After acquisition, SPECT data were reconstructed with HiSPECT software using \( \gamma \)-energies of 140.5 keV ± 10%. In advance, the SPECT camera was calibrated using a phantom filled with a known radioactive amount of \(^{99m}\)Tc allowing the determination of the accumulated radioactivity in a defined volume of tissue. The obtained data were analyzed using VivoQuant post-processing program. Renal \(^{99m}\)Tc-DMSA uptake was determined in a defined volume covering the size of a kidney. The determined activities were decay-corrected and expressed as percentage of injected activity (% IA) per kidney.
3.2.4.2.2. Determination of blood urea nitrogen and creatinine in blood plasma

The renal function parameters blood urea nitrogen and creatinine were determined in blood plasma in week 8, 19 and 26 after radiofolate application. Plasma samples were prepared by centrifugation of blood (~200 µL per mouse) which was sampled from the sublingual vein into a heparinized vial (Microvette 200, Sarstedt, Nümbrecht, Germany). The parameters were analyzed with a Fuji Dri-Chem 4000i analyzer (Polymed Medical Center AG, Glattbrugg, Switzerland) using 10 µL of blood plasma per measurement.

3.2.5. Statistical analysis

Data are presented as mean ± standard. Statistics was conducted by using one-way ANOVA with Bonferroni’s multiple comparison post-test (GraphPad Prism, version 5.01).

3.3. Results

3.3.1. Tissue distribution of radiofolates after co-application of captopril

A potential impact of captopril on the tissue distribution of radiofolates was investigated in KB tumor-bearing mice by SPECT/CT. Mice injected with the radiofolate only (group A1) showed tumor-to-kidney ratios of ~1 between 4 h and 24 h after radioactivity application (Fig. 3.4, Fig. 3.5). Comparable tumor-to-kidney ratios were observed for mice which were co-treated with captopril applied in the drinking water (0.1 mg/mL), starting 24 h (group B1), 48 h (group C1) and 72 h (group D1) before radiofolate injection. In group D1 the tumor-to-kidney ratio was ~1.5 for two mice and ~0.9 for two other mice, 24 h after injection of the radiofolate (Fig. 3.4). Oral application of captopril in the drinking water at high dosages (0.25 mg/mL), which was started 72 h prior to radiofolate injection, resulted in a tumor-to-kidney ratio of ~1.8 as observed in SPECT/CT images 24 h after radiofolate application (Fig. 3.4). Mice, which received the radiofolate co-applied with captopril i.p. at various dosages (group F1-H1), showed tumor-to-kidney ratios of ~0.8-1 at 4 h after radioactivity application (Fig. 3.5).
Fig. 3.4. Tumor-to-kidney ratios of the radiofolate $^{177}$Lu-cm10 (24 h p.i.) in control mice (group A1) and mice co-treated with various dosages of captopril applied in the drinking water (groups B1-E1) obtained by SPECT/CT imaging (A). A representative SPECT/CT image of each group is presented (B). Radioactivity uptake was observed in KB tumor xenografts (yellow arrows) and kidneys (green arrows).

Fig. 3.5. Tumor-to-kidney ratios of the radiofolate $^{177}$Lu-cm10 (4 h p.i.) in control mice and in mice i.p. injected with various dosages of captopril obtained by SPECT/CT imaging.
3.3.2. Tissue distribution of radiofolates after co-application of amifostine

A potential impact of amifostine on the biodistribution of radiofolates was studied in KB tumor-bearing mice using SPECT/CT. Tumor-to-kidney ratios of control mice injected with only $^{177}$Lu-cm09 (group A2) were ~1 (Fig. 3.6, A). Mice which received the radiofolate and three co-injections of amifostine (each time 2.5 mg, group B2) showed tumor-to-kidney ratios of ~1 around 24 h after radiofolate application (Fig. 3.6, B).

![Fig. 3.6. SPECT/CT images of a control mouse only injected with the radiofolate $^{177}$Lu-cm09 (A) and a mouse which was co-injected with amifostine (B). The images were acquired 24 h after radiofolate injection. Radioactivity accumulation was observed in KB tumor xenografts (yellow arrows) and kidneys (green arrows).](image)

3.3.3. Renal side effects after co-treatment with $^{177}$Lu-cm09 and amifostine

3.3.3.1. Renal uptake of $^{99m}$Tc-DMSA

In a long-term study (study 3), kidney function was monitored in mice which received a therapeutic dose of the radiofolate only (group B3) or in combination with amifostine (group C3). The results were compared to untreated control mice of group A3. Renal uptake of $^{99m}$Tc-DMSA, as a measure of renal function, was determined non-invasively using SPECT imaging. In week 15 after therapy start, the uptake of $^{99m}$Tc-DMSA per kidney was determined to be 10 ± 1.7% IA in control mice (Fig. 3.7, B). At that time, the renal uptake of $^{99m}$Tc-DMSA was reduced to 8.5 ± 1.1% IA/kidney in mice of group B3, which were injected with $^{177}$Lu-cm09, and to 7.4 ± 0.9% IA/kidney in mice of group C3, which received in addition amifostine. The renal uptake of $^{99m}$Tc-DMSA progressively decreased in these animals (week
At all times, renal uptake of $^{99m}$Tc-DMSA was not significantly different for mice injected with 20 MBq of $^{177}$Lu-cm09 (group B3) and mice which were co-treated with amifostine (group C3; Fig. 3.7).

![Fig. 3.7](image)

**Fig. 3.7.** Average $^{99m}$Tc-DMSA uptake per kidney (% IA) determined in week 4 (A), 15 (B), 22 (C) and 30 (D) after injection of the radiofolate alone or in combination with amifostine. Control mice were injected with only saline. Five mice of group C3 had to be euthanized before week 30 after radiofolate injection due to reached end-point criteria. ** P < 0.01, *** P < 0.001.

### 3.3.3.2. Renal plasma parameters

In study 3, the investigation of blood urea nitrogen and creatinine in blood plasma revealed comparable levels for control mice and mice injected with 20 MBq of $^{177}$Lu-cm09 (group B3) alone or in combination with amifostine (group C3) in week 8 and 19 after radiofolate application (Table 3.3). Approximately 26 weeks after radiofolate injection, the levels of blood urea nitrogen of mice of groups B3 and C3 were comparable (B3: $21 \pm 7.2$ mM; C3: $21 \pm 10$ mM) but significantly different from those of control animals (A3: $8.5 \pm 0.8$ mM, ...
Mice treated with $^{177}$Lu-cm09 (groups B3 and C3) showed also significantly increased blood urea nitrogen levels ($P < 0.001$) at terminal state. Creatinine plasma levels of mice which received radionuclide therapy did not differ from those of control mice at any time of the study (Table 3.3).

**Table 3.3.** Plasma levels of blood urea nitrogen and creatinine of control mice and mice injected with $^{177}$Lu-cm09 alone or in combination with amifostine. The parameters were determined in week 8, 19, 26 after radiofolate injection and before euthanasia (terminal).

<table>
<thead>
<tr>
<th>Week</th>
<th>Control Group A3</th>
<th>$^{177}$Lu-cm09 (20 MBq) Group B3: w/o amifostine</th>
<th>Group C3: with amifostine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood urea nitrogen [mM]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.8 ± 0.8 (n = 7)</td>
<td>8.3 ± 0.6 (n = 4)</td>
<td>10 ± 1.0 (n = 6)</td>
</tr>
<tr>
<td>19</td>
<td>9.8 ± 0.6 (n = 9)</td>
<td>9.3 ± 2.5 (n = 6)</td>
<td>12 ± 1.0 (n = 6)</td>
</tr>
<tr>
<td>26</td>
<td>8.5 ± 0.8 (n = 9)</td>
<td>21 ± 7.2* (n = 5)</td>
<td>21 ± 10* (n = 6)</td>
</tr>
<tr>
<td>terminal</td>
<td>8.4 ± 1.4 (n = 9)</td>
<td>38 ± 4.1*** (n = 4)</td>
<td>37 ± 9.9*** (n = 4)</td>
</tr>
<tr>
<td></td>
<td>Creatinine [µM]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&lt;18 (n = 3)</td>
<td>&lt;18 (n = 3)</td>
<td>&lt;18 (n = 2)</td>
</tr>
<tr>
<td></td>
<td>21 ± 3.5 (n = 4)</td>
<td>18 (n = 1)</td>
<td>35 ± 14 (n = 4)</td>
</tr>
<tr>
<td>19</td>
<td>26 ± 9.6 (n = 8)</td>
<td>&lt;18 (n = 2)</td>
<td>&lt;18 (n = 2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 ± 0 (n = 3)</td>
<td>23 ± 2.1 (n = 4)</td>
</tr>
<tr>
<td>26</td>
<td>&lt;18 (n = 4)</td>
<td>33 ± 7.6 (n = 5)</td>
<td>32 ± 5.9 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>20 ± 1.7 (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terminal</td>
<td>&lt;18 (n = 3)</td>
<td>&lt;18 (n = 2)</td>
<td>37 ± 15 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>24 ± 5.1 (n = 6)</td>
<td>63 ± 46 (n = 2)</td>
<td></td>
</tr>
</tbody>
</table>

Statistics: Comparison of treated animals to control; * $P < 0.05$, *** $P < 0.001$.
Detection limits: Blood urea nitrogen: 50 mM; creatinine: 18 µM.

### 3.3.3.3. Body weight and survival

The relative body weight of mice which received 20 MBq of $^{177}$Lu-cm09 only (group B3) and mice co-treated with amifostine (group C3) was comparable over the whole time of investigation (Fig. 3.8, A). Mice of group B3 reached maximum body weight 28 weeks after radiofolate injection (+28%). The highest body weight of mice of group C3 was recorded in week 32 (+25%). Control mice showed maximum body weight in week 28 (+35%). At the end
of the study more than 50% of mice which received 20 MBq of $^{177}$Lu-cm09 (group B3) were still alive (Fig. 3.8, B). Mice which were co-treated with 20 MBq of $^{177}$Lu-cm09 and amifostine (group C3) survived in average 29 weeks.

![Fig. 3.8. Average relative body weight (A) and mean survival time (B) after the injection of 20 MBq of $^{177}$Lu-cm09 alone (group B3) or in combination with amifostine (group C3). Control mice (group A3) were injected with only saline.](image)

### 3.4. Discussion

From a histological and functional perspective it was found that renal toxicity occurs in a dose- and time-dependent manner after application of therapeutic radiofolates as observed in preclinical studies performed by our group [107]. The co-application of radioprotectors during radiofolate therapy was considered as a feasible approach to enhance the tolerability of the renal tissue towards ionizing radiation.

The herein performed SPECT/CT studies revealed that the application of the ACE inhibitor captopril improved the tissue distribution of albumin-binding radiofolates (Fig. 3.4). High dosages of captopril applied in the drinking water (0.25 mg/mL) over 72 h before radiofolate injection (group E1) increased the tumor-to-kidney ratio of radiofolates ~2-fold in comparison to mice which only received the radiofolate (group A1). Quantitative SPECT studies revealed that the tumor-to-kidney ratio was mainly increased due to significantly higher tumor accumulation of the radiofolate and not due to a reduced retention of radioactivity in the kidneys. However, the mice showed signs of dehydration and lost body weight which was prohibitive for further use of this therapy scheme. Reduced captopril
concentrations in the drinking water (0.1 mg/mL instead of 0.25 mg/mL) were well tolerated by the animals and resulted also in improved tumor-to-kidney ratios of the radiofolate, if mice were pretreated with captopril over 72 h before radiofolate injection (group D1). However, the effect was not as significant as after application of high dosages of captopril (group E1). Moreover, the tissue distribution of the radiofolate was only enhanced in some animals and, hence, the interindividual variability was high (Fig. 3.4). In further SPECT studies, we showed that pretreatment with captopril applied in the drinking water <72 h was not sufficient to improve the tumor-to-kidney ratio of the radiofolate (groups B1 and C1). Also i.p. application of captopril at various dosages (groups F1-H1) did not influence the tumor-to-kidney ratio of the radiofolate. ACE inhibitors such as captopril are effective antihypertensive drugs with known renoprotective effects [17, 124, 125]. The application of ACE inhibitors leads to dilatation of intrarenal efferent vessels and, hence, induces a reduction of filtration pressure [124]. Thus, the observed improvement of the tumor-to-kidney ratio might be a consequence of reduced renal filtration of the albumin-binding radiofolate and, hence, prolonged circulation of the radioconjugate in the blood. It is, however, described that the effect of captopril on the glomerular filtration rate is different in each patient which requires individual dose finding [124]. Therefore, the herein observed low reproducibility might be a consequence of variable responses to captopril in each mouse. As it was impossible to monitor blood pressure in individual mice under the giving housing conditions, we did not further investigate the impact of captopril on the blood pressure and the effect it had possibly caused on the tissue distribution of radiofolates. However, captopril and other RAS inhibitors were reported to reduce renal toxicity after therapy with ionizing radiation [77, 78, 125, 126]. Therefore, it is still worthwhile to test the potential renoprotective effect of captopril during radiofolate therapy in long-term mouse studies independent on the effect on the tissue distribution which was found at high dosage.

Amifostine co-treatment had no impact on the tissue distribution of radiofolates as shown in SPECT/CT studies (Fig. 3.6). Long-term investigations performed over eight months revealed that renal $^{99m}$Tc-DMSA uptake and the plasma parameters, blood urea nitrogen and creatinine, were comparable for mice injected with 20 MBq of $^{177}$Lu-cm09 (group B3) and mice which were co-treated with amifostine (group C3; Fig. 3.7, Table 3.3). Mice of both groups (B3 and C3) showed reduced kidney uptake of $^{99m}$Tc-DMSA 15 weeks after radiofolate application compared to untreated mice (group A3). Moreover, levels of blood urea nitrogen were increased 26 weeks after radiofolate injection in comparison to untreated control mice. These tests indicated impaired kidney function of treated mice. Body weights of mice of groups B3 and C3 were slightly lower compared to control mice, and in group B3 as well as C3 some of the animals reached an end-point criterion before termination of the study (Fig. 3.8). Our results indicated that amifostine did not preserve the kidney function during
therapy with radiofolates at the applied dosage of three-times 2.5 mg. The active form of amifostine (WR-1065) is reported to reach maximal accumulation in kidneys within minutes after amifostine injection and the renal concentration has been found to be reduced to 50% after 1-2 h in rats [127]. Renal retention of $^{177}$Lu-cm09 is, however, very high and only after 10 d the majority of radioactivity is cleared from the kidneys [107]. Hence, multiple injections of amifostine might be necessary to protect kidneys during therapy with the albumin-binding radiofolate $^{177}$Lu-cm09. Possibly, a daily application of amifostine up to 10 d after injection of $^{177}$Lu-cm09 would protect kidneys from radiation damage.

The herein obtained data with amifostine were different to what was previously observed with radiolabeled somatostatin analogs. A co-treatment of rats with a $^{177}$Lu-radiolabeled somatostatin analog and amifostine, which was applied daily over one week, successfully reduced functional renal damage [73]. Moreover, it was demonstrated that amifostine competes with somatostatin analogs in renal tubules for reabsorption and, therefore, reduces the renal uptake of these derivatives [74]. Most probably, the observed renoprotective effect of amifostine is due to the reduced renal dose burden and not due to its function as a radioprotector.

In our study, creatinine levels of untreated and treated mice were comparable over the whole time of investigation In contrast, the measured blood urea nitrogen levels increased over time in treated mice. Hence, determination of blood urea nitrogen levels was more sensitive than the measurement of creatinine. A similar observation was already previously reported in the literature [128].

### 3.5. Conclusion

In this study, we found that amifostine had no impact on the tissue distribution of radiofolates. Moreover, it did also not protect kidneys from radiation damage in mice treated with high quantities of radiofolates. Due to the fast clearance of amifostine, it may be necessary to administer it at multiple injections in order to induce radioprotective effects after radionuclide therapy with long-circulating radiofolates. Unexpectedly, we found that captopril improved in some mice the tumor-to-kidney ratio of radiofolates by a factor of ~2 while it had no effect in others. This high interindividual variability was considered as prohibitive for further investigations of this combination treatment. It may be worthwhile to study retrospectively a potentially improved tumor-to-kidney ratio in patients treated with radiolabeled somatostatin analogs who received at the same time antihypertensive drugs. Such analysis may shed light on the role of the blood pressure regarding renal damage after radionuclide therapy.
CHAPTER 4

A Comparative Study about Renal Toxic Side Effects after \( \alpha \)- and \( \beta^- \)-Therapy

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Parts of this chapter will be published in a modified version.

**Author contributions:**

Stephanie Haller performed the radiolabeling, in vivo studies, analyzed and interpreted the data and wrote the chapter. Katharina A. Domnanich, Christiaan Vermeulen and Nicholas P. van der Meulen provided the radionuclides. Peter Bernhardt performed dosimetric calculations. Giovanni Pellegrini stained and analyzed tissue samples. Roger Schibli reviewed the manuscript. Cristina Müller supervised the study and reviewed the manuscript.
4.1. Introduction

Radionuclide therapy of cancer with β-particle-emitting radionuclides (e.g. $^{177}$Lu, $^{90}$Y, $^{186}$Re, $^{131}$I) is successfully applied in clinical trials [7, 8]. These days, somatostatin analogs (e.g. $^{177}$Lu/$^{90}$Y-DOTA-[Tyr$^3$]-octreotate ($^{177}$Lu/$^{90}$Y-DOTATATE)) and other small molecules (e.g. $^{186}$Re-hydroxyethylidene diphosphonate ($^{186}$Re-HEDP)) radiolabeled with β-particles and $[^{131}]$I$\text{NaI}$ are applied on a regular basis for cancer treatment in clinics. In contrast, clinical experiences with α-therapy are scarce due to limited availability of α-particle-emitting radionuclides with suitable decay characteristics [129]. Most critical are the short half-lives of numerous α-particle emitters and/or the formation of α-emitting daughter radionuclides in their decay chain [130]. These daughter radionuclides can cause toxic effects to healthy tissues if their distribution is not controlled. The treatment of bone metastasis in prostate cancer patients by use of $^{223}$RaCl$_2$ (Xofigo®, formerly Alpharadin®) was so far the most successful clinical application of α-therapy [9].

The application of α-particle emitters is very promising for tumor treatment since these radionuclides have a high potential for inducing cell death. In several preclinical trials it was shown that peptides, radiolabeled with the α-emitters $^{213}$Bi or $^{225}$Ac, were therapeutically more effective in comparison to peptides radiolabeled with β-particle emitters such as $^{177}$Lu [131-133]. A potential benefit of α-particles over β-particles was recently also demonstrated in the mouse model using an albumin-binding folate (cm09) radiolabeled with $^{149}$Tb and $^{161}$Tb [10, 134, 135]. Moreover, preclinical and clinical studies indicated that α-particle-emitting radionuclides might be successful in the treatment of cancer types which are resistant towards chemo- and radiotherapy including β-radionuclide therapy [85, 136, 137].

The high biological effectiveness of α-particles can be explained by the short tissue range (0.04-0.1 mm, 5-10 cell diameters) and the high linear energy transfer (LET, ~80 keV/µM). These properties lead to beneficial cancer treatment if the radiopharmaceutical is uniformly distributed at the tumor site and at the same time impairment of surrounding healthy tissue is limited [5]. In contrast, β-particles have a longer tissue path-length (0.05-12 mm, up to hundreds of cell diameters), but the LET is ~400-fold lower (~0.2 keV/µM) [5]. Therefore, the application of β-particles might be advantageous for therapy of larger tumors and metastases, but at the same time the risk for irradiation of non-targeted tissues is enhanced due to the "crossfire" effect. Tissue damage caused by α-particles is even more potentiated as its effects are independent on cell cycle progression and oxygen levels which are often low in tumors [7].

Targeted radionuclide therapy with small molecular weight radiopharmaceuticals (e.g. vitamin- or peptide-based radioconjugates) comprise the risk for renal toxic side effects as
these derivatives show high retention in the renal cortex [63, 91]. To date, little is known about the toxic side effects of targeted α-therapy to the kidneys.

In this work, we had the unique opportunity to have α- and β- emitting radionuclides of the same element available, namely 149Tb (α-particle emitter) and 161Tb (β-particle emitter; Table 4.1). This allowed us to compare potential toxic side effects to the kidneys after application of targeted α- and β- radionuclide therapy using chemically identical folic acid radioconjugates (149Tb-cm09 and 161Tb-cm09). We investigated the kidney function of non-tumor-bearing mice after the injection of various radioactive quantities of 149Tb-cm09 and 161Tb-cm09 over eight months. At different time points, blood urea nitrogen and creatinine was determined in blood plasma and the kidney uptake of 99mTc-DMSA was investigated as a non-invasive kidney function test. At the study end, morphological changes of the renal tissue were evaluated and potential differences among damage after α- and β- radionuclide therapy were assessed.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Emission type</th>
<th>E_α [MeV]</th>
<th>E_β−av [keV]</th>
<th>Max. tissue range of α/β− [mm]</th>
<th>T_{1/2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>149Tb</td>
<td>α, γ, β⁺</td>
<td>3.97</td>
<td>-</td>
<td>0.028</td>
<td>4.1 h</td>
</tr>
<tr>
<td>161Tb</td>
<td>β−, Auger, γ</td>
<td>-</td>
<td>154</td>
<td>1.7</td>
<td>6.9 d</td>
</tr>
</tbody>
</table>

4.2. Materials and methods

4.2.1. Radionuclides

149Tb was produced at the on-line isotope mass separation facility ISOLDE (CERN, Geneva, Switzerland) as previously reported [10, 134, 140]. In brief, it was produced by high-energy proton-induced spallation of tantalum foil targets and released from the target, using surface ionization and resonant laser ionization. The ionized spallation products were separated according to their mass-to-charge ratio as previously described in the literature [140, 141]. Spallation products of mass number 149 were collected on a zinc-coated gold foil and shipped to PSI for separation of the desired 149Tb by cation exchange chromatography as previously reported [134]. After separation, 149Tb was made available in a solution of α-hydroxyisobutyric acid (α-HIBA, pH ~4.5) at an activity concentration of ~3-6 MBq/100 µL.

No-carrier added 161Tb was produced at the high-flux neutron reactor of the Institut Laue-Langevin (ILL, Grenoble, France) using enriched 160Gd targets. Separation of 161Tb from the
CHAPTER 4

target material was carried out at PSI by means of cation exchange chromatography as previously reported [14]. $^{161}$Tb was available in 0.05 M hydrochloric acid (HCl, pH $\sim$1.5) at an activity concentration of 2-5 GBq/100 µL.

4.2.2. Preparation of $^{149}$Tb-cm09 and $^{161}$Tb-cm09

$^{149}$Tb in $\alpha$-HIBA (0.13 M, pH $\sim$4.5, 330 µL, $\sim$20 MBq) was directly mixed with the DOTA-folate conjugate cm09 (14 µL, 1 mM) resulting in a specific activity of $\sim$1.4 MBq/nmol. $^{161}$Tb in HCl (0.05 M, pH $\sim$1.5, 45 µL, $\sim$300 MBq) was added to a mixture of cm09 (15 µL, 1 mM), HCl (0.05 M, 55 µL) and sodium acetate (0.5 M, pH 8, 20 µL) resulting in a specific activity of 20 MBq/nmol and a reaction pH of $\sim$4.5. The reaction mixtures were heated for 10 min at 95°C. After reaction, sodium diethylenetriaminepentaacetic acid (Na-DTPA, 10 µL, 5 mM, pH 5) was added for complexation of potential traces of unreacted $^{149}$Tb(III) or $^{161}$Tb(III). Quality control was performed by high performance liquid chromatography (HPLC) using a C-18 reversed phase column (XTerra™ MS C18, 5 µm, 15 cm x 4.6 cm, Waters). The mobile phase consisted of aqueous 0.1% trifluoroacetic acid (A) and methanol (B) with a linear gradient from 5% B to 80% B over 25 min at a flow rate of 1 mL/min.

4.2.3. Animal studies

The in vivo experiments were approved by the local veterinarian department and conducted in accordance with the Swiss law of animal protection. Female athymic nude mice (CD-1 Foxn1/nu, 5-7-week-old) were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were fed with a folate-deficient rodent diet (ssniff Spezialdiäten GmbH, Soest, Germany) ad libitum starting one week prior to the injection of the radiofolate. The folate-free diet was replaced by a standard rodent diet (Kliba Nafag, Kaiseraugst, Switzerland) three weeks after application of the radiofolates.

4.2.3.1. Dosimetry

Dosimetric calculations were performed on the basis of biodistribution data previously obtained by our group with $^{161}$Tb-cm09 in KB tumor xenografted mice [10, 135]. To estimate the equivalent absorbed radiation dose of $^{149}$Tb-cm09 and $^{161}$Tb-cm09 in tumor xenografts and the renal tissue, the following calculations were made: (i) the cumulative radioactivity was calculated by fitting a bi-exponential curve to the non-decay corrected biodistribution data (% IA/g). The areas under the curve (AUCs, MBq·s) were
determined by integrating the bi-exponential function to infinity. (ii) The mean absorbed dose
to tumor xenografts and kidneys was assessed for a tumor size of ~40-50 mg and a kidney
mass of ~125 mg at the time of injection. The absorbed fraction to tumors and kidneys with
the radioactivity uniformly distributed was simulated by PENELope [97]. (iii) The mean
absorbed dose (Gy/MBq) was calculated by multiplying the AUC normalized to 1 MBq
injected activity (s) with the absorbed fraction and the emitted energy per decay for $^{149}$Tb and
$^{161}$Tb (ENSDF decay data in the MIRD format, www.nndc.bnl.gov) and multiplied with a
conversion factor.

4.2.3.2. Long-term side effects after application of $^{149}$Tb-cm09 and $^{161}$Tb-cm09

In a long-term experiment the side effects after application of $^{149}$Tb-cm09 and $^{161}$Tb-cm09 at
therapeutic doses were investigated in nude mice without tumors over eight months. Control
mice were injected with only saline (group A, n = 12). Mice of group B were injected with
2.5 MBq of $^{149}$Tb-cm09 (1.8 nmol, n = 6) and mice of group C were administered with 5 MBq
of $^{149}$Tb-cm09 (3.6 nmol, n = 2). Three different groups of mice were injected with 10 MBq
(group D, 0.5 nmol, n = 6), 20 MBq (group E, 1 nmol, n = 12) or 30 MBq of $^{161}$Tb-cm09
(group F, 1.5 nmol, n = 6). End-point criteria were defined as (i) body weight loss >15% of
the initial body weight or (ii) signs of unease. The body weights were determined at the day
of radiofolate injection followed by a measurement once a week. The relative body weight
(RBW) was calculated according to the equation $\text{RWB} = \frac{W_x}{W_0}$ in which $W_x$ is the weight at
day x and $W_0$ is the weight at day 0. RBW of each group was indicated as the average of the
individual RBW of each mouse.

4.2.3.2.1. Quantification of renal uptake of $^{99m}$Tc-DMSA using SPECT

The renal function was monitored by the determination of the renal uptake of $^{99m}$Tc-
dimercaptosuccinic acid ($^{99m}$Tc-DMSA) by quantitative single photon emission computed
tomography (SPECT) [100]. For this purpose DMSA (TechneScan® DMSA, Mallinckrodt
Pharmaceuticals, Petten, The Netherlands) was radiolabeled with $[^{99m}\text{Tc}]\text{NaTcO}_4$ which was
eluted from a $^{99m}$Mo/$^{99m}$Tc-generator (Mallinckrodt Pharmaceuticals, Petten, The Netherlands).
The animals were i.v. injected with 30-40 MBq of $^{99m}$Tc-DMSA 15, 22 and 30 weeks after
injection of the radiofolates. SPECT acquisitions were performed 2 h after the injection of
$^{99m}$Tc-DMSA with 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 collimator of nine 1.4 mm-diameter pinholes and a thickness
of 10 mm. The energy peak for the camera was set at 140.5 keV ± 10%. The SPECT images
were acquired with Nucline Software (version 1.02, Bioscan Inc., Poway, U.S.). The scan range was 3 cm which resulted in a scan time of about 7 min. After the performance of the SPECT scan, the data were reconstructed with HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany). The NanoSPECT was calibrated in advance with a phantom filled with a known radioactive amount of $^{99m}$Tc allowing the determination of the accumulated radioactivity in a defined volume of tissue. The uptake of $^{99m}$Tc-DMSA in the kidneys was determined in a defined volume covering the size of a kidney using VivoQuant post-processing program (version 1.23, inviCRO Imaging Services and Software, Boston, U.S.). The determined activities were decay-corrected and expressed as percentage of injected activity (% IA) per kidney.

4.2.3.2.2. Determination of blood plasma parameters

Plasma parameters such as creatinine, blood urea nitrogen, alkaline phosphatase and total bilirubin were measured in week 8, 19, 26 and before euthanasia. For this purpose blood samples were drawn from the sublingual vein of each mouse, collected in heparinized vials (Microvette 200, Sarstedt, Nümbrecht, Germany) and centrifuged to get plasma. Blood plasma parameters were analyzed using a Fuji Dri-Chem 4000i analyzer (Polymed Medical Center AG, Glattbrugg, Switzerland).

4.2.3.2.3. Kidney size and morphology

Terminally collected kidneys were weighed, fixed in 4% buffered formalin (Formafix buffered 4%, Formafix Switzerland AG, Hittnau, Switzerland) and embedded in paraffin. Sections (3-5 µm thick) were prepared, mounted on glass slides, deparaffinized in xylene, rehydrated through graded alcohols and stained with hematoxylin and eosin (HE) for the histological examination, which was performed by an independent pathologist. Histochemical stains such as periodic acid-Schiff (PAS), Masson trichrome or phosphotungstic acid hematoxylin (PTAH) were applied on selected tissue samples. Each compartment (glomeruli, tubules, interstitium and blood vessels) was evaluated separately to assess renal damage. A semi-quantitative scoring of 0-4 was employed, indicating normal histology (0), minimal (1), mild (2), moderate (3) or severe (4) renal damage.

4.2.3.2.4. Determination of white blood cell death by FACS

Fluorescence activated cell sorting (FACS; Guava EasyCyte Plus Flow Cytometer System, Merck Millipore, Darmstadt, Germany) was used to count the number of white blood cells in
week 8. Blood samples were diluted in PBS (pH 7.4, 1:5) and then mixed 1:10 with Guava ViaCount Reagent (Merck Millipore, Darmstadt, Germany). Before analysis the samples were incubated at room temperature for 10 min.

4.2.4. Statistical analysis

Data are presented as mean ± standard. Statistics was conducted by using one-way ANOVA with Bonferroni’s multiple comparison post-test (GraphPad Prism, version 5.01).

4.3. Results

4.3.1. Radiosynthesis of $^{149}$Tb-cm09 and $^{161}$Tb-cm09

Radiolabeling of cm09 with $^{149}$Tb (~1.4 MBq/nmol) and $^{161}$Tb (~20 MBq/nmol) resulted in a radiochemical purity of >98% (Fig. 4.1). Only trace amounts (<2%) of $^{149}$Tb(III) and $^{161}$Tb(III), respectively, were detected.

Fig. 4.1. HPLC chromatograms showing the γ-traces of $^{149}$Tb-cm09 (Rt = 19 min, red) and of $^{161}$Tb-cm09 (Rt = 19 min, blue). Unconjugated $^{149}$Tb(III) and $^{161}$Tb(III), respectively, was detected at a retention time of ~3 min.

4.3.2. Renal dose estimations for $^{149}$Tb-cm09 and $^{161}$Tb-cm09

The absorbed kidney dose was estimated to be ~0.66 Gy/MBq for $^{149}$Tb-cm09. In the case of $^{161}$Tb-cm09 the absorbed kidney dose was determined to be ~3.0 Gy/MBq for $^{161}$Tb-cm09 (Table 4.2).
**Table 4.2.** Calculated absorbed kidney dose in nude mice after injection of different quantities of $^{149}$Tb-cm09 and $^{161}$Tb-cm09, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>$^{149}$Tb-cm09</th>
<th>$^{161}$Tb-cm09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected activity [MBq]</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Kidney dose&lt;sup&gt;1)&lt;/sup&gt; [Gy]</td>
<td>~1.7</td>
<td>~3.3</td>
</tr>
</tbody>
</table>

<sup>1</sup> The higher biological effectiveness of $\alpha$-particles compared to $\beta$-particles is not considered in these calculations.

### 4.3.3. Long-term damage to the kidneys

#### 4.3.3.1. Monitoring of body weight and survival

The average relative body weight of animals injected with 2.5 MBq or 5 MBq of $^{149}$Tb-cm09 (groups B and C) or 10 MBq or 20 MBq of $^{161}$Tb-cm09 (groups D and E) was approximately 10% lower compared to control mice (group A) between weeks 16 and 35 (Fig. 4.2, A). Animals injected with high radioactive quantities of $^{161}$Tb-cm09 (group F) showed body weight loss up to 6% of the initial body weight at week 17. Additionally, the mean survival of these mice (group F) was reduced to 17 weeks (Fig. 4.2, B). Mice injected with 20 MBq of $^{161}$Tb-cm09 (group E) showed an average survival time of 31 weeks. Animals injected with $^{149}$Tb-cm09 (groups B and C) and animals that had received low quantities of $^{161}$Tb-cm09 (group D) survived until the end of the study eight months after injection of the radioconjugates.
4.3.3.2. Renal uptake of $^{99m}$Tc-DMSA

Animals injected with 20 MBq or 30 MBq of $^{161}$Tb-cm09 (groups E and F) showed a significantly decreased renal uptake of $^{99m}$Tc-DMSA ($P < 0.05$) compared to control mice (group A) at 100 d after radiofolate injection (week 15; Fig. 4.3, A). Mice injected with lower quantities of $^{161}$Tb-cm09 (10 MBq, group D) showed a significant progressive reduction of renal $^{99m}$Tc-DMSA uptake ($P < 0.05$) in week 22 and 30 (150 d p.i and 210 d p.i.; Fig. 4.3, B and C), whilst no significant change was observed at earlier times (week 15). The kidney uptake of $^{99m}$Tc-DMSA was comparable for mice injected with 2.5 MBq or 5 MBq of $^{149}$Tb-cm09 (groups B and C) and control animals (group A) at all investigated time points.
Fig. 4.3. Average $^{99m}$Tc-DMSA uptake per kidney (% IA) in week 15 (A), 22 (B) and 30 (C) after injection of various quantities of $^{149}$Tb-cm09 and $^{161}$Tb-cm09. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.3.3.3. Renal plasma parameters

Blood urea nitrogen levels were significantly increased ($P < 0.001$) in animals injected with 30 MBq of $^{161}$Tb-cm09 (group F, 42 ± 8.1 mM) compared to control mice (group A, 8.6 ± 1.2 mM) shortly before euthanasia (Table 4.3). At time of euthanasia also creatinine levels were significantly elevated (77 ± 17 µM, $P < 0.05$) in mice of group F in comparison to control mice (28 ± 10 µM). For all other animals which received $^{149}$Tb-cm09 (groups B and C) or lower quantities of $^{161}$Tb-cm09 (groups D and E), blood urea nitrogen and creatinine levels were comparable to untreated control mice at each time of investigation in this study (Table 4.3).
Table 4.3. Values of plasma parameters from mice injected with various radioactive amounts of $^{149}$Tb-cm09 and $^{161}$Tb-cm09. Blood urea nitrogen and creatinine levels were measured in week 8, 19, 26 after therapy start and before euthanasia (terminal).

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>$^{149}$Tb-cm09</th>
<th>$^{161}$Tb-cm09</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B: 2.5 MBq</td>
<td>Group C: 5.0 MBq</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group A</td>
<td>Group B: 2.5 MBq</td>
<td>Group C: 5.0 MBq</td>
</tr>
<tr>
<td></td>
<td>&lt;18</td>
<td>&lt;18</td>
<td>&lt;18</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 6)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group A</td>
<td>Group B: 2.5 MBq</td>
<td>Group C: 5.0 MBq</td>
</tr>
<tr>
<td></td>
<td>&lt;18</td>
<td>26 ± 5.7</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 4)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>26</td>
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<td>Group A</td>
<td>Group B: 2.5 MBq</td>
<td>Group C: 5.0 MBq</td>
</tr>
<tr>
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<td>&lt;18</td>
<td>21 ± 2.9</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>(n = 7)</td>
<td>(n = 3)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>terminal</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group A</td>
<td>Group B: 2.5 MBq</td>
<td>Group C: 5.0 MBq</td>
</tr>
<tr>
<td></td>
<td>&lt;18</td>
<td>50 ± 50</td>
<td>&lt;18</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 4)</td>
<td>(n = 1)</td>
</tr>
</tbody>
</table>

Abbreviations: n.d., not determined (already euthanized).

Statistics: Comparison of treated animals to control; * P < 0.05, *** P < 0.001.

Detection limits: Blood urea nitrogen: 50 mM; creatinine: 18 µM.
4.3.3.4. Renal histopathology

Investigation of renal tissue sections revealed morphological changes of the cortex consistent with radiation nephropathy. These findings ranged from slight glomerular and tubular injury (e.g. mild reduction of glomerular capillaries and tubular atrophy) to severe effacement of the renal architecture. Severe damage was represented by glomerular shrinkage and glomerulosclerosis, extensive tubular atrophy and interstitial fibrosis which led to cortical parenchymal collapse (Fig. 4.4). Morphological changes were observed in all animals which received $^{149}$Tb-cm09 and $^{161}$Tb-cm09, with a generally dose-dependent increased severity.

Fig. 4.4. Microscopic images (magnification 1.25x) of transversal sections of the kidneys stained with hematoxylin-eosin (HE). Cortex width, indicated by bidirectional arrows, is clearly reduced in the kidneys exhibiting severe renal damage (mean score of ~3; F). A, untreated mouse; B, 2.5 MBq $^{149}$Tb-cm09 (group B); C, 5 MBq $^{149}$Tb-cm09 (group C); D, 10 MBq $^{161}$Tb-cm09 (group D); E, 20 MBq $^{161}$Tb-cm09 (group E); F, 30 MBq $^{161}$Tb-cm09 (group F).
Mice injected with 2.5 MBq of $^{149}$Tb-cm09 (group B) and animals treated with 10 MBq of $^{161}$Tb-cm09 (group D) showed minimal renal injury which was comparable in both groups (mean score group B: 1.2 and group D: 1.3). The exposure to 5 MBq of $^{149}$Tb-cm09 (group C) resulted in mild renal injury in both tested animals (average score was 2). Animals of group E, that had received 20 MBq of $^{161}$Tb-cm09 (~60 Gy), exhibited variable renal toxicity with histological scores ranging from 1 to 3 (average score was 2). The most pronounced morphological changes (mean score of 3) were observed in mice which were injected with 30 MBq of $^{161}$Tb-cm09 (~90 Gy). These mice showed severe glomerular and tubular atrophy (Fig. 4.5, Fig. 4.6).

**Fig. 4.5.** Hematoxylin-eosin (HE)- and periodic acid-Schiff (PAS)-stained renal cortex presenting the different degrees of glomerular injury. Representative microscopic images (magnification 40x) of a control mouse (A), a mouse which received 2.5 MBq of $^{149}$Tb-cm09 (B) and a mouse which was injected with 30 MBq of $^{161}$Tb-cm09 (C) are shown. Glomeruli (G) in the control animal exhibited numerous capillaries filled with erythrocytes (green arrows). Radiation-induced glomerular injury is characterized by the deposition of faintly or intensely PAS-positive, eosinophilic, amorphous material within the glomerular tuft (black arrows) and by reduced numbers of glomerular capillaries. Atrophic glomeruli (yellow arrow) were observed in severely injured kidneys (mean score of ~3).
CHAPTER 4

Fig. 4.6. Hematoxylin-eosin (HE)-stained renal cortex with different degrees of tubular injury. Representative microscopic images (magnification 10x and 40x) of a control mouse (A), a mouse which received 2.5 MBq of $^{149}$Tb-cm09 (B) and a mouse which was injected with 30 MBq of $^{161}$Tb-cm09 (C) are shown. Kidneys exposed to radiation exhibit patchy to large diffuse areas of tubular atrophy and collapse (blue arrow). Numerous tubules in markedly damaged kidneys (mean score of ~3) are dilated (yellow arrows) and contain protein casts (red arrows).

4.3.4. Non-renal side effects

4.3.4.1. Determination of total bilirubin and alkaline phosphatase

Mice treated with 30 MBq of $^{161}$Tb-cm09 showed significantly increased plasma levels of total bilirubin and alkaline phosphatase ($P < 0.001$) short before euthanasia (Fig. 4.7). For all other mice which received $^{149}$Tb-cm09 (groups B and C) or lower quantities of $^{161}$Tb-cm09 (groups D and E) total bilirubin and alkaline phosphatase levels were comparable to untreated control mice at any time of the study.
4.3.4.2. Determination of viable white blood cells

Approximately 8 weeks (50 d) after application of $^{149}$Tb-cm09 or $^{161}$Tb-cm9 no significant difference ($P > 0.05$) in the number of viable, apoptotic and dead white blood cells was observed among the groups (Table 4.4).

Table 4.4. Counting of viable, apoptotic and dead white blood cells (WBCs) 50 d (week 8) after the injection of $^{161}$Tb-cm09 and $^{161}$Tb-cm09.

<table>
<thead>
<tr>
<th></th>
<th>Number of mice</th>
<th>Viable WBCs [%]</th>
<th>Apoptotic WBCs [%]</th>
<th>Dead WBCs [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>10</td>
<td>97 ± 3.3</td>
<td>1.8 ± 1.8</td>
<td>1.0 ± 1.9</td>
</tr>
<tr>
<td><strong>$^{149}$Tb-cm09</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B (2.5 MBq)</td>
<td>6</td>
<td>97 ± 1.6</td>
<td>2.0 ± 1.1</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>Group C (5.0 MBq)</td>
<td>2</td>
<td>98 ± 1.9</td>
<td>2.0 ± 1.7</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td><strong>$^{161}$Tb-cm09</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D (10 MBq)</td>
<td>4</td>
<td>95 ± 1.7</td>
<td>1.9 ± 0.5</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>Group E (20 MBq)</td>
<td>6</td>
<td>96 ± 2.5</td>
<td>2.3 ± 2.1</td>
<td>1.8 ± 1.2</td>
</tr>
<tr>
<td>Group F (30 MBq)</td>
<td>6</td>
<td>97 ± 1.4</td>
<td>2.1 ± 0.8</td>
<td>1.3 ± 0.9</td>
</tr>
</tbody>
</table>
4.4. Discussion

It was recently demonstrated by our group that the application of $^{149}$Tb-cm09 at a tumor dose of $\sim$1.6 Gy (injection of 3 MBq) resulted in comparable anti-tumor effects in the mouse as $^{161}$Tb-cm09 at a tumor dose of $\sim$22 Gy (injection of 10 MBq) [134, 135]. In this thesis, therefore, the renal toxic side effects of $^{149}$Tb-cm09 ($\alpha$-therapy) and $^{161}$Tb-cm09 ($\beta$'-therapy) were compared considering tumor doses of $\sim$1.6 Gy ($\alpha$) and $\sim$22 Gy ($\beta$') as therapeutically equivalent.

As a renal function test, the kidney uptake of $^{99m}$Tc-DMSA was determined non-invasively using SPECT [100, 101]. This test revealed impaired kidney function in mice at approximately 22 weeks after exposure to $^{161}$Tb-cm09 at a renal dose of $\sim$30 Gy (group D, injection of 10 MBq; Fig. 4.3). In contrast, mice treated with $^{149}$Tb-cm09 at renal dose of $\sim$1.7 Gy (group B, injection of 2.5 MBq) did not show reduced renal uptake of $^{99m}$Tc-DMSA at any time of the study, indicating preservation of normal kidney function. Comparing $^{149}$Tb-cm09 (2.5 MBq) and $^{161}$Tb-cm09 (10 MBq) at estimated therapeutically equivalent doses, it seemed that the renal function was slightly less impaired in the case of $^{149}$Tb-therapy. This was, however, not confirmed in morphological studies. At the study end, pathological changes of the renal tissue were observed for all animals injected with $^{149}$Tb-cm09 or $^{161}$Tb-cm09. The severity of the observed damage was comparable for animals of groups B and D (mean score of 1.2 and 1.3, respectively). Based on the obtained data conclusions about whether $^{149}$Tb-cm09 would result in similar or increased renal damage compared to $^{161}$Tb-cm09 are not yet possible.

It was recently demonstrated that $^{177}$Lu-radiopeptides ($\beta$'-therapy) mainly led to glomerulopathy in mice, whereas $^{213}$Bi-labeled peptides ($\alpha$-therapy) primarily resulted in tubular degeneration [131]. The herein performed morphological studies did not reveal distinct differences in suborgan damage distribution after $\alpha$-therapy and $\beta$'-therapy. The discrepancy between the results of our study and those reported in the literature are not completely understood. However, it has to be considered that the number of mice in each cohort used in the $^{149}$Tb-cm09 study was very small ($n = 6$ (2.5 MBq) and $n = 2$ (5 MBq), respectively) due to limited availability of $^{149}$Tb. In the future, it will be necessary to determine the maximum tolerated dose of $^{149}$Tb-cm09 and $^{161}$Tb-cm09 with larger cohorts. This would then allow investigating specific differences between effects and side effects of $\alpha$- and $\beta$'-radiofolate therapy.

Determination of viable white blood cells did not show significant changes in any investigated group eight weeks after radioactivity application. In previous studies using $^{177}$Lu-radiolabeled antibodies reduction in the number of white blood cells was observed four and eight weeks after treatment start [142]. Hence, our data indicated that $\alpha$- and $\beta$'-radiofolate
therapy with long-circulating albumin-binding derivatives do not impact the production of white blood cells at the applied doses.

In this study, it was demonstrated that the application of 30 MBq of $^{161}$Tb-cm09 (group F, ~90 Gy) led to most pronounced changes in renal function and morphology (mean score ~3). Renal uptake of $^{99m}$Tc-DMSA was already ~4.5-fold lower in these mice compared to untreated control animals around 3-4 months after radiofolate injection (Fig. 4.3). Moreover, blood urea nitrogen and creatinine were significantly increased in comparison to untreated control animals (Table 4.3). These changes correlated microscopically with widespread glomerular and tubular alterations, such as obliteration of the glomerular tufts and glomerulosclerosis and parenchymal collapse with tubular dilatation and tubular casts (Fig. 4.4-4.6). Furthermore, interstitial fibrosis and nephritis was observed in these mice.

Animals injected with high quantities of $^{161}$Tb-cm09 (group F) showed in addition increased total bilirubin and alkaline phosphatase plasma levels before euthanasia (Fig. 4.7). Both parameters are indicative for impaired liver function as described previously [106]. This toxicity might be a result of cross-organ doses from the source organ kidney to hepatic tissue. Mice of group F also showed a constant drop in body weight from week 11 on and a reduced survival compared to control mice (Fig. 4.2). This indicated a general impairment of the health status of these animals and suggested that 30 MBq of $^{161}$Tb-cm09 exceeded the maximum tolerated dose.

The herein performed dose-escalating studies with $^{161}$Tb-cm09 (10 MBq, 20 MBq and 30 MBq) revealed that the severity of radiation nephropathy and the onset of impaired renal function are dose-dependent. These observations are in line with what was recently observed with $^{177}$Lu-cm09 [107]. The same is also described for radionuclide therapy with peptides in rodents and humans [56, 90, 91].

4.5. Conclusion

Based on this pilot study, final conclusions about the severity of side effects caused by $\alpha$-therapy in comparison to $\beta$-therapy are not yet possible. Further investigations with larger cohorts of mice will be necessary in the future. This study clearly revealed, however, time- and dose-dependent nephrotoxicity after therapy with $^{161}$Tb-cm09 which is in line with our previous study performed with the same folate conjugate radiolabeled with the clinically established $^{177}$Lu.
CHAPTER 5

Combination of Glucocorticoids with Therapeutic Radiopharmaceuticals: Beneficial Effects or Contraindication?

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Parts of this chapter will be published in a modified version.

Author contributions:
Stephanie Haller planned and coordinated the study, performed in vitro and in vivo experiments, analyzed and interpreted the data and wrote the chapter. Claudia Neuhaus assisted experimental work. Roger Schibli reviewed the manuscript. Cristina Müller supervised the study and reviewed the manuscript.
5.1. Introduction

Glucocorticoids (GCs) such as dexamethasone and methylprednisolone are of great value for the treatment of lymphoid malignancies due to an effective induction of cell apoptosis [143, 144]. Moreover, application of GCs during anti-cancer therapies is often advantageous in terms of controlling nausea and emesis, protecting healthy tissue from cytotoxic side effects and reducing symptoms caused by the advanced disease such as inflammation, pain, oedema or anorexia [145]. However, it is also known that GCs can induce apoptosis resistance towards cytotoxic therapies in cells of solid tumors [145-150].

Despite extensive investigations, the mechanisms responsible for these pro- and anti-apoptotic effects of GCs are only partially understood. So far, there is clear evidence that both actions of GCs are dependent on functional glucocorticoid receptors (GCRs) [146]. In the literature are several mechanisms suggested to be responsible for the GC-mediated apoptosis resistance (reviewed in [143, 146]). For instance, up-regulation of anti-apoptotic factors and down-regulation of pro-apoptotic molecules, such as caspases, might be responsible for this effect. Moreover, it was previously shown that the application of GCs can lead to a cell cycle arrest in the G1 phase [150]. At this phase of the cell cycle, cells are relatively resistant towards radiation therapy. Furthermore, the G1-block leads to slower cell growth and therefore reduced efficacy of anti-cancer drugs which are most successful when applied to rapidly dividing cells [146, 150]. Another disadvantage of GCs is their immunosuppressive effect when administered at high doses to cancer patients [146]. This might compromise the endogenous immune defence mechanism against the disease and, as a consequence, increase the incidence of metastases [150-153].

Literature reports clearly demonstrate that GC-induced apoptotic and anti-apoptotic effects are neither restricted to a specific cell line nor to an anti-cancer treatment [145, 150]. The development of resistance towards chemotherapeutics and ionizing irradiation when combined with GCs was observed in vitro and in vivo for various tumor types including lung, cervical, ovarian, breast and prostate cancer [150, 154, 155]. In vitro studies performed to investigate the effect of dexamethasone on the therapeutic response of cancer cells to radiation are summarized in Table 5.1.
Table 5.1. Impact of dexamethasone on the effectiveness of external radiation treatment tested in vitro in various tumor cell lines. Except as noted otherwise, the cell lines are of human origin.

<table>
<thead>
<tr>
<th>Tumor origin</th>
<th>Cell line</th>
<th>Dexamethasone [µM]</th>
<th>Radioresistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervix</td>
<td>C4-1</td>
<td>1</td>
<td>yes</td>
<td>[156, 157]</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>0.01-1</td>
<td>yes</td>
<td>[154, 157, 158]</td>
</tr>
<tr>
<td></td>
<td>C-33A</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td></td>
<td>SW756</td>
<td>1</td>
<td>yes</td>
<td>[157]</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>1</td>
<td>yes</td>
<td>[154]</td>
</tr>
<tr>
<td>Ovary</td>
<td>Primary(^1)</td>
<td>0.1-10</td>
<td>yes</td>
<td>[155]</td>
</tr>
<tr>
<td>Breast</td>
<td>MCF-7</td>
<td>125</td>
<td>no</td>
<td>[160]</td>
</tr>
<tr>
<td>Prostate</td>
<td>PC-3</td>
<td>0.1-1</td>
<td>yes/no</td>
<td>[159, 161]</td>
</tr>
<tr>
<td></td>
<td>DU-145</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td>Lung</td>
<td>H1-57</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td></td>
<td>H2-26</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td>Colon</td>
<td>CoL-600</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td>Brain</td>
<td>LN308</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td></td>
<td>RT489 (rat)</td>
<td>125</td>
<td>no</td>
<td>[160]</td>
</tr>
<tr>
<td></td>
<td>U 118</td>
<td>1</td>
<td>no</td>
<td>[162]</td>
</tr>
<tr>
<td></td>
<td>U 251</td>
<td>1</td>
<td>no</td>
<td>[162]</td>
</tr>
<tr>
<td>Bladder</td>
<td>EJ</td>
<td>1</td>
<td>no</td>
<td>[162]</td>
</tr>
<tr>
<td>Head/neck</td>
<td>SQ-20B</td>
<td>1</td>
<td>yes</td>
<td>[159]</td>
</tr>
<tr>
<td>Bile duct</td>
<td>EBD-1</td>
<td>1</td>
<td>no</td>
<td>[159]</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>FSa 1233 (mouse)</td>
<td>125</td>
<td>no</td>
<td>[160]</td>
</tr>
</tbody>
</table>

\(^1\) Freshly isolated.

Besides the combined application of GCs and chemotherapeutics or external beam radiation therapy, also co-application of GCs and targeted radiopharmaceuticals is of clinical relevance. Nowadays, somatostatin-based radioconjugates, such as \(^{177}\text{Lu-DOTA-}[\text{Tyr}^3]\)-octreotide (\(^{177}\text{Lu-DOTATOC}\)), are routinely employed in combination with GCs in humans for therapy of somatostatin receptor (SSTR)-expressing tumors [163-165].

The folate receptor (FR) is frequently overexpressed in a broad variety of human cancer types and at the same time its expression in healthy tissues is limited [41]. Therefore, the FR is a promising target for radionuclide tumor therapy. The potential of such an approach has
recently been demonstrated in a preclinical study in which tumor xenografts of mice were successfully treated with an albumin-binding radiofolate [51]. In the literature, it is described that GCs up-regulate the FR expression level in tumor cells [166]. The impact of this up-regulation on the therapeutic outcome of FR-targeted therapeutics was not yet investigated.

The first goal of this study was to explore the impact of FR up-regulation in cancer cells by GCs on the cellular uptake of radiofolates in vitro and in vivo. It was also investigated whether such an up-regulation would positively impact the anti-cancer effect. For this purpose, cell internalization and viability studies were performed with FR-positive KB and IGROV-1 tumor cells in vitro by applying various concentrations of dexamethasone or methylprednisolone and a folate radiolabeled with the therapeutic radionuclide $^{177}$Lu ($^{177}$Lu-cm10). The impact of dexamethasone on the tumor uptake and anti-tumor effect of $^{177}$Lu-cm10 in vivo was studied in KB tumor-bearing mice by performing single photon emission computed tomography (SPECT) and therapy studies, respectively. Secondly, we investigated the effect of GCs on the uptake and therapeutic effect of other targeting agents than folic acid-based derivatives in vitro. Internalization and cell viability studies were performed after co-applying radiolabeled somatostatin ($^{177}$Lu-DOTATOC) or bombesin analogs ($^{177}$Lu-DOTA-BN-4) to AR42J (SSTR-positive) and PC-3 (gastrin-releasing peptide (GRP)-positive) tumor cells in combination with GCs.

5.2. Materials and methods

5.2.1. Reagents

The RPMI 1640 cell culture medium was obtained from Bioconcept Amimed (Allschwil, Switzerland) and the folate-free RPMI cell culture medium (FFRPMI, without folic acid, vitamin B$_{12}$ and phenol red) was purchased from Cell Culture Technologies GmbH (Gravesano, Switzerland). The cell culture media were supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics (penicillin 100 IU/mL, streptomycin 100 mg/mL, fungizone 0.25 µg/mL) from Bioconcept Amimed (Allschwil, Switzerland). Except as noted otherwise, cell culture media were used including the supplements.

No-carrier added $^{177}$LuCl$_3$ was purchased from Isotope Technologies Garching (ITG GmbH, Garching, Germany). The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-folate conjugate cm10 was kindly provided by Merck & Cie (Schaffhausen, Switzerland). DOTA-[Tyr$^3$]-octreotide (DOTATOC) was purchased from ABX GmbH (ABX advanced chemical compounds, Radeberg, Germany). The DOTA-bombesin analog DOTA-BN-4 was a kind gift from Prof. Dr. Dirk A. Tourwé (Department of Organic Chemistry, Free
University of Brussels, 1050 Brussels, Belgium). For radiolabeling, metal-free hydrochloric acid (HCl 30% suprapur®) was obtained from Merck (Zug, Switzerland) and sodium acetate (Na-acetate, Puratronic 99.9985%) was optioned from Alfa Aesar GmbH (Karlsruhe, Germany). Sodium diethylenetriaminepentaacetic acid (Na-DTPA, sodium salt) was purchased from Fluka Chemie AG (Buchs, Switzerland).

Phosphate buffered saline (PBS, pH 7.2) was prepared using 0.21 g monopotassium phosphate (KH₂PO₄), 9 g sodium chloride (NaCl) and 0.48 g disodium phosphate (Na₂HPO₄ x 2H₂O) per 1 L of Milli-Q water. PBS (pH 7.4) was prepared in a volume of 1 L containing 5.8 g sodium chloride (NaCl), 6 g disodium phosphate (Na₂HPO₄ x 2H₂O), 1.5 g monosodium phosphate (NaH₂PO₄ x H₂O). The pH was adjusted by using sodium hydroxide (NaOH, 5 M).

Dexamethasone and methylprednisolone were used from commercially available ampoules for injection purposes (Dexamethason Helvepharm™, Helvepharm, Frauenfeld, Switzerland and Solu-Medrol® SAB, Pfizer AG, Zurich, Switzerland, respectively). The compounds were diluted in cell culture medium, NaCl 0.9% or drinking water depending on the study.

5.2.2. Cell culture

KB cells (human cervical carcinoma cells [96], subclone of HeLa cells, ACC-136, FR-positive [167]) and PC-3 (human prostate cancer cells, ACC-465, gastrin-releasing peptide (GRP)-positive [168]) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). AR42J cells (rat pancreatic cancer cells, Cat-No° 93100618, SSTR-positive [169]) were obtained from Health Protection Agency Culture Collections (Salisbury, U.K.). IGROV-1 cells (human ovarian cancer cells, FR-positive [167]) were kindly provided by Dr. Gerrit Jansen (Department of Rheumathology, Free University Medical Center, Amsterdam, The Netherlands). The cells were cultured as monolayers at 37°C in humidified atmosphere containing 5% CO₂. KB and IGROV-1 cells were cultured in FFRPMI cell culture medium. AR42J and PC-3 cells were cultured in RPMI 1640 cell culture medium. Routine cell culture was performed twice a week.

5.2.3. Radiosynthesis

The radiolabeling of cm10, DOTATOC and DOTA-BN-4 was performed according to standard labeling procedures at a specific activity of 50 MBq/nmol. The conjugates were mixed with a solution of HCl (0.05 M) and Na-acetate (0.5 M) at pH 4.5. In case of cm10 and DOTATOC the radiolabeling mixture was incubated for 10 min at 95°C. The radiolabeling of DOTA-BN-4 was performed over 15 min at 95°C. Na-DTPA solution (5 mM, pH 5, 10 µL) was
added for complexation of potential traces of unreacted $^{177}$Lu(III). Quality control was performed by high performance liquid chromatography (HPLC, Merck Hitachi, Darmstadt, Germany) on a C-18 reversed phase column (XTerra™ MS C-18, 5 µm, 15 cm x 4.6 cm, Waters, Milford, MA, U.S.) as previously described [51]. The mobile phase consisted of Milli-Q water with 0.1% trifluoroacetic acid (A) and acetonitrile (B) with a linear gradient from 5% B to 80% of B over 15 min at a flow rate of 1 mL/min. For cell uptake studies the radiolabeled derivatives were purified via HPLC using 0.1% trifluoroacetic acid (A) and methanol (C) with a linear gradient from 5% B to 80% of C over 25 min as mobile phase. The radiochemical yield was always >95%.

5.2.4. In vitro studies

5.2.4.1. Internalization studies

For uptake studies, KB, IGROV-1, AR42J and PC-3 cells were seeded in 12-well plates to grow overnight. In the case of KB and IGROV-1 cells, 0.7 x 10^6 cells were seeded in 2 mL FFRPMI. AR42J cells (1 x 10^6 cells in 2 mL) and PC-3 cells (0.7 x10^6 cells in 2 mL) were seeded in RPMI medium. In all cases some of the wells were additionally supplemented with dexamethasone or methylprednisolone (100 nM). The next day, purified $^{177}$Lu-cm10 (37.5 kBq, 1.5 pmol) was added to KB and IGROV-1 cells, $^{177}$Lu-DOTATOC (37.5 kBq, 1.5 pmol) to AR42J cells and $^{177}$Lu-DOTA-BN-4 (37.5 kBq, 0.75 pmol) to PC-3 cells followed by incubation of the cells for 2 h or 4 h at 37°C. Afterwards, the cells were washed three times with PBS (pH 7.4) to determine the total uptake of the radiopharmaceuticals. To measure the total internalized fraction, KB and IGROV-1 cells were washed with an acidic buffer (150 mM NaCl, 38.5 mM acetic acid, pH 3) to release the FR-bound fraction from the cell surface [51]. Removal of receptor-bound $^{177}$Lu-DOTATOC and $^{177}$Lu-DOTA-BN-4, respectively, was achieved by incubating the cells with an acidic buffer of a different composition (50 mM glycine, pH 2.8) for 30 min. Subsequently, the cells were lysed in NaOH (1 M). Receptor-bound and internalized activities were measured in the y-counter (Wizard 3, Automatic Gamma Counter, Perkin Elmer 1480, Waltham, MA, U.S.). The concentrations of proteins were determined for each sample using Micro BCA™ Protein Assay Kit (Thermo Scientific Prod, Rockford, U.S.) enabling a standardization of the measured radioactivity to a defined quantity of proteins. Uptake and internalization of all samples are indicated as percentage of radioactivity uptake in control cells incubated with the radiopharmaceutical only (arbitrary set to 100%).
5.2.4.2. Cell viability studies

The cell viability after exposure to GCs and radiopharmaceuticals was investigated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [170]. For studying the impact of dexamethasone and methylprednisolone alone on the cell viability of KB, IGROV-1 and PC-3 cells around 1500 cells were seeded in 200 µL cell culture medium in 96-well plates. In the case of AR42J cells, around 7500 cells were seeded per well. The next day, dexamethasone or methylprednisolone (0-10 µM) was added and the cells were then allowed to grow at 37°C for one week.

For comparison of the effect of the radiopharmaceuticals on the cell viability with and without a co-treatment of GCs another experimental setup was used. In brief, cells were seeded in 96-well plates (KB, IGROV-1, PC-3: 2500 cells per well, AR42J: 7500 cells per well) in cell culture medium and some of the cells were co-incubated with dexamethasone or methylprednisolone (100 nM). The following day the cells were exposed to the radiopharmaceuticals (KB: 0.01-5 MBq/mL $^{177}$Lu-cm10, IGROV-1: 0.01-40 MBq/mL $^{177}$Lu-cm10, AR42J: 0.01-40 MBq/mL $^{177}$Lu-DOTATOC and PC-3: 0.01-20 MBq/mL $^{177}$Lu-DOTA-BN-4) for 4 h at 37°C. Control cells were incubated only with cell medium without additives. The radiopharmaceuticals were diluted in cell medium without additives, except in the case of $^{177}$Lu-DOTATOC and $^{177}$Lu-DOTA-BN-4 addition of 1% FCS to RPMI medium was necessary to reduce unspecific binding. After incubation, the cells were washed once with PBS (pH 7.2) followed by addition of cell culture medium. Again parts of the cells were co-incubated with dexamethasone or methylprednisolone (100 nM). The cells were then incubated for 6 d at 37°C without changing cell culture medium. The same experimental setting was used for investigating the effect of $^{177}$LuCl$_3$ (0-40 MBq/mL) on the cell viability. The assay was analyzed as previously described using a MTT reagent (5 mg/mL dissolved in PBS (pH 7.4)) and a microplate reader (560 nm, Victor™ X3, Perkin Elmer, Waltham, MA, U.S.) [171]. The cell viability was quantified by expressing the measured absorbance of the test sample as percentage of the absorbance of control cell samples (= 100%).

5.2.5. Animal studies

The in vivo experiments were approved by the local veterinarian department and conducted in accordance with the Swiss law of animal protection. Female athymic nude mice (CD-1 Foxn1/nu, 5-7 week-old, Charles River Laboratories, Sulzfeld, Germany) were fed ad libitum with a folate-deficient rodent diet (ssniff Spezialdiäten GmbH, Soest, Germany) starting one week before tumor cell inoculation [122].
5.2.5.1. SPECT/CT

For SPECT/CT imaging studies, mice were inoculated with KB tumor cells (5 x 10^6 cells in 100 µL PBS pH 7.2) into the subcutis of each shoulder. Three weeks after tumor cell inoculation, mice were subcutaneously (s.c.) injected four times (day -4, -3, 0 and 1) with 1.25 µg dexamethasone (in 100 µL NaCl 0.9%) or NaCl 0.9% (100 µL) in the case of control mice. At day 0, the animals were in addition intravenously (i.v.) injected with ^177^Lu-cm10 (45-55 MBq, 1.8-2.2 nmol). SPECT/CT acquisitions were performed 4 h, 24 h and 168 h after the injection of the radiofolate with a four-head multiplexing multipinhole small-animal SPECT/CT scanner (NanoSPECT/CT, Mediso Medical Imaging Systems, Budapest, Hungary). Each head was equipped with a tungsten collimator of nine 1.4-mm-diameter pinholes. SPECT/CT images were obtained using Nucline Software (version 1.02, Bioscan Inc., Poway, California, U.S.). The time per view was 30 s resulting in a scan time of ~25 min. CT scans were acquired with the integrated CT scanner using a tube voltage of 55 kVp and an exposure time of 1 s per view. After the performance of the SPECT and CT scans, the data were reconstructed with HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany) using γ-energies for ^177^Lu of 56.1 keV ± 10%, 112.9 keV ± 10% and 208.4 keV ± 10%. SPECT and CT data were automatically co-registered as both modalities share the same axis of rotation. The fused data sets were analyzed and Gauss post-reconstruction filtering was performed using the VivoQuant post-processing software (version 1.23, inviCRO Imaging Services and Software, Boston, U.S.). The SPECT camera was calibrated in advance with a phantom filled with a known quantity of ^177^Lu allowing a quantification of the accumulated radioactivity in a defined volume. The average uptake of radioactivity in tumors and kidneys was determined with VivoQuant post-processing program by using an approximate volume covering complete size of a single tumor or kidney. The uptake of radiofolates in tumors and kidneys of control animals was arbitrary set to 100%.

5.2.5.2. Therapy study in KB tumor-bearing mice

Mice were inoculated with KB tumor cells (4.5 x 10^6 cells in 100 µL PBS) under the skin of each shoulder. The designs of pilot study 1 and 2 are described in Table 5.2. Body weights and tumor volumes were determined at day 0 followed by a measurement three times per week until completion of the study. Endpoint criteria were defined as (i) body weight loss of >15% of the initial body weight, (ii) a tumor volume of >1000 mm³, (iii) active ulceration of the tumor xenograft or (iv) signs of unease. The body weights are presented as the average of the individual relative body weight (RBW = W_x/W_0; W_x: weight at day x; W_0: weight at day 0). The tumor volume (V) was determined by measuring two perpendicular diameters with a caliper and calculated according to the following equation: \[ V = 0.5 \times (L \times W^2) \], where L is the
length and W is the width of the tumor [98]. The values were expressed as the average of the individual relative tumor volumes (RTV = \( V_x/V_0 \); \( V_x \): volume at day \( x \); \( V_0 \): volume at day 0). The effectiveness of the different treatments was described by calculating the percentages of tumor growth inhibition (% TGI), using the equation \[100-((T/C) \times 100)\], where \( T \) is the mean RTV of treated mice and \( C \) is the mean RTV of control mice at the time of euthanasia of the first mouse of the control group [99].

Table 5.2. Design of the two pilot studies applying dexamethasone and \(^{177}\text{Lu-cm10}\) (20 MBq, 1 nmol) in nude mice xenografted with KB tumors. In pilot study 1 dexamethasone was injected s.c. and in pilot study 2 dexamethasone was applied in the drinking water (0.3-1.5 µg/mL). A water consumption of 3-5 mL per day was assumed [123].

<table>
<thead>
<tr>
<th>Pilot study 1</th>
<th>Group</th>
<th>Number of mice</th>
<th>Tumor cell inoculation</th>
<th>Dexamethasone (s.c.)</th>
<th>(^{177}\text{Lu-cm10}) (i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>3</td>
<td>day -4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1</td>
<td>3</td>
<td>day -4</td>
<td>-</td>
<td>-</td>
<td>20 MBq (day 0)</td>
</tr>
<tr>
<td>C1</td>
<td>3</td>
<td>day -4</td>
<td>1.25 µg (day -4, -3, 0, 1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D1</td>
<td>3</td>
<td>day -4</td>
<td>1.25 µg (day -4, -3, 0, 1)</td>
<td>20 MBq (day 0)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pilot study 2</th>
<th>Group</th>
<th>Number of mice</th>
<th>Tumor cell inoculation</th>
<th>Dexamethasone (oral)</th>
<th>(^{177}\text{Lu-cm10}) (i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>3</td>
<td>day -6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>3</td>
<td>day -6</td>
<td>-</td>
<td>-</td>
<td>20 MBq (day 0)</td>
</tr>
<tr>
<td>C2</td>
<td>3</td>
<td>day -6</td>
<td>(\sim 1.2) µg/d (day -2 until end of study)</td>
<td>20 MBq (day 0)</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>3</td>
<td>day -6</td>
<td>(\sim 6) µg/d (day -2 until day 7) (\sim 1.2) µg/d (day 7 until end of study)</td>
<td>20 MBq (day 0)</td>
<td></td>
</tr>
</tbody>
</table>

5.2.6. Statistical analysis

Data are presented as mean ± standard. Statistics was conducted by using one-way ANOVA with Bonferroni’s multiple comparison post-test (GraphPad Prism, version 5.01).
5.3. Results

5.3.1. Impact of GCs on cellular uptake of radiopharmaceuticals

In order to investigate whether GCs influence the cellular uptake of radiolabeled folic acid conjugates ($^{177}$Lu-cm10), somatostatin ($^{177}$Lu-DOTATOC) and bombesin analogs ($^{177}$Lu-DOTA-BN-4), cell internalization studies were performed in KB, IGROV-1, AR42J and PC-3 tumor cells, respectively. Uptake of $^{177}$Lu-cm10 in KB tumor cells was comparable for control cells incubated with only the radiofolate for 2 h (arbitrary set to 100%) and cells co-exposed to dexamethasone (99 ± 14%) or methylprednisolone (100 ± 7%; Fig. 5.1, A). In the case of AR42J cells, the uptake of $^{177}$Lu-DOTATOC after 2 h was similar for control cells (arbitrary set to 100%) and cells simultaneously incubated with dexamethasone (119 ± 7%) or methylprednisolone (108 ± 4%; Fig. 5.1, B). Also, for IGROV-1 and PC-3 cells the uptake of the targeted radiopharmaceuticals was comparable with and without co-incubation of dexamethasone or methylprednisolone (Fig. 5.1, C/D).
For investigating the effect of dexamethasone and methylprednisolone on the cell viability of KB, IGROV-1, AR42J and PC-3 tumor cells, MTT assays were performed. The incubation of KB, IGROV-1 and PC-3 cells with various concentrations of dexamethasone and methylprednisolone (0.1-10 µM) for one week resulted in a cell viability which was comparable (P > 0.05) to untreated control cells (~100%) (Fig. 5.2, A/C/D; Table 5.3). AR42J cells incubated for one week with 100 nM of dexamethasone or methylprednisolone showed reduced cell viability of 63 ± 6% (P < 0.005) and 38 ± 2% (P < 0.005), respectively (Fig. 5.2, B).
5.3.3. In vitro radioresistance after co-incubation with GCs

The impact of GCs on the potential anti-cancer effect of $^{177}$Lu-radiopharmaceuticals was investigated in vitro using MTT assay. KB tumor cells only incubated with $^{177}$Lu-cm10 (5 MBq/mL) for 4 h showed a viability of 6 ± 5% compared to untreated control cells (100%). A simultaneous incubation of the cells with dexamethasone or methylprednisolone resulted in a cell viability of 38 ± 5% and 32 ± 9%, respectively, which was significantly increased (P < 0.005) compared to cells only incubated with the radiofolate (Fig. 5.3, Table 5.3). Exposure of AR42J cells to $^{177}$Lu-DOTATOC (20 MBq/mL) reduced the number of viable cells to 3 ± 0.5%. In comparison to that, co-incubation with dexamethasone or methylprednisolone resulted in significantly increased (P < 0.005) cell viability of 37 ± 11% and 16 ± 2%, respectively. The cell viability of IGROV-1 was similar (P > 0.05) after incubation with $^{177}$Lu-cm10 (40 MBq/mL) only and $^{177}$Lu-cm10 (40 MBq/mL) together with GCs. PC-3 cells exposed to $^{177}$Lu-DOTA-BN-4 or $^{177}$Lu-DOTA-BN-4 combined with GCs resulted in similar (P > 0.05) cell viabilities. The application of high concentrations of the
radiopharmaceutical (≥20 MBq/mL) did not inhibit the viability of IGROV-1 and PC-3 tumor cells to ~10%.

In order to study whether the observed effects of GCs on radiation-induced cell viability inhibition is independent on the targeting system, cell viability studies were performed by co-exposing cancer cells to $^{177}$LuCl$_3$ and GCs. KB cells only incubated with $^{177}$LuCl$_3$ (5 MBq/mL) showed a reduction of the cell viability to $11 \pm 7\%$ compared to untreated control cells (100%). If KB cells were co-treated with 100 nM dexamethasone or methylprednisolone the cell viability was determined to be $37 \pm 6\%$ and $41 \pm 5\%$, respectively, which was significantly increased ($P < 0.005$) compared to cells only exposed to the radiofolate (Fig. 5.4, Table 5.3). In the case of AR42J cells, incubation with $^{177}$LuCl$_3$ (1 MBq/mL) led to a viability of $8 \pm 3\%$, whereas a co-exposure to dexamethasone or methylprednisolone (100 nM) resulted in significantly increased ($P < 0.005$) cell viability of $52 \pm 15\%$ and $48 \pm 18\%$, respectively. IGROV-1 and PC-3 tumor cells showed comparable ($P > 0.05$) viabilities upon incubation with $^{177}$LuCl$_3$ without or by co-incubation with GCs. In contrast to KB and AR42J tumor cells, high concentrations of $^{177}$LuCl$_3$ (≥20 MBq/mL) were necessary to inhibit the viability of IGROV-1 and PC-3 tumor cells to ~10% of untreated control cells (100%).
Fig. 5.4. Cell viability of KB, AR42J, IGROV-1 and PC-3 tumor cells after exposure to $^{177}$LuCl$_3$ only or after incubating simultaneously with $^{177}$LuCl$_3$ and GCs. The cells were incubated for one week with 100 nM dexamethasone or methylprednisolone, respectively. In addition, at day 2 the cells were exposed to $^{177}$LuCl$_3$ for 4 h. *** P < 0.005.

Table 5.3. Results of in vitro studies with various tumor cell lines incubated with only glucocorticoids (GCs) or co-exposed to GCs and radiation.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor origin</th>
<th>Species</th>
<th>Growth inhibition by GCs</th>
<th>Radioresistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>cervix</td>
<td>human</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>AR42J</td>
<td>pancreas</td>
<td>rat</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>IGROV-1</td>
<td>ovary</td>
<td>human</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>PC-3</td>
<td>prostate</td>
<td>human</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

5.3.4. Tissue distribution of $^{177}$Lu-cm10 after co-treatment with dexamethasone

In vitro tests revealed that GCs do not inhibit the growth of KB tumor cells but lead to radioresistance development (Table 5.3). Therefore, the combination of dexamethasone and the radiofolate $^{177}$Lu-cm10 was studied in vivo.

The impact of dexamethasone co-application on the biodistribution of $^{177}$Lu-cm10 was investigated in KB tumor-bearing mice by SPECT/CT. The mouse injected with the radiofolate only ($n = 1$) and the mouse co-treated with dexamethasone ($n = 1$) showed comparable uptake of $^{177}$Lu-cm10 in kidneys and tumors 24 h p.i. (Fig. 5.5). Compared to
control mice (arbitrary set to 100%) the renal uptake was ~105% and the tumor uptake was ~95% in the case of co-treatment with dexamethasone.

Fig. 5.5. SPECT/CT images acquired 24 h after the injection of $^{177}$Lu-cm10 only (A) or after co-treatment with dexamethasone (B). Radioactivity accumulated in KB tumor xenografts (yellow arrows) and kidneys (green arrows).

5.3.5. Anti-tumor effect of $^{177}$Lu-cm10 combined with dexamethasone

Therapy studies in KB tumor-bearing mice were performed in order to test the impact of dexamethasone on the therapeutic outcome of the treatment with $^{177}$Lu-cm10. Two different application routes of dexamethasone (s.c. (study 1) and oral (study 2)) were investigated. In the literature the application of GCs in the drinking water, s.c. as well as i.p., is described in the context of GC-induced apoptosis resistance [155, 172-175]. As shown in pilot study 1, the tumor growth of control animals (group A1) and mice administered with dexamethasone (s.c., group C1) was comparable (Fig. 5.6, A; Table 5.4). KB tumor-bearing mice treated with 20 MBq of $^{177}$Lu-cm10 (group B1) showed a tumor growth delay, compared to control mice (TGI ~96%), which was similar to mice s.c. co-treated with dexamethasone (group D1,
TGI ~98%). Also oral administration of dexamethasone (groups C2 and D2) had no impact on the tumor growth of mice treated with $^{177}$Lu-cm10 as shown in pilot study 2 (Fig. 5.6, C; Table 5.4). High oral dosages of dexamethasone (~6 µg/d) led to a significant body weight loss within 7 d (Fig. 5.6, D). After reduction of dexamethasone dosage to ~1.2 µg/d, these mice gained body weight again.

Table 5.4. Results of pilot study 1 and 2 using $^{177}$Lu-cm10 (20 MBq, 1 nmol) and various applications schemes of dexamethasone. In pilot study 1 dexamethasone was injected s.c. and in pilot study 2 dexamethasone was applied in the drinking water (0.3-1.5 µg/mL). A water consumption of 3-5 mL per day was assumed [123].

### Pilot study 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Dexamethasone (s.c.)</th>
<th>$^{177}$Lu-cm10 (i.v.)</th>
<th>TGI</th>
<th>Additional survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1</td>
<td>-</td>
<td>20 MBq (day 0)</td>
<td>96%</td>
<td>+9%</td>
</tr>
<tr>
<td>C1</td>
<td>1.25 µg (day -4, -3, 0, 1)</td>
<td>-</td>
<td>17%</td>
<td>+9%</td>
</tr>
<tr>
<td>D1</td>
<td>1.25 µg (day -4, -3, 0, 1)</td>
<td>20 MBq (day 0)</td>
<td>98%</td>
<td>+9%</td>
</tr>
</tbody>
</table>

### Pilot study 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Dexamethasone (oral)</th>
<th>$^{177}$Lu-cm10 (i.v.)</th>
<th>TGI</th>
<th>Additional survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>~1.2 µg/d (day -2 until end of study)</td>
<td>20 MBq (day 0)</td>
<td>97%</td>
<td>+94%</td>
</tr>
<tr>
<td>C2</td>
<td>~6 µg/d (day -2 until day 7) ~1.2 µg/d (day 7 until end of study)</td>
<td>20 MBq (day 0)</td>
<td>94%</td>
<td>+94%</td>
</tr>
<tr>
<td>D2</td>
<td>~6 µg/d (day -2 until day 7) ~1.2 µg/d (day 7 until end of study)</td>
<td>20 MBq (day 0)</td>
<td>94%</td>
<td>+94%</td>
</tr>
</tbody>
</table>

Abbreviations: TGI, tumor growth inhibition.

1) Undefined survival: ≥50% of mice were still alive at end of the study.
5.4. Discussion

In the past, GCs have been demonstrated to induce resistance towards diverse cytotoxic drugs and external beam irradiation in various carcinoma cells cultured in vitro, grown as xenografts in rodents as well as after isolation from fresh patient samples [147, 150, 176]. Therefore, the use of GCs in patients with solid tumors undergoing cytotoxic therapy is controversial discussed in the literature [145, 146, 150]. Herein, it was found that GCs applied in combination with targeted radionuclide therapy might also cause radioresistance.

**Fig. 5.6.** Tumor growth curves indicated as relative tumor volumes (RTVs, A and C) and relative body weights (RBWs, B and D) of KB tumor-bearing mice after treatment with $^{177}$Lu-cm10 alone or in combination with dexamethasone (DEX) investigated in pilot study 1 (A and B) and 2 (C and D). The average tumor volumes and body weights of each group are shown until the first mouse reached an end-point criterion.
In cell viability studies KB (FR-positive) and AR42J (SSTR-positive) tumor cells were protected from irradiation damage of radiolabeled folic acid derivatives and somatostatin analogs, respectively, after co-exposure to dexamethasone or methylprednisolone (100 nM; Fig. 5.3). The same effect was observed upon exposure of the cells to GCs and $^{177}$Lu when it was applied as $^{177}$LuCl$_3$. In its cationic version $^{177}$Lu is transported through the cellular membrane unspecifically. Hence, radioresistance development was independent of the targeting agent. Co-incubation with lower concentrations of GCs (1 nM instead of 100 nM) did not result in the development of radioresistance (data not shown). This implies that a certain GC concentration (in this setting ~100 nM) is necessary to induce radioresistance in tumor cells. This concentration is comparable to GC plasma levels in cancer patients who are co-treated with GCs [145].

In IGROV-1 (FR-positive) and PC-3 (GRP-positive) tumor cells the development of radioresistance after co-incubation with GCs was absent, even if the concentration of the GC was increased to 1 µM. GCs act by binding to GCRs in the cell interior [146]. Hence, the presence of GCRs at low levels, in mutated or non-functional forms, or the lack of this receptor might be responsible for the absence of radioresistance development in tumor cells such as IGROV-1 and PC-3 cells. Moreover, in these cell lines high radioactive quantities of $^{177}$LuCl$_3$ ($\geq$20 MBq/mL) were necessary to inhibit the cell viability to ~10%. In contrast, KB and AR42J cells developed radioresistance upon GC exposure and were more prone to radiation damage which was shown by the fact that low radioactive quantities of $^{177}$LuCl$_3$ (5 MBq/mL and 1 MBq/mL, respectively) were sufficient to reduce cell viability to ~10%. On the basis of our in vitro experiments, we hypothesize that more radiosensitive tumor cells such as KB and AR42J cells are more prone to develop radioresistance than tumor cells which are less sensitive to radiation damage such as IGROV-1 and PC-3 cells.

In order to study the observed impact of GCs on the actions of radiofolates in vivo, we performed studies in KB tumor-bearing mice. SPECT/CT studies performed in KB xenografted nude mice revealed that the in vivo tissue distribution of radiofolates was not changed if the animal was co-treated with dexamethasone confirming what was observed in vitro (Fig. 5.5). In therapy studies, no effect of dexamethasone on the tumor growth after application of therapeutic quantities of the radiofolate was determined (Fig. 5.6, Table 5.4). The absence of radioresistance development of KB tumors in vivo was in contradiction to our in vitro results. Further studies will be necessary to explain why the effect observed in vitro was not translatable to the in vivo situation. For instance, it will be essential to study the GCR expression in KB tumors grown in nude mice by immunohistochemical analysis since the levels of GCRs are crucial for GC-induced apoptosis resistance. Moreover, it will be necessary to find the maximum tolerated dose of dexamethasone in the mouse model used.
in this study as the applied amount of dexamethasone was possibly not sufficient and/or the administration route not ideal to obtain the necessary GC concentration at tumor site.

**5.5. Conclusion**

Based on our data, it can be concluded that some tumor types develop radioresistance upon exposure to GCs while others don’t. We found that GC-mediated radioresistance is cell type-specific, but independent on the targeting strategy which is used to deliver the radioactivity to the tumor cells. In future, it will be essential to investigate the GR levels and the effect of GCs on potential cell cycle arrest in different tumor cell lines and correlate it with their response to GCs exposure in terms of radioresistance development. Moreover, it appears important to recognize that, despite beneficial effects of GCs during treatment of cancer patients, potential induction of resistance towards radionuclide therapy might occur. Therefore, it would be interesting to study clinical data of patients who were treated with targeted radiopharmaceuticals and GCs, retrospectively.
Are Auger Electrons Suitable for Targeted Radionuclide Therapy? - First In Vitro Results with Nuclear-Localizing Peptides

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This chapter will be published in a modified version.

Author contributions:
Stephanie Haller designed and established the in vitro assays, carried out the radiolabelings, in vitro and vivo studies, performed data analysis and interpretation and wrote the manuscript. Fiona P. Frank and Raffaella M. Schmid assisted experimental work. Katharina A. Domnanich, Christiaan Vermeulen and Nicholas P. van der Meulen provided $^{161}$Tb. Roger Schibli reviewed the manuscript. Cristina Müller supervised the study and reviewed the manuscript.
6.1. Introduction

Auger/conversion electrons have a high linear energy transfer (LET) of ~4-26 keV/µm and a tissue range of only ~2-500 nm [5]. Hence, these electrons deposit energy over only subcellular dimensions what is suitable for the treatment of single cancer cells. The localization of Auger/conversion electron-emitting radionuclides in perinuclear or nuclear area is essential [11, 83]. In the past, it was demonstrated in several preclinical studies that the integration of a nuclear localization signal (NLS) into the targeted radiopharmaceutical is a possibility to transport it into the cell nucleus [84, 177-181]. NLS are short hydrophilic peptide sequences necessary for the transport of peptides >40 kDa across the nuclear pore complex [182]. The best characterized NLS is the peptide sequence PKKKRKV132 discovered in the simian virus 40 large tumor antigen [183]. The translocation across the nuclear membrane is mediated by the carrier protein importin β which interacts with the nuclear pore complex [184]. This transport is unidirectional [182].

![Fig. 6.1. Transport of a radioconjugate derivatized with a nuclear localization signal (NLS) from cytoplasm to the cell nucleus. The NLS binds to the cytosolic carrier importin α which forms a complex with the transport protein importin β. This trimeric complex is then transported across the nuclear membrane via the nuclear pore complex. In the nucleus, the complex dissociates and importin α and β are independently recycled back to cytoplasm. The targeted radiopharmaceutical is deposited in the nucleus. This figure was adapted from Lange et al. [184].](image-url)
Recently, the clinically employed somatostatin analog DOTA-[Tyr³]-octreotide (DOTATOC) was derivatized at various positions with PKKKRKV\textsuperscript{132} [185]. Among the different synthesized DOTATOC analogs, it was MG136 containing a NLS at the N-terminus which revealed to be most promising (Fig. 6.2) [84]. This derivative was radiolabeled with \(^{111}\text{In}\) and tested in vitro [84]. It showed high affinity ($K_d = 7.4 \pm 1.3 \text{ nM}$) to somatostatin receptors (SSTRs) which was similar to the affinity of \(^{111}\text{In}\)-DOTATOC ($K_d = 2.5 \pm 0.51 \text{ nM}$). Moreover, nuclear uptake and prolonged cellular retention were observed in cell experiments performed with transfected HEK cells which expressed SSTR2A.

![Chemical structures of the somatostatin analogs DOTATOC (A) and MG136 (B). Both derivatives consist of TOC (D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Thr(ol)) as the targeting ligand (blue) and DOTA (green) as a chelator for complexation of radiometals. MG136 contains in addition a nuclear localization signal (NLS, PKKKRKV\textsuperscript{132}, red) for nuclear targeting.](image)

In clinics, peptides radiolabeled with \(^{177}\text{Lu}\) are routinely used in cancer patients for targeted radionuclide therapy [7, 8]. Recently, \(^{161}\text{Tb}\) was proposed as an alternative to \(^{177}\text{Lu}\) because of additional emission of Auger/conversion electrons which might be beneficial for targeted radionuclide therapy [14]. Both, \(^{161}\text{Tb}\) and \(^{177}\text{Lu}\), are radiolanthanides with comparable coordination chemistry what allows using the same chelators and, hence,
targeting agents. In addition, their half-lives and $\beta^-$-energies are similar (Table 6.1). In preclinical studies, it was shown by our group that folate receptor (FR)-targeted tumor therapy with $^{161}$Tb was slightly more effective than with $^{177}$Lu [135]. Moreover, investigation of renal toxic side effects revealed that damage to the kidneys was comparable for $^{161}$Tb- and $^{177}$Lu-radiofolates if applied at the same radioactive quantity (chapter 2 and 4).

Table 6.1. Characteristics of $^{177}$Lu and $^{161}$Tb [15, 139].

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Emission type</th>
<th>Auger/ conversion electron</th>
<th>$E_{\beta^{\text{av}}}$ [keV]</th>
<th>$E_{\gamma}$ [keV]</th>
<th>Max. tissue range of $\beta^-$ [mm]</th>
<th>$T_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{177}$Lu</td>
<td>$\beta^-$, $\gamma$</td>
<td>-</td>
<td>134</td>
<td>113, 208</td>
<td>1.5</td>
<td>6.64 d</td>
</tr>
<tr>
<td>$^{161}$Tb</td>
<td>$\beta^-$, $\gamma$</td>
<td>yes</td>
<td>154</td>
<td>49, 75</td>
<td>1.7</td>
<td>6.89 d</td>
</tr>
</tbody>
</table>

The goal of this study was to develop an experimental setting to allow studying the Auger electron effect. Moreover, this test system should be applicable to investigate the impact of the radioconjugate’s subcellular localization on the anti-tumor effect. For this purpose, DOTATOC and its analog MG136 containing a NLS were radiolabeled with commercially available $^{177}$Lu. In vitro characterization of the two radiopeptides, including binding affinity, internalization/externalization and nuclear uptake studies, were performed. Moreover, the cytotoxic potential of the radioligands was investigated in vitro. For in vivo characterization single photon emission computed tomography (SPECT) and biodistribution studies were performed in AR42J tumor-bearing mice. In addition, preliminary results using the Auger/conversion electron-emitting radionuclide $^{161}$Tb were obtained in vitro.

6.2. Material and methods

6.2.1. Radiosynthesis

DOTA-[Tyr$^3$]-octreotide (DOTATOC) was obtained from Advanced Biochemical Compounds GmbH (ABX, Radeberg, Germany). The DOTATOC analog MG136 was kindly provided by Prof. Helmut R. Mäcke (Department of Radiology, University Hospital of Basel, Switzerland). No-carrier added $^{177}$LuCl$_3$ was purchased from Isotope Technologies Garching GmbH (Munich, Germany). No-carrier added $^{161}$TbCl$_3$ was produced at PSI as previously reported [14]. Radiolabeling of DOTATOC and MG136 was performed according to a standard
labeling procedure. For the preparation of the radiopeptides a stock solution of DOTATOC or MG136 (10 µL, 1 mM) was mixed with hydrochloric acid (HCl, 100-x µL, 0.05 M), sodium acetate (Na-acetate, 20 µL, 0.5 M) and the radionuclide (300 MBq in a volume of x µL). Depending on the experiment, additional amounts of peptide were added to obtain the required specific activity. The reaction mixture was then incubated for 15 min at 95°C. Sodium diethylene triamine pentaacetic acid (Na-DTPA, 10 µL, 5 mM, pH 5) was added to the reaction mixture for complexation of potential traces of $^{177}$Lu(III) or $^{161}$Tb(III), respectively. Quality control was performed by high-performance liquid chromatography (HPLC, Merck Hitachi, Darmstadt, Germany). The system was equipped with a C-18 reversed phase column (XTerra™ MS C-18, 5 µm, 15 cm x 4.6 cm, Waters). The mobile phase consisted of an aqueous 0.1% trifluoroacetic acid solution (A) and acetonitrile (B) with a linear gradient from 5% B to 80% of B over 15 min at a flow rate of 1 mL/min.

### 6.2.2. Cell culture

AR42J cells (rat pancreatic cancer cells, Cat-N° 93100618) were purchased from Health Protection Agency Culture Collections (Salisbury, U.K.) and PC-3 cells (human prostate cancer cells, ACC-465) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). AR42J cells are used as a model cell line for studies with somatostatin analogs as they express all five subtypes of the SSTR [169]. The cells were cultured as monolayers at 37°C in humidified atmosphere containing 5% CO$_2$. Both cell lines were cultured in RPMI 1640 cell culture medium (Bioconcept Amimed, Allschwil, Switzerland) supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics (penicillin 100 IU/mL, streptomycin 100 mg/mL, fungizone 0.25 µg/mL) from Bioconcept Amimed (Allschwil, Switzerland). Routine cell culture work was conducted twice a week. For all cell studies the polystyrene well plates were coated with poly-L-lysine (Sigma-Aldrich, St. Louis, U.S.) to prevent unspecific binding of the radiopeptides. In brief, sterile poly-L-lysine solution (0.5 mg/mL dissolved in Milli-Q water) was applied to the well plates at a volume that bottom and walls of the wells were covered, followed by incubation at 37°C for ~2-5 h. Afterwards, the poly-L-lysine solution was removed and the wells were washed twice with sterile phosphate buffered saline (PBS, pH 7.2). After drying, the plates were directly used for an experiment or stored at 4°C. For all in vitro assays cell culture medium containing 1% FCS (referred herein as assay medium) was used.
6.2.3. In vitro studies

6.2.3.1. Saturation binding assay

The affinity of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 to SSTRs expressed on AR42J cells was determined in saturation binding assays ($n \geq 3$). For this purpose, AR42J cells were seeded at a density of $1 \times 10^6$ cells per well (in 2 mL cell culture medium) in poly-L-lysine-coated 12-well plates and incubated overnight to obtain good cell adhesion. The next day, medium was removed and cells were washed once with 1 mL PBS (pH 7.4). Next, 975 µL of assay medium or 975 µL of assay medium containing 1 µM DOTA-[NaI$^3$]-octreotide (DOTANOC, blocking agent, Advanced Biochemical Compounds GmbH, ABX, Radeberg, Germany) were added to the wells. The cells were then incubated for 30 min at 37°C to obtain complete blockade of the SSTRs. Afterwards, increasing concentrations (0.1 nM to 1000 nM) of $^{177}$Lu-DOTATOC/DOTATOC or $^{177}$Lu-MG136/MG136 (specific activity of 20 MBq/nmol) were added to the cells, followed by incubation of 1 h at 37°C. For each radioligand concentration duplicates were prepared for specific and nonspecific binding. Before lysing the cells in sodium hydroxide (NaOH 1 M), the cells were washed twice with ice-cold PBS (pH 7.4). The cell lysate was measured in a $\gamma$-counter (Wizard 3, Automatic Gamma Counter, Perkin Elmer 1480, Waltham, MA, U.S.) for determination of bound fraction. The specific binding was calculated as the difference of measured radioactivity in the bound fraction and the fraction which was co-exposed to 1 µM DOTANOC. $K_d$ values were calculated using GraphPad Prism software (version 5.01).

6.2.3.2. Internalization studies

Internalization studies were performed according to a previously published procedure [186]. AR42J (1 x $10^6$) and PC-3 cells (0.6 x $10^6$) were seeded in 2 mL cell culture medium at confluence in poly-L-lysine-coated 12-well plates and left overnight to attach. When different radiopeptides were compared, the well plates were prepared the same day using the same cell suspension. The next day, the cells were washed once with PBS (pH 7.4). For blocking experiments the cells were pre-incubated with 1 µM DOTANOC for 30 min at 37°C. $^{177}$Lu-DOTATOC or $^{177}$Lu-MG136 (15 kBq, 0.75 pmol, 25 µL) were added to the each well containing 975 µL of assay medium. After incubation for 30 min, 2 h and 4 h at 37°C, the cells were washed three times with ice-cold PBS (pH 7.4) to determine the total uptake of the radiopeptides. The internalized fraction was determined by washing the cells once with ice-cold PBS (pH 7.4), followed by an acid-wash with glycine buffer (50 mM, pH 2.8) for 30 min and then the cells were again washed once with ice-cold PBS (pH 7.4). The acid-wash allows distinguishing between membrane-bound (acid-releasable) and internalized (acid-
resistant) fraction. The cells were lysed with 1 mL NaOH (1 M) and transferred to 4 mL-tubes for counting in a γ-counter. In order to standardize the measured radioactivity to the average of 0.3 mg protein per well, the protein concentration was determined using Micro BCA™ Protein Assay Kit (Thermo Scientific Prod, Rockford, U.S.). The internalization rate of both radioligands was determined in three independent experiments.

6.2.3.3. Externalization studies

Externalization studies were conducted as previously described [186]. AR42J cells were seeded in poly-L-lysine-coated 12-well plates (1 x 10^6 cells in 2 mL cell culture medium) and incubated overnight allowing cell adhesion. The next day, the cells were incubated for 2 h at 37°C with ^177^Lu-DOTATOC or ^177^Lu-MG136 (15 kBq, 0.75 pmol) diluted in assay medium to allow maximal internalization. After incubation, the supernatant was discarded and the cells were washed once with ice-cold PBS (pH 7.4) and once for 30 min with glycine buffer (50 mM, pH 2.8) followed by PBS washing. The cells were then incubated again in assay medium (1 mL) at 37°C for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 6 h (one plate per time point using 6 wells). During the experiment the assay medium was exchanged in every plate after 15 min, 30 min, 1 h, 2 h, 3 h and 4 h to prevent reuptake of the externalized fraction. For determining the externalized fraction, the supernatant was discarded after 0 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h or 6 h, respectively, and the cells were washed twice with ice-cold PBS. The cells were then lysed in NaOH (1 M) for counting the radioactivity in a γ-counter. The concentration of proteins was determined for each sample using Micro BCA™ Protein Assay Kit in order to standardize measured radioactivity to the average of 0.2 mg protein per well. The externalization rate of both radioligands was determined in three independent experiments.

6.2.3.4. Nuclei isolation assay

For determination of the uptake of ^177^Lu-DOTATOC and ^177^Lu-MG136, respectively, into the cell nucleus a nuclei isolation kit (Nuclei EZ Prep Kit, Sigma-Aldrich, St. Louis, U.S.) was used. AR42J (10 x 10^6) and PC-3 cells (5 x 10^6) were seeded in poly-L-lysine-coated Petri dishes using 15 mL of cell culture medium and incubated overnight at 37°C. The next day, the medium was discarded, the cells were rinsed once with PBS (pH 7.4) and 19.5 mL of assay medium was added. ^177^Lu-DOTATOC and ^177^Lu-MG136, respectively, were added at a volume of 0.5 mL (2.5 MBq, 50 pmol) and the cells were then incubated for 30 min, 2 h or 4 h at 37°C. After these time period, the Petri dishes were washed four times with ice-cold PBS (pH 7.4). Subsequently, the cell nuclei and cytoplasm/membrane fractions were harvested by
following the instructions of the manufacturer. In brief, 4 mL of ice-cold Nuclei EZ lysis buffer was added to each Petri dish in order to lyse the cells. The cell lysate was then centrifuged for 5 min at 500 rcf at 4°C. The supernatant containing cytoplasm/membrane fractions was transferred to a tube for counting radioactivity in a γ-counter. The pellet was resuspended in 4 mL ice-cold Nuclei EZ lysis buffer by vortexing briefly at moderate speed and then centrifuged again for 5 min at 500 rcf at 4°C. The supernatant which contained residual cytoplasm/membrane fractions was collected for counting radioactivity and the pellet was resuspended in 200 µL Nuclei EZ storage buffer before transferring into a tube for radioactivity measurement in the γ-counter. The measured radioactivity of nuclei and cytoplasm/cell membrane fractions was assumed to be together 100% cellular uptake. Nuclear uptake was expressed as percentage of total cellular uptake. The collected fractions were stained with trypan blue solution (0.4%, Sigma-Aldrich, St. Louis, U.S.) and analyzed using a microscope in order to test whether nuclei were properly separated from other cell fragments. The nuclei isolation assay was performed for each radioligand and time point twice.

6.2.3.5. Clonogenic assay

The capability of a single AR42J cell to grow into a colony upon exposure to $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 was determined by performing clonogenic assays [187]. Poly-L-lysine-coated 6-well plates were in addition coated with Matrigel® (Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix, Corning Inc., New York, U.S.) to allow proper formation of colonies. For this purpose, 300 µL Matrigel® (2 mg/mL diluted in RPMI cell medium without additives) were added to each well and then the plates were incubated at 37°C for at least 30 min to solidify the Matrigel®. AR42J cells were seeded at a density of 2000 cells per well in 2 mL cell culture medium and incubated overnight at 37°C. The next day, the medium was removed and the cells were incubated for 2 h at 37°C with 1 mL of $^{177}$Lu-DOTATOC or $^{177}$Lu-MG136 diluted with assay medium at activity concentrations of 0.1 MBq/mL to 5 MBq/mL. The peptides were radiolabeled at a specific activity of 30 MBq/nmol. The same procedure was applied to untreated control cells just without exposing to radiopeptides. After incubation, the supernatant was discarded and the cells were washed once with PBS (pH 7.4) before cell culture medium was added at a volume of 2 mL. Two weeks later the medium was removed and the wells were washed once with 1 mL PBS (pH 7.4). Then, the colonies were stained using 800 µL of a crystal violet solution (0.5% crystal violet, 6% glutaraldehyde in water). The number of colonies (>0.1 mm) was determined by placing the well plates on a grid (0.5 cm x 0.5 cm) and counting manually the
colonies in five selected squares using a microscope. The plating efficiency (PE) and surviving fraction (SF) were calculated according to the following formulas [187]:

\[
PE = \frac{\text{number of colonies formed (untreated)}}{\text{number of cells seeded}} \cdot 100\%
\]

\[
SF = \frac{\text{number of colonies formed after treatment}}{\text{number of cells seeded} \cdot PE} \cdot 100\%
\]

The SF upon exposure to various radioactivity concentrations of the radioligands was determined in two independent experiments using triplicates in each experiment.

6.2.3.6. MTT assay

The cell viability after incubation with the radiopeptides was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [170]. The MTT assay is an alternative to clonogenic assay for determining cytotoxicity upon exposure to ionizing radiation [188]. A total of 7500 AR42J and 2500 PC-3 cells in 200 µL cell culture medium were seeded in each well of a poly-L-lysine-coated 96-well plate. After incubation overnight allowing cell adhesion, the medium was removed and the cells were incubated with DOTATOC or MG136 radiolabeled with either \(^{177}\text{Lu}\) or \(^{161}\text{Tb}\) at a specific activity of 30 MBq/nmol. Per well 200 µL of the radioligand solution were added and for each concentration 12 wells were used. The applied radioactivity concentrations varied between 0.1 MBq/mL and 40 MBq/mL. After an incubation time of 2 h at 37°C, the cells were washed once with PBS (pH 7.4) followed by addition of 200 µL cell culture medium. The cells were then allowed to grow for 6 d at 37°C without changing cell culture medium. The assay was analyzed as previously described [171]. In brief, cells were incubated with MTT reagent (ultra-pure grade, Amresco, Solon, U.S.; 5 mg/mL dissolved in PBS pH 7.4) for 3-5 h, followed by dissolving of the formed formazan crystals in dimethyl sulfoxide (DMSO, Merck KGaA, Darmstadt, Germany). The absorption was measured at 560 nm with a microplate reader (560 nm, Victor™ X3, Perkin Elmer, Waltham, MA, U.S.). The absorbance measured for untreated control cells was assumed to be 100% cell viability. The cell viability of treated cells was expressed as percentage of the absorbance of control cells. Data analysis and dose-response-fitting was carried out using GraphPad Prism software (version 5.01). The cell viability inhibition was calculated as the radioactivity concentration which was necessary to reduce AR42J cell viability to 50% of untreated control cells (IC\(_{50}\)). IC\(_{50}\) values were determined in at least three independent experiments.
6.2.4. Animal studies

The performed in vivo studies were approved by the local veterinarian department and conducted in accordance with the Swiss law of animal protection. For all experiments female athymic nude mice (CD-1 Foxn1/nu, 5-7 week-old, Charles River Laboratories, Sulzfeld, Germany) were used. For the induction of tumor xenografts, mice were inoculated with $5 \times 10^6$ AR42J cells in 100 µL PBS (pH 7.2) into the subcutis of each shoulder. The tumors were allowed to grow for approximately two weeks. Radioligands were diluted in PBS (pH 7.4) containing 0.5% bovine serum albumin (BSA).

6.2.4.1. SPECT/CT imaging

In vivo SPECT/computed tomography (CT) studies were conducted 30 min, 2 h and 4 h after the injection of mice with $^{177}$Lu-DOTATOC or $^{177}$Lu-MG136 (30 MBq, 0.6 nmol) using a four-head multiplexing multipinhole small-animal SPECT/CT scanner (NanoSPECT/CT, Mediso Medical Imaging Systems, Budapest, Hungary). Each head was outfitted with a tungsten collimator of nine 1.4-mm-diameter pinholes and a thickness of 10 mm. SPECT/CT images were acquired using Nucline Software (version 1.02, Bioscan Inc., Poway, California, U.S.). CT images were obtained with the integrated CT scanner using a tube voltage of 55 kVp and an exposure time of 1 s per view. After the acquisitions, the data were reconstructed with HiSPECT software (version 1.4.3049, Scivis GmbH, Göttingen, Germany) using γ-energies for $^{177}$Lu of 56.1 keV ± 10%, 112.9 keV ± 10% and 208.4 keV ± 10%. SPECT and CT data were automatically co-registered because both modalities shared the same axis of rotation. The fused data sets were analyzed and Gauss post-reconstruction filtering was performed with VivoQuant post-processing software (version 1.23, inviCRO Imaging Services and Software, Boston, U.S.).

6.2.4.2. Biodistribution studies

For biodistribution, mice were injected with $^{177}$Lu-DOTATOC or $^{177}$Lu-MG136 (5 MBq, 0.6 nmol) into a lateral tail vein. For determination of unspecific tissue uptake, mice were co-injected with excess DOTANOC (~80 µg/mouse). The animals were euthanized 30 min, 2 h or 4 h after application of the radiopeptides. Selected tissues and organs were harvested, weighed, and counted for radioactivity using a γ-counter. Also, a defined volume of the original injection solution was counted for its radioactivity at the same time. The results are presented as percentage of injected activity per gram of tissue mass (% IA/g). Biodistribution studies were performed in triplicate.
6.2.5. Statistical analysis

Data are presented as mean ± standard. Statistics was conducted by using one-way ANOVA with Bonferroni’s multiple comparison post-test (GraphPad Prism, version 5.01).

6.3. Results

6.3.1. Binding affinity of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136

The binding affinities ($K_d$ values) of $^{177}$Lu-DOTATOC/DOTATOC and $^{177}$Lu-MG136/MG136 to SSTR-expressing AR42J cells were determined as $11 \pm 2.2 \text{ nM}$ and $8.7 \pm 2.4 \text{ nM}$, respectively. These $K_d$ values were not significantly different indicating that the receptor affinity was not affected by the NLS in MG136.

6.3.2. Cellular retention of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136

The cellular internalization rate of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 reached a steady state after ~2 h. After 2 h of incubation, 5.7 ± 2.1% and 8.6 ± 2.5% of the total added radioactivity were detected in AR42J cells for $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136, respectively (Fig. 6.3). In the case of $^{177}$Lu-DOTATOC, ~58% of the initially internalized fraction was externalized from the cells 6 h later (Fig. 6.3). At that time, the externalized fraction of $^{177}$Lu-MG136 was determined to be only ~24%. In SSTR-negative PC-3 cells no cellular uptake (<0.5% of the added radioactivity) of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 was detected (data not shown).

Fig. 6.3. Total internalized fraction of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 in AR42J cells considering the externalization rate. The cells were incubated with the radioligands for 2 h and subsequently the externalized fractions were determined over 6 h.
6.3.3. Nuclear uptake of ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136

Nuclei of AR42J and PC-3 cells were successfully isolated upon incubation with ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136 as proved by trypan blue staining (Fig. 6.4, A). After 2 h incubation of AR42J cells with ¹⁷⁷Lu-DOTATOC the nuclear uptake was 0.30 ± 0.28% of total cellular uptake, which means <0.05% of total added radioactivity (Fig. 6.4, B). In the case of ¹⁷⁷Lu-MG136, the nuclear uptake was 4.4 ± 2.3% which corresponds to ~0.7% of total added radioactivity. In the case of PC-3 cells the nuclear uptake expressed as percentage of total added activity was <0.02% for both, ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136 (data not shown).

![Fig. 6.4. Determination of nuclear uptake of ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136 in AR42J cells. Trypan blue staining of isolated nuclei fraction proved successful isolation of the nuclei (A). The fractions isolated with the Nuclei EZ Prep Kit contained large amounts of single (round spots, green arrow) or aggregated nuclei (red arrow) and only traces of other cell fragments (fibers, yellow arrow). Nuclear uptake of ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136 in AR42J cells was expressed as percentage of total cellular uptake (B). * P < 0.05.](image)

6.3.4. Cytotoxicity of ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136

The ability of a single AR42J cell to grow into a colony upon exposure to ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136 for 2 h was assessed by colony forming assays. The plating efficiency of AR42J cells was determined to be ~7%. Figure 6.5 presents the cell survival of AR42J cells after incubation with various radioactivity concentrations (0.1-5 MBq/mL) of ¹⁷⁷Lu-DOTATOC and ¹⁷⁷Lu-MG136, respectively. The survival fraction expressed as percentage of survival compared to untreated control cells was 71 ± 7.9% after exposure to ¹⁷⁷Lu-DOTATOC (0.5 MBq/mL) and 26 ± 4.0% after incubation with ¹⁷⁷Lu-MG136 (0.5 MBq/mL). Using a 2-fold higher concentration of these radiopeptides (1 MBq/mL) resulted in a survival fraction of 48 ± 13% for ¹⁷⁷Lu-DOTATOC and 8.2 ± 4.9% for ¹⁷⁷Lu-MG136.
Fig. 6.5. AR42J cell survival upon exposure to increasing radioactivity concentrations of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 determined by colony forming assays. Survival values are shown as percentage of survival compared to untreated control cells. *** $P < 0.001$.

The metabolic activity and, hence, the viability of AR42J cells was investigated by MTT assays after incubation with increasing radioactivity concentrations of DOTATOC and MG136 radiolabeled with $^{177}$Lu or $^{161}$Tb. The radioactivity concentration which was necessary to reduce AR42J cell viability to 50% of untreated control cells (IC$_{50}$) was 13 ± 6.3 MBq/mL for $^{177}$Lu-DOTATOC but only 5.4 ± 2.1 MBq/mL for $^{177}$Lu-MG136 (Fig. 6.6, A). For $^{161}$Tb-DOTATOC the IC$_{50}$ value was determined as 11 ± 4.6 MBq/mL but only 1.5 ± 0.28 MBq/mL for $^{161}$Tb-MG136 (Fig. 6.6, B). The cell viability of PC-3 cells was still ~100% upon exposure to a radioactivity concentration of ≤7.5 MBq/mL of the radiopeptides (data not shown).

Fig. 6.6. AR42J cell viability upon exposure to increasing radioactivity concentrations of $^{177}$Lu-DOTATOC/$^{177}$Lu-MG136 (A) and $^{161}$Tb-DOTATOC/$^{161}$Tb-MG136 (B) determined by MTT assay. Cell viability is presented as percentage of cell viability compared to untreated control cells. The radioactivity concentrations which were necessary to reduce cell viability to 50% of control cells (IC$_{50}$) were 13 ± 6.3 MBq/mL ($^{177}$Lu-DOTATOC), 5.4 ± 2.1 MBq/mL ($^{177}$Lu-MG136), 11 ± 4.6 MBq/mL ($^{161}$Tb-DOTATOC) and 1.5 ± 0.28 MBq/mL ($^{161}$Tb-MG136).
6.3.5. Tissue distribution of $^{177}\text{Lu}$-DOTATOC and $^{177}\text{Lu}$-MG136

SPECT/CT studies were performed 2 h after the injection of $^{177}\text{Lu}$-DOTATOC and $^{177}\text{Lu}$-MG136, respectively (Fig. 6.7). In the case of $^{177}\text{Lu}$-DOTATOC high amounts of radioactivity were found in AR42J tumor xenografts and in the kidneys. The administration of $^{177}\text{Lu}$-MG136 resulted in comparable radioactivity uptake in AR42J tumor xenografts. The renal uptake of $^{177}\text{Lu}$-MG136 was significantly higher compared to $^{177}\text{Lu}$-DOTATOC and, in addition, substantial amounts of radioactivity were found in the liver.

![Fig. 6.7. In vivo SPECT/CT images of AR42J tumor-bearing mice 2 h after injection of $^{177}\text{Lu}$-DOTATOC (A) and $^{177}\text{Lu}$-MG136 (B). Tumor xenografts, kidneys and liver are indicated with yellow, green and white arrows, respectively.](image)

Biodistribution studies with $^{177}\text{Lu}$-DOTATOC and $^{177}\text{Lu}$-MG136 revealed comparable accumulation of radioactivity in AR42J tumor xenografts at all investigated time points (Fig. 6.8, Table 6.2). The maximum uptake was $13 \pm 0.83\% \text{ IA/g}$ (30 min p.i.) for $^{177}\text{Lu}$-DOTATOC and $10 \pm 1.4\% \text{ IA/g}$ (4 h p.i.) for $^{177}\text{Lu}$-MG136 (Table 6.2). Uptake of both radiopeptides in tumors could be blocked by co-injecting excess DOTANOC. The renal uptake of $^{177}\text{Lu}$-DOTATOC was $12 \pm 1.9\% \text{ IA/g}$ 30 min after injection and $8.7 \pm 1.2\% \text{ IA/g}$ after 4 h. In the case of $^{177}\text{Lu}$-MG136 the kidney accumulation was $96 \pm 20\% \text{ IA/g}$ 30 min after application and increased to $103 \pm 8.9\% \text{ IA/g}$ after 4 h. A significant accumulation of radioactivity ($25 \pm 8.3\% \text{ IA/g}$, 30 min p.i.) was also found in the liver of mice injected with $^{177}\text{Lu}$-MG136 (Fig. 6.8, Table 6.2). Radioactivity accumulation in the liver of mice which received $^{177}\text{Lu}$-DOTATOC was negligible at all investigated time points (<0.5% IA/g). The uptake in kidneys and liver could not be blocked with excess DOTANOC. In all other tissues...
such as blood, muscle, bone and pancreas retention of radioactivity was low (<6% IA/g) and decreased further over time (Table 6.2).

**Fig. 6.8.** Accumulation (% IA/g tissue) of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 in kidneys, liver and AR42J tumor xenografts after 30 min, 2 h and 4 h. For blockade experiments mice were co-injected with DOTANOC (80 µg/mouse).
Table 6.2. Biodistribution of $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 in AR42J tumor-bearing mice.

<table>
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<th>$^{177}$Lu-MG136</th>
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</thead>
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<tr>
<td></td>
<td>30 min p.i.</td>
<td>2 h p.i.</td>
<td>4 h p.i.</td>
<td>2 h p.i.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>(blockade)</td>
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<td>Blood</td>
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<td>0.07 ± 0.03</td>
<td>0.03 ± 0.00</td>
<td>0.15 ± 0.06</td>
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<td>Spleen</td>
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<td>0.16 ± 0.06</td>
<td>0.10 ± 0.01</td>
<td>0.15 ± 0.04</td>
</tr>
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<td>Stomach</td>
<td>1.9 ± 0.33</td>
<td>1.4 ± 0.29</td>
<td>0.90 ± 0.07</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>Kidneys</td>
<td>12 ± 1.9</td>
<td>9.3 ± 1.9</td>
<td>8.7 ± 1.2</td>
<td>20 ± 2.7</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.0 ± 1.1</td>
<td>0.28 ± 0.11</td>
<td>0.13 ± 0.04</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>0.32 ± 0.04</td>
<td>0.15 ± 0.03</td>
<td>0.12 ± 0.02</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>Bone</td>
<td>0.38 ± 0.20</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.19 ± 0.05</td>
<td>0.03 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.64 ± 0.22</td>
<td>0.25 ± 0.03</td>
<td>0.25 ± 0.08</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.4 ± 0.34</td>
<td>1.3 ± 0.17</td>
<td>0.91 ± 0.31</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Tumor</td>
<td>13 ± 0.83</td>
<td>11 ± 1.5</td>
<td>11 ± 0.4</td>
<td>0.30 ± 0.07</td>
</tr>
</tbody>
</table>

|                    | 30 min p.i.        | 2 h p.i.           | 4 h p.i.           | 2 h p.i.         |
|                    |                   |                    |                    | (blockade)       |
| Blood              | 1.1 ± 0.31         | 0.09 ± 0.01        | 0.05 ± 0.01        | 0.25 ± 0.10      |
| Lung               | 2.5 ± 0.29         | 1.1 ± 0.22         | 1.2 ± 0.19         | 1.26 ± 0.23      |
| Spleen             | 5.5 ± 1.4          | 5.2 ± 1.3          | 5.0 ± 0.42         | 3.7 ± 0.20       |
| Stomach            | 2.2 ± 0.21         | 1.6 ± 0.21         | 1.4 ± 0.46         | 0.34 ± 0.04      |
| Kidneys            | 96 ± 20            | 99 ± 15            | 103 ± 8.9          | 90 ± 13          |
| Small intestine    | 0.84 ± 0.14        | 0.44 ± 0.06        | 0.42 ± 0.07        | 0.41 ± 0.02      |
| Liver              | 25 ± 8.3           | 28 ± 6.5           | 31 ± 4.1           | 18 ± 6.4         |
| Bone               | 1.7 ± 0.55         | 1.5 ± 0.71         | 1.2 ± 0.32         | 1.04 ± 0.50      |
| Muscle             | 0.28 ± 0.08        | 0.08 ± 0.02        | 0.05 ± 0.01        | 0.11 ± 0.01      |
| Adrenal            | 1.8 ± 0.24         | 2.0 ± 0.96         | 1.1 ± 0.19         | 1.7 ± 0.48       |
| Pancreas           | 2.1 ± 0.25         | 1.3 ± 0.25         | 1.2 ± 0.05         | 0.2 ± 0.01       |
| Tumor              | 8.7 ± 1.8          | 8.0 ± 1.4          | 10 ± 1.4           | 0.53 ± 0.14      |

Values [%IA/g] represent the mean ± SD of data from three animals per cohort.
6.4. Discussion

The combined emission of $\beta^-$-particles and Auger/conversion electrons of $^{161}\text{Tb}$ is of high interest for optimizing targeted radionuclide tumor therapy. The use of $\beta^-$-particles is efficient for the treatment of larger metastases, whereas Auger/conversion electrons are advantageous for therapy of single cancer cells [6].

In this work, an experimental setting using DOTATOC and its NLS-derivatized counterpart MG136 was established in AR42J tumor cells (SSTR-positive). These peptides were used as model compounds for investigating the Auger electron effect and for studying the impact of the subcellular deposition of a radiopharmaceutical. During the establishment of these assays, it was found that MG136 sticks to the used polystyrene plates most probably due to the cationic properties of the NLS. To prevent unspecific cell irradiation, it was necessary to avoid sticking of the radiopeptides to the well plates. Tests applying various compounds to coat polystyrene plates revealed that cationic poly-L-lysine prevents unspecific binding of MG136 to the plates. Herein, the radionuclide $^{177}\text{Lu}$ was used to establish the assays since the chemical properties of the radiolanthanides $^{177}\text{Lu}$ and $^{161}\text{Tb}$ are similar, but $^{177}\text{Lu}$ was commercially available from ITG.

$K_d$ determinations revealed that both, $^{177}\text{Lu}$-DOTATOC (11 ± 2.2 nM) and $^{177}\text{Lu}$-MG136 (8.7 ± 2.4 nM), have high affinities to SSTRs expressed on AR42J cells. These values were slightly different compared to previously published data for $^{111}\text{In}$-DOTATOC (2.5 ± 0.51 nM) and $^{111}\text{In}$-MG136 (7.4 ± 1.3 nM), but in agreement regarding the fact that both derivatives bound equally well to the receptor [84]. Possibly, the values are not identical as the used cell line and radionuclide were different. Cellular uptake and retention studies demonstrated that externalization of $^{177}\text{Lu}$-MG136 was not as pronounced as it was the case for $^{177}\text{Lu}$-DOTATOC (Fig. 6.3). This observation might be explained by nuclear retention of $^{177}\text{Lu}$-MG136 as it was shown in cell nuclei isolation studies (Fig. 6.4). These data confirmed what was previously published by Ginj et al. [84].

The therapeutic potential of the two radiopeptides was investigated in vitro by performing colony forming and MTT assays [170, 187, 188]. Both assays demonstrated that $^{177}\text{Lu}$-MG136 was superior in reducing cell survival and viability to $^{177}\text{Lu}$-DOTATOC (Fig. 6.5, Fig. 6.6, A). Clonogenic assays demonstrated that $^{177}\text{Lu}$-MG136 reduced AR42J cell survival ~6-fold more efficiently than $^{177}\text{Lu}$-DOTATOC if applied at 1 MBq/mL radioactivity concentration. Moreover, MTT tests revealed that the radioactivity concentration for reducing cell viability by 50% was ~2.5-fold lower in the case of $^{177}\text{Lu}$-MG136 ($IC_{50} = 5.4 \pm 2.1 \text{MBq/mL}$) than for $^{177}\text{Lu}$-DOTATOC ($IC_{50} = 13 \pm 6.3 \text{MBq/mL}$). MTT tests performed with SSTR-negative PC-3 cells revealed that the observed effects were SSTR-specific. This data indicated that the nuclear deposition and, hence, the increased cellular retention of the NLS-
peptide radiolabeled with $^{177}$Lu improved therapeutic effect in vitro. This data are in agreement with previous statements predicating that nuclear deposition of a radiopharmaceutical is advantageous independent on the tissue range of the applied radionuclide [5].

During this work, preliminary cell viability studies were also performed with the Auger electron-emitting radionuclide $^{161}$Tb. MTT assays revealed that $^{161}$Tb-MG136 reduced AR42J cell viability ~7.5-fold more efficiently than $^{161}$Tb-DOTATOC (Fig. 6.6, B). The herein performed cell viability studies demonstrated a comparable trend for $^{161}$Tb- and $^{177}$Lu-radiolabeled peptides, but a more significant difference of inhibiting cell viability was found in the case of $^{161}$Tb. This result was expected since deposition of Auger/conversion electrons emitted by $^{161}$Tb in the nucleus is more damaging to the cell than $\beta^-$-particle radiation. This finding is in agreement with previous studies performed with $^{123/125}$I and $^{111}$In [11, 13, 83, 189].

However, for an appropriate comparison of data obtained with these two radiolanthanides, it will be essential to estimate the applied cellular dose. Moreover, it will be necessary to perform the herein established assays with a pure Auger/conversion electron emitter such as $^{165}$Er, in order to finally answer whether a nuclear deposition is crucial for the cell damaging effects of Auger/conversion electrons.

SPECT/CT studies as well as biodistribution studies performed in AR42J tumor-bearing mice revealed that the tumor uptake was comparable for $^{177}$Lu-DOTATOC and $^{177}$Lu-MG136 (Fig. 6.7, Fig. 6.8). The tumor uptake of both radiopeptides was SSTR-specific as its accumulation could be blocked by co-injection of excess amount of DOTANOC. However, renal uptake of $^{177}$Lu-MG136 was ~10-fold higher than after injection of $^{177}$Lu-DOTATOC which would be prohibitive for a therapeutic application of this radioconjugate. For $^{177}$Lu-MG136 an unspecific liver uptake was observed, which was absent in the case of $^{177}$Lu-DOTATOC (Table 6.2). The obtained data showed that the increased number of cationic amino acids, as a consequence of the integrated NLS, resulted in enhanced renal and hepatic uptake of $^{177}$Lu-MG136. This observations is in line with previous literature reports demonstrating elevated accumulation of radiopeptides in kidneys and liver after introducing positively charged amino acids into the peptide structure [190, 191]. For future in vivo application of this new radiopeptide, it will be crucial to test whether the co-application of cationic amino acids also effectively reduces the renal uptake of radiolabeled MG136, as it is known to be the case for commonly used radiolabeled somatostatin analogs [56]. The observed liver uptake (18 ± 6.4% IA/g, 4 h p.i.) might be less of a problem since the hepatic tissue is not as radiosensitive as the kidneys [58]. However, uptake of NLS derivatives in healthy tissues potentially results also in higher off-target toxicity which is unfavorable and needs to be addressed in future studies.
6.5. Conclusion

The data of this work indicate that deposition of $^{161}$Tb-radioconjugates in the cell nucleus might enhance the therapeutic effect caused by Auger/conversion electron emission. Besides, studies performed with the radionuclide $^{177}$Lu, which lacks the emission of short-ranging high-LET particles, demonstrated that nuclear deposition of therapeutic radiopharmaceuticals might be in general favorable due to prolonged cellular retention. Hence, nuclear targeting might be a promising concept for targeted radionuclide therapy in general, but in particular if short-ranging Auger/conversion electron-emitting radionuclides are applied.
CHAPTER 7

Conclusion and Outlook
In this thesis, it was confirmed that prolongation of the blood circulation time of radiofolates by binding to albumin is a successful approach to improve the tumor-to-kidney ratio of accumulated radioactivity. As a consequence of the improved tissue distribution, the renal dose burden could be reduced and, at the same time, the anti-tumor effect was enhanced during therapy. Our studies successfully demonstrated complete tumor remission in tumor-bearing mice after application of $^{177}$Lu-radiolabeled albumin-binding folate at a tumor dose of ~28 Gy. However, at this therapeutic dose the renal dose burden was ~46 Gy which resulted in impaired renal function and morphological changes of the kidneys. Long-term studies performed over eight months revealed time- and dose-dependent radionephrotoxicity in mice and a renal threshold dose of ~23 Gy to prevent kidney toxicity after radiofolate therapy. Hence, doses of radiofolates which do not harm the kidneys are not sufficient to obtain complete tumor remission. This data evidently demonstrated that the kidneys are the dose-limiting organs during tumor therapy with radiolabeled folates.

To reduce the risk of damage to the kidneys during radionuclide therapy, we studied a potential benefit of co-administering the antihypertensive drug captopril and amifostine as radioprotective agents. It was previously demonstrated that inhibitors of the renin-angiotensin system like captopril are beneficial in the mitigation of radiation nephropathy [75, 77, 78, 125]. SPECT/CT studies revealed that captopril co-treatment improved in some animals the tumor-to-kidney ratio of albumin-binding radiofolates by a factor of ~2, whilst in other animals the tissue distribution was unchanged. Due to the high interindividual variability and, therefore, low reproducibility of the desired effect, this phenomenon was not further investigated. It is likely that the effect of captopril on the blood pressure and, hence, on the renal filtration pressure is dose-dependent and different in each animal. However, it would be worthwhile to investigate this effect retrospectively in patients who were co-treated with targeted radiopharmaceuticals and antihypertensive drugs such as captopril. These studies would indicate if patients could potentially profit from a combined therapy with radiofolates and antihypertensive drugs in the future. Beside its potential impact on the tissue distribution of targeted radiopharmaceuticals, captopril could possibly act as a radioprotector. It will be crucial to test as next the potential renoprotective effect of captopril after radiofolate therapy in long-term mouse studies. In contrast to captopril, the clinically used chemo- and radioprotector amifostine did not influence the tissue distribution profile of radiofolates. In long-term studies performed over eight months it was demonstrated that amifostine application did not protect the mouse kidneys from renal damage after application of $^{177}$Lu-radiolabeled albumin-binding folate at a renal dose of ~46 Gy. Based on our data, it remained unclear whether amifostine does not have protective effects during radiofolate therapy at all, or whether the application of amifostine 4 h, 24 h and 48 h after radiofolate injection (each time 2.5 mg) was not sufficient. Amifostine shows fast renal clearance [127].
Hence, it might be worthwhile to test whether daily injections of amifostine up to 10 d after radiofolate application, until radioactivity is almost completely cleared from the kidneys, would result in renoprotective effects.

The results of a comparative pilot study performed in mice over eight months was not conclusive regarding the question whether α-therapy (\(^{149}\text{Tb}\)-cm09) or β-therapy (\(^{161}\text{Tb}\)-cm09) is more nephrotoxic. The emission of α-particles results in a highly localized energy deposition which is favorable for the treatment of small tumors and metastases. Regarding undesired side effects, it is so far not clear, whether α-particles would also be more toxic to healthy renal tissue. The small cohorts of mice used in our study, as a consequence of the limited availability of \(^{149}\text{Tb}\), did not allow appropriate investigations in this respect. In the future, it will be essential to investigate specific differences between effects and side effects of α- and β-radiofolate therapy in large cohorts of mice. To this end, using \(^{149}\text{Tb}\) and \(^{161}\text{Tb}\) would present a unique opportunity to perform such comparative studies because they can be employed to prepare chemically identical radioconjugates.

It was previously shown by our group that tumor treatment with \(^{161}\text{Tb}\) (\(E_{\beta^-}\text{av} = 154\) keV, Auger/conversion electrons) was favorable over \(^{177}\text{Lu}\)-radiofolate therapy (\(E_{\beta^-}\text{av} = 134\) keV) in KB and IGROV-1 (both FR-positive) tumor mouse models. Moreover, the performed long-term studies with \(^{161}\text{Tb}\)- and \(^{177}\text{Lu}\)-radiolabels over eight months demonstrated that the renal functional impairment was comparable at therapeutic doses which led to slightly superior anti-tumor effects of \(^{161}\text{Tb}\)-radiofolate. This data imply that the emission of additional Auger/conversion electrons in the case of \(^{161}\text{Tb}\) might improve the anti-tumor effect whilst the risk for radionephrotoxicity remains unchanged. The potential benefit of the Auger electron effect was studied in a side project of this thesis using a model radiopeptide which targets the cell nucleus (MG136) after radiolabeling with \(^{161}\text{Tb}\) and \(^{177}\text{Lu}\). Our results revealed that the emission of Auger/conversion electrons might be most beneficial when deposited in the cell nucleus. In this study, \(^{161}\text{Tb}\) was used as an Auger/conversion electron emitter which also emits β-particles (\(E_{\beta^-}\text{av} = 154\) keV) with a slightly higher β-energy compared to \(^{177}\text{Lu}\) (\(E_{\beta^-}\text{av} = 134\) keV). Hence, it is not clear which particle emission is mainly responsible for the observed effect. To unambiguously determine a potential benefit of \(^{161}\text{Tb}\) over \(^{177}\text{Lu}\), it will be necessary to calculate the applied cellular doses upon exposure to the \(^{161}\text{Tb}\)- and \(^{177}\text{Lu}\)-radiolabeled targeting agents. Moreover, it will be essential to test cell viability inhibition after applying pure Auger/conversion electron emitters such as \(^{165}\text{Er}\) (\(T_{1/2} = 10.4\) h, \(E_{\text{electrons}}: 5.33\) keV (65.6%) and 38.4 keV (4.8%) [15]) to find out more about the Auger electron effect. The herein performed in vitro studies showed that nuclear targeting with a nuclear localization signal leads to increased cellular retention and, hence, to an improved therapeutic effect. Therefore, translation of this principle to other cell-internalizing radiopharmaceuticals might be a possibility to improve the anti-tumor effect during targeted
radionuclide therapy, particularly if Auger/conversion electron-emitting radionuclides are applied (Fig. 8.1).

The long-term studies performed during this thesis demonstrated that determination of renal $^{99m}$Tc-DMSA uptake is more sensitive to detect impaired renal function than the measurement of blood urea nitrogen and creatinine in the blood plasma. However, also $^{99m}$Tc-DMSA uptake studies cannot detect renal damage earlier than 3 months after therapy start in the mouse. Moreover, this method requires complex handling of the animals, is time-consuming and expensive. It would be of a great benefit to identify renal biomarkers which indicate kidney damage at an earlier stage (<3 months) and which would be easily detectable. Such a biomarker would simplify renal toxicity studies in preclinical trials and would prevent the necessity of keeping mice for several months. Several such parameters, including urinary clusterin, cystatin C, $\beta_2$-microglobulin, kidney injury molecule-1 or retinol binding protein 4, were proposed in the literature [104, 128, 192]. Moreover, the measurement of total protein and albumin in the urine might be a useful marker for renal injury as recently demonstrated [128, 193]. For exploring the option of early detection of renal damage, it will be necessary to correlate data of long-term damage, as performed in our studies, with potential changes of kidney markers at an early stage. The identification of reliable biomarkers for early detection of kidney injury would allow taking measure regarding dose reduction or discontinuation of treatment at an early stage also in human patients.

In this work, it was shown in vitro that despite of FR up-regulation by GCs the therapeutic effect of radiofolates was not enhanced. In contrast, it was incidentally discovered during in vitro experiments that FR-positive KB cells exposed to GCs became insensitive towards ionizing radiation. In FR-positive IGROV-1 cells such radioresistant effects were, however, not observed. In order to investigate a potential impact of GCs on radiofolates’ therapy in vivo, studies in KB tumor-bearing mice were performed. In these studies the GC dexamethasone did not influence the tumor uptake of radiofolates as it was expected from our in vitro experiments. However, the performed in vivo studies could not prove the effect of developing radioresistance upon GC exposure as it was observed in vitro. It is assumed that studies reported in the literature, which proved GC-induced chemoresistance [172], have to be adapted for our experimental setting. Hence, it will be necessary to find an appropriate application scheme of dexamethasone to demonstrate the development of radioresistance in mice. Further in vitro investigations with AR42J and PC-3 tumor cells using radiolabeled somatostatin and bombesin analogs, respectively, showed that the development of radioresistance upon exposure to GCs is cell type specific but independent on the targeting agent. The determination of glucocorticoid receptor levels by immunohistochemical analysis might shed light on better understanding the observed differences between various cell lines. In the future, it will be essential to study this phenomenon in humans retrospectively by
analyzing clinical data of patients who underwent radiotherapy with or without co-application of GCs. GCs are routinely applied to cancer patients and, therefore, it is of interest to understand potential interactions of these drugs with radionuclide therapy.

For optimizing radionuclide therapy with small molecular weight radiopharmaceuticals, it will be necessary to evaluate further radionuclides with variable decay characteristics in order to identify the most favorable one for therapeutic application (Fig. 8.1). In this respect, the investigation of “matched pairs” of radionuclides of the same element but with different decay characteristics is promising since radiolabeling of compounds results in chemically identical radiopharmaceuticals. These radioconjugates would allow radionuclide-based imaging and therapy at the same time (“radiotheragnostics”). Moreover, exact dose estimations of therapeutic radiopharmaceuticals become feasible. Promising candidates of matched radionuclides include scandium (\(^{43/44/47}\)Sc) and copper radionuclides (\(^{64/67}\)Cu, Table 8.1).

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>(E_{\beta^{+}\text{av}}) [keV]</th>
<th>(E_{\gamma}) [keV]</th>
<th>(E_{\beta^{-}\text{av}}) [keV]</th>
<th>(T_{1/2})</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{43})Sc</td>
<td>476</td>
<td>-</td>
<td>-</td>
<td>3.89 h</td>
<td>PET</td>
</tr>
<tr>
<td>(^{44})Sc</td>
<td>632</td>
<td>-</td>
<td>-</td>
<td>3.97 h</td>
<td>PET</td>
</tr>
<tr>
<td>(^{47})Sc</td>
<td>-</td>
<td>159</td>
<td>162</td>
<td>3.35 d</td>
<td>SPECT, therapy</td>
</tr>
<tr>
<td>(^{64})Cu</td>
<td>278</td>
<td>-</td>
<td>191</td>
<td>12.7 h</td>
<td>PET, therapy</td>
</tr>
<tr>
<td>(^{67})Cu</td>
<td>-</td>
<td>91, 93, 185</td>
<td>141</td>
<td>2.58 d</td>
<td>SPECT, therapy</td>
</tr>
</tbody>
</table>

Abbreviations: PET, positron emission tomography; SPECT, single photon emission computed tomography.

The use of \(^{43/44}\)Sc for positron emission tomography (PET) and the application of \(^{47}\)Sc for therapy are promising as recently demonstrated in the mouse model [194, 195]. \(^{43}\)Sc, \(^{44}\)Sc and \(^{47}\)Sc can be stably coordinated by conventional DOTA chelators which is advantageous as numerous radiopharmaceuticals, including clinically used somatostatin analogs, are derivatized with DOTA [196]. \(^{64}\)Cu and \(^{67}\)Cu were employed successfully in numerous preclinical studies using different targeting agents [197]. As previously demonstrated, copper radionuclides show optimal complexation with NODAGA [196, 198]. Hence, derivatization of targeting agents of interest with this chelator is essential. Both, \(^{47}\)Sc (\(T_{1/2} = 3.35\) d) and \(^{67}\)Cu
(T_{1/2} = 2.58 \text{ d}), have half-lives which are in particular suitable for therapeutic application of small molecular weight radiopharmaceuticals.

Fig. 8.1. Possible strategies for improved tumor therapy with radiopharmaceuticals which comprise potential risk for radionephrotoxicity. NLS, nuclear localization signal; RAS, renin-angiotensin system.


