Radiopeptides for Targeted Tumour Therapy and the Kidney

Radiopeptiden voor doelgerichte tumortherapie en de nier

Marleen Melis

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Radiopeptides for Targeted Tumour Therapy and the Kidney

Radiopeptiden voor doelgerichte tumortherapie en de nier

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Promotoren:	Prof.dr.ir. M. de Jong Prof.dr. E.P. Krenning Prof.dr. O.C. Boerman
Overige leden:	Prof.dr. F.J. Beekman Prof.dr. T.J. Visser Prof.dr. R.R. de Krijger

Voor mijn moeder
Nel Melis-Versluis
(† 8-7-2007)

En ook voor andere vrouwen,
zoals mijn grootmoeders,
die niet de kans hebben
gekregen of genomen
hun talenten te ontplooien

Radiopeptides for targeted tumour therapy and the kidney

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General introduction

Chapter 1

Introduction and outline of the thesis

Chapter 1.1

Introduction

One of the main causes of morbidity and mortality in the modern world is cancer [1]. According to World Health Organization (WHO) reports the worldwide mortality rate due to malignant neoplasms is 12.5%, but in the Western world this is about 30% in the 45-75 year age group. In The Netherlands the prevalence of different types of cancer varies between sexes; amongst men prostate cancer (21%) and amongst women breast cancer (33%) are the most frequently diagnosed types, expressed as percentage of all registered tumour patients. Lung (16% men, 8% women) and colorectal carcinomas (13%) are major cancers as well (The Netherlands Cancer Registry). In this respect the incidence of cancers from neuroendocrine origin, the main tumour type discussed in this thesis, is very low; less than 0.1% [2]. Because of slow progression of the disease the prevalence is much higher, however.

Many different clinical tools are available to detect tumours. Several imaging modalities can be employed to visualize tumours and their metastases, including radiography using X-rays, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and nuclear imaging. The latter includes gamma (γ)-radiation, both planar- and single-photon emission computed tomography- (SPECT) imaging and positron (β^+) emission tomography (PET). For therapy surgery is the first option, followed by (adjuvant) chemotherapy, external beam radiation therapy (EBRT), radionuclide therapy, or combinations thereof. The ideal situation during therapy would be that only, or at least primarily, tumour cells are removed, damaged or killed, while harmful side effects on normal cells and tissues are reduced to a minimum. Any undesirable effects may be unavoidable though.

Over the last decades progress has been achieved concerning specific targeting of tumour cells. This concept is based on the presence of biomarkers expressed only, mainly or in high density on malignant cells in comparison to healthy cells. Targeting agents with specific ligand-binding properties to these biomarkers, can be applied as tumour targeting tools for diagnostic or therapeutic use. Diagnostic imaging is feasible when γ- or β^+-emitting radionuclides or other imaging tags are labelled to tumour-specific antibodies or peptides. These antibodies and peptides can have a therapeutic effect as such, but when conjugated with a therapeutic alpha (α)- or beta (β^-)-emitting radionuclide or other cytotoxic compound DNA damage can be induced to destroy tumour cells expressing the biomarkers, while sparing healthy tissues.

1. Tools for targeted tumour therapy: focus on monoclonal antibodies and peptides

1.1 Monoclonal antibodies

The technique developed by Köhler and Milstein in 1975 to prepare monoclonal antibodies (mAb) with predefined specificity offered the possibility to produce a huge variety of specific antibodies [3]. Antibody-secreting B-lymphocytes were isolated from animal spleen cells in cell culture and immortalized by fusion with a cancer cell line; forming so-called hybridomas. Originating from one hybridoma cell, continuous production and secretion of many mAb in large amounts became available. MAbs specifically identify one epitope, such as receptors or structural components of the cell membrane. The CD (Cluster of Differentiation) series of antibodies targeting white blood cells are numbered up to 350 recently [4]. These antibodies are being used as diagnostic markers in many diseases where immune functions need to be monitored, such as in HIV infection, but also to characterize different subtypes of leukaemia. Also applications of mAbs in e.g. characterization of malignant tissues and for pregnancy tests were developed.

Therapeutic use of mAbs is employed to treat diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and a variety of cancers, including non-Hodgkin's lymphoma, colorectal cancer, head and neck cancer and breast cancer [5]. More than 20 products have been approved by the Food and Drug Administration (FDA) and over 200 mAbs are currently in clinical trials [6]. The therapeutic efficacy of mAbs, especially in solid tumours, appeared to be limited though, therefore radionuclides or toxic drugs were conjugated to several mAbs to enhance their therapeutic results [7]. Treatment of non-Hodgin lymphoma patients with ibritumomab tiuxitan (Zevalin®) and tositumomab (Bexxar®), both anti CD20 mAbs radiolabelled with

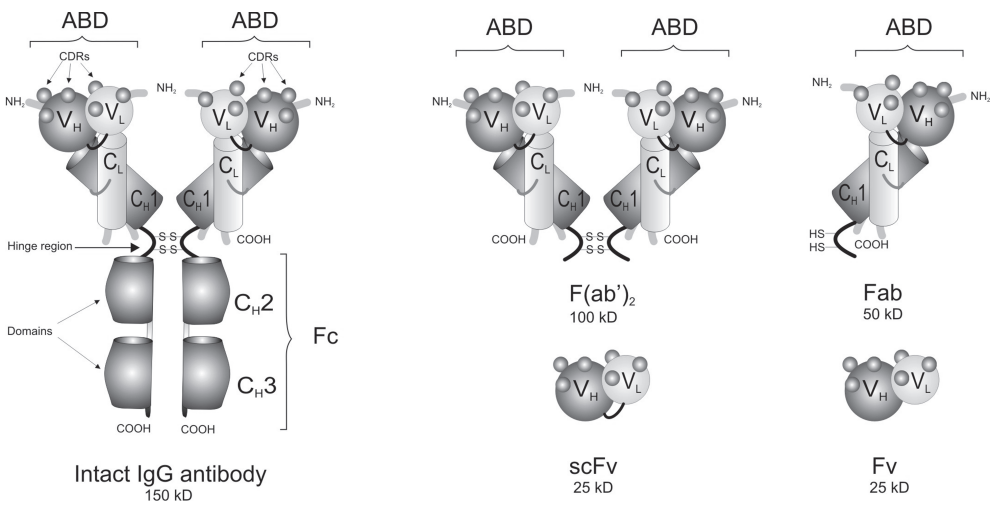


Figure 1, adapted from Majidi et al. [5]. Graphical representation of intact immunoglobulin G (IgG) antibody and antibody fragments. ABD: antigen-binding domain; Fab: antibody fragment; Fv: variable fragment; ScFv: single chain variable fragment; VH: Variable domains of heavy chain; VL: Variable domains of light chain; CH1-3: constant regions of heavy chain; CL: constant region of light chain.

$^{90}Y/^{111}In$ and ^{131}I respectively, results in good response rates, higher than those with unlabelled mAbs [4, 5]. This result reflects one of the major successes of radionuclide therapy for tumour therapy.

Antibodies are large (~150 kDa) globular plasma proteins consisting of two identical heavy chains and light chains connected by disulfide bonds, as shown in Figure 1. Drawbacks of the large size of mAbs are instability and relatively long circulation times and poor tissue penetration. When radiolabelled mAbs are administered this may result in a high radiation dose to the bone marrow, causing decreased normal blood cell counts and increased incidence of myelodysplastic syndrome (MDS) or even leukaemia [8].

Therefore research into application of smaller F(ab) or $F(ab)_2$ fragments of mAbs (~50 kDa or ~100 kDa) and preparation of minibodies (110 kDa) or diabodies (55 kDa) with more favourable pharmacokinetic properties is ongoing. Engineered synthetic small and stable Ab fragments such as single chain antibodies (ScFv) prepared by phage display technique (~25 kDa), nanobodies (15 kDa) derived from heavy chains of camelids, and so-called Affibody molecules (~7 kDa) are therefore promising new targeting tools [5, 9-13]. Generally, reduced size of antibody fragments facilitates blood clearance resulting in higher tumour-to-background ratios.

Radiolabelled nanobodies and Affibody molecules specifically targeting breast tumour cells, present examples of recent progress and opens new perspectives for clinical applications of antibody-based tumour diagnosis and therapy [14, 15].

1.2 Peptides

Since the late 1980's peptides, synthesized to mimic natural ligands of receptors, are being developed as tools for specific tumour cell targeting. Different kinds of receptors e.g. hormone-, growth factor- or folate receptors, are often overexpressed on tumour cells, enabling discrimination between malignant and normal tissues [16-20]. The number of amino acid residues needed to synthesize these biologically active peptides varies between four to more than fifty, with a molecular size ranging between 0.5 to 6 kDa. The much smaller size of tumour-specific peptide analogues compared to that of antibodies reveals several advantages: rapid tissue penetration combined with a rapid clearance; no antigenicity; easy synthesis and simple to be modified chemically [20]. Moreover, peptides can be easily conjugated with chelates to enable radiolabelling with metallic radionuclides.

Tumours originating from functioning neuroendocrine cells often show overexpression of receptors for peptide hormones such as somatostatin, glucagon like peptide (GLP-1) or gastrin. The group of neuroendocrine tumours (NETs) is quite heterogeneous. The classification according to WHO and European Neuroendocrine Tumour Society (ENETS) guidelines are based on a combination of clinical and pathological features specific for the organ the tumours originate from and on production/secretion of hormones [21-23]. Functioning endocrine tumours are named according to respective hormones that are produced, such as insulinomas, gastrinomas or carcinoids when serotonin is secreted. Five subtypes of the somatostatin receptors are known of which subtype 2 (sst2) is predominantly expressed in NETs, although subtype 3 and 5 overexpression has been described in several cases as well, e.g. in paraganglioma [20, 24, 25].

The incidence of NETs is very low (~2/100.000 per year) [2]. Moreover, these tumours often remain undetected for a long period, because generally they grow relatively slowly. Upon diagnosis many patients present with metastasized disease [26], which limits the possibilities to cure these patients using surgery or EBRT, whereas chemotherapy offers only limited beneficial effects [27]. This urged development of new tools to improve both diagnostics and therapy for NETs. Somatostatin, gastrin and other peptide analogues seemed to offer promising perspectives in this respect when tagged with radionuclides either for diagnostic imaging or radionuclide therapy [20, 28].

The small cyclic peptide hormone somatostatin shows inhibitory effects on secretion of various hormones. Two native somatostatin structures occur; a 14 and a 28 amino acid sequence, that are rapidly degraded upon injection, however. Octreotide, consisting of 8 amino acids (~1 kDa) in a cyclic structure, is a still biologically active and stable somatostatin analogue. Insertion of two D-residues instead of the natural occurring L-amino acids provided improved stability. Several octreotide derivatives, e.g. Sandostatin-LAR (long-acting release), are being applied intramuscularly to successfully reduce hormone-based clinical symptoms such as flushing and diarrhoea, thereby improving quality of life of patients suffering from hormone producing NETs. Sandostatin-LAR treatment resulted in less than 10% of the patients in objective tumour regression though [29].

Over the years many variants of the eight amino acid sequence of octreotide have been developed (some examples shown in Table 1). In Tyr^3-octreotide the phenylalanine (Phe) residue at position 3 has been replaced by tyrosine (Tyr). When in addition the threoninol group at the C-terminus (position 8) has been exchanged by threonine Tyr^3-octreotate is obtained, showing higher affinity for sst2 than octreotide [30]. By insertion of 1-Nal at position 3 instead of Tyr, the somatostatin analogues Nal^3-octreotide (NOC) or Nal^3-octreotate (NATE) were obtained [31, 32]. The characteristics regarding receptor specificity and affinity vary among the various somatostatin analogues [30].

Position:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Octreotide	D-Phe	Cys	Phe	D-Trp	Lys	Thr	Cys	Thr(ol)
Tyr^3-octreotide	D-Phe	Cys	Tyr	D-Trp	Lys	Thr	Cys	Thr(ol)
Tyr^3-octreotate	D-Phe	Cys	Tyr	D-Trp	Lys	Thr	Cys	Thr
NOC	D-Phe	Cys	1-Nal	D-Trp	Lys	Thr	Cys	Thr(ol)
NATE	D-Phe	Cys	1-Nal	D-Trp	Lys	Thr	Cys	Thr

Table 1: Several 8 amino acid somatostatin analogues. The SS-bridge between the two cysteine (Cys) residues at position 2 and 7 forms the ring of the cyclic peptide.

Next to a variety of somatostatin analogues, other radiopeptide analogues of ligands specifically binding to hormone receptors have been generated targeting several human tumours, as reviewed by Reubi et al. [20]. Examples comprise gastrin releasing peptide (GRP) or bombesin analogues that can be applied in patients with breast or prostate carcinoma as GRP-receptors are often over-expressed on these tumour cells [33, 34]. Neurotensin analogues may target

e.g. exocrine pancreatic tumours [20, 35]. GLP-1 analogues, such as exendin-3 and 4, were developed to target GLP-1 receptors being over-expressed in nearly all cases of insulinoma, in a high incidence in gastrinomas, and in 30% of cases of carcinoids [36-39]. Cholecystokinin (CCK) or gastrin analogues have been developed to specifically target CCK2-receptors over-expressed in most insulinomas, but also in the majority of medullary thyroid cancers (MTC), stromal ovarian cancers, astrocytomas, and small cell lung carcinomas (SCLC) [40-42]. Several stable radiolabelled GRP, neurotensin, GLP-1 and CCK peptide analogues have been developed recently, primarily for diagnostic use. Examples are shown in Table 2. The molecular sizes, net charges and renal uptake and retention of these peptide analogues vary largely.

Peptide ligands	Peptide receptor expressing tumours (a.o.)	Analogues
Bombesin, GRP	Breast, prostate	Demobesin, AMBA [33, 34, 43]
Neurotensin	Exocrine pancreas	MP2530 [35]
GLP-1	Insulinoma, gastrinoma	exendin 3, 4 [37, 38]
CCK2/gastrin	Neuroendocrine, MTC, SCLC	MG0-11, CCK8 [41, 42, 44]

Table 2: Overview of peptide ligands, other than somatostatin, the tumours targeted and examples of developed peptide analogues.

2. Radiolabelling of peptide analogues

^{123}I-iodination of the Tyr residue in octreotide was the first route applied to create radiolabelled somatostatin analogues for diagnostic purposes [17, 45]. Rapid degradation of these iodinated ligands and therefore fast washout of radioactivity from targeted cells, plus the hepatobiliary excretion of the radiopeptide demonstrated that this compound was a suboptimal imaging agent, however [46]. Therefore ^{111}In-labelling was performed using octreotide chelated with diethylenetriaminepentaacetic acid (DTPA, Figure 2) at the N-terminus. ^{111}In-DTPA-octreotide showed improved cellular retention in comparison with the radioiodinated peptide, because of internalization of the receptor-ligand complex and retention of the radionuclide in lysosomes [47]. Furthermore the fast renal excretion of ^{111}In-DTPA-octreotide facilitated clear visualization of liver metastases during scintigraphy [48]. Promising preclinical studies with ^{111}In-DTPA-octreotide in rats were readily followed by the first diagnostic imaging of patients suffering from tumours of neuroendocrine origin.

To enable stable conjugation of therapeutic radionuclides to peptide analogues the cyclic chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, Figure 2) was being linked to peptides. DTPA is especially suited for ^{111}In-labelling; ^{90}Y, ^{177}Lu, ^{68}Ga, and ^{111}In all can be stably complexed by DOTA. In the latter case a heating procedure (>90^{0}C) is needed to chelate the trivalent metal ions into the DOTA 'cage'.

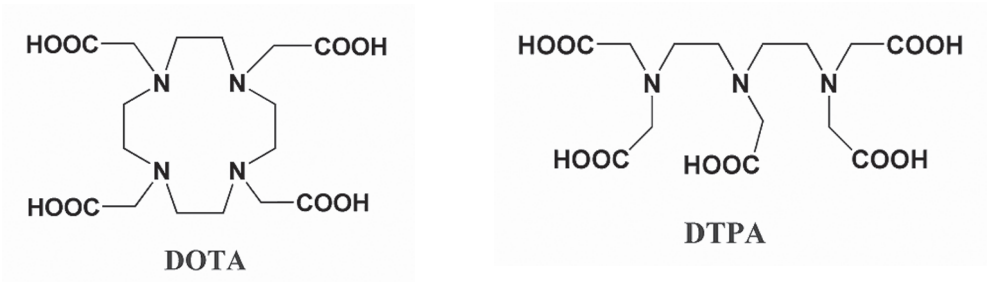


Figure 2: Molecular structure of the chelators DTPA (diethylenetriaminepentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).

Dependent on the physical radiation characteristics of the respective radionuclides used (listed in Table 3), radiolabelled peptide analogues are suited either for diagnostic (γ or β^+ radiation) or for therapeutic purposes (Auger/conversion electrons or β^--radiation).

Radionuclide	Radiation	γ energy (keV)	Max. particle energy (keV)	Max. range	Half-life
^{111}In	γ-radiation, Augers/CE	171 and 245	3 and 19 (Augers) 114 and 218 (CE)	10 µm (Augers) 0.5 mm (CE)	2.8 d
^{90}Y	β^--radiation		2281	12 mm	2.7 d
^{177}Lu	γ-radiation, β^--radiation	113 and 208	430	2 mm	6.7 d
^{68}Ga	β^+-radiation		1920	9 mm	68 min
^{18}F	β^+-radiation		635	2 mm	110 min

Table 3: Characteristics of various radionuclides.

Octreoscan®, ^{111}In-DTPA-octreotide, is being used as the gold standard in diagnostic γ-camera imaging to monitor the disease in patients with NET since the introduction in the early nineties of the previous century [48, 49]. An example of Octreoscan scintigraphy is shown in Figure 3. Also PET imaging using ^{68}Ga- or ^{18}F-labelled somatostatin analogues is being performed to localise tumours and/or metastases with a higher sensitivity than using SPECT. PET has a higher resolution and enables accurate quantification of the tumour uptake, an indication of the magnitude of tumour somatostatin receptor expression [50-55].

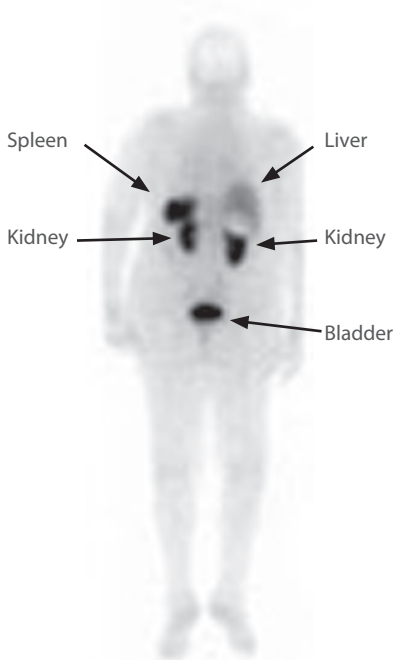


Figure 3: Visualization of physiological uptake of ^{111}In-DTPA-octreotide during Octreoscan scintigraphy in spleen, liver, kidneys and bladder (posterior view).

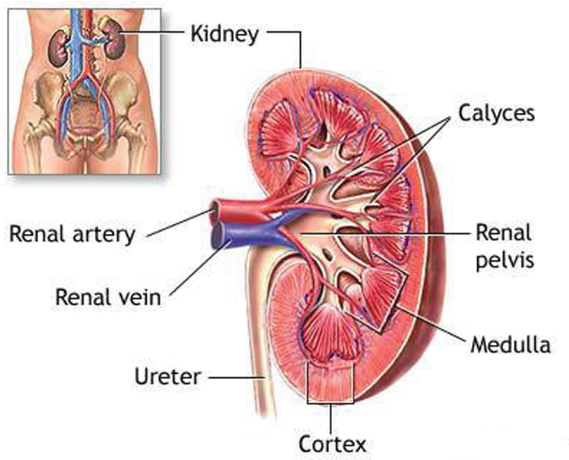
Peptide receptor radionuclide therapy (PRRT) was first accomplished with ^{111}In-DTPA-octreotide. It was hypothesized that ^{111}In could be effective in radionuclide therapy because ^{111}In emits Auger- and conversion electrons next to γ-radiation. Indeed, beneficial effects were observed in patients with metastasized disease that were treated with high activity doses of ^{111}In-DTPA-octreotide [56]. Stable disease, minor or partial tumour remission was found in 50% of 40 evaluable patients. Radiolabelling with the therapeutic β^--radiation emitting radionuclides ^{90}Y and ^{177}Lu of either Tyr^3-octreotide or Tyr^3-octreotate, accelerated the application of PRRT in many centres. More pronounced therapeutic effects were achieved than after ^{111}In PRRT as recently reviewed [57-61]. Although inter-study comparison is difficult because of variations among treatment protocols concerning patient selection, inclusion criteria and definitions of responses, it can be stated that significant survival benefit and improvement of quality of life is achieved after PRRT with ^{90}Y-DOTA,Tyr^3-octreotide or ^{177}Lu-DOTA,Tyr^3-octreotate in patient groups not eligible for other treatment options [62]. Although complete remission was achieved in a minority of the patients, minor and partial remissions, i.e. 25-50% or >50% reduction of tumour size according tot SWOG criteria, has been described in 10-40% of the cases. Progression of disease was still encountered in approximately 20% of patients, while the remainder of the patients achieved stable disease.

Two clinical trials in a few patients to perform therapy with gastrin- and bombesin-analogues have been performed recently as well. ^{90}Y-labelled minigastrin was administered to MTC patients, and ^{177}Lu-AMBA to patients with prostate cancer [44, 63].

3. Renal uptake and retention of radiopeptides

The kidneys are very important organs to maintain homeostasis regarding salt balance and blood pressure. Moreover, the blood is filtered in the kidneys about twenty five times a day and waste molecules are collected in excess water to be excreted into the urine.

A:



B:

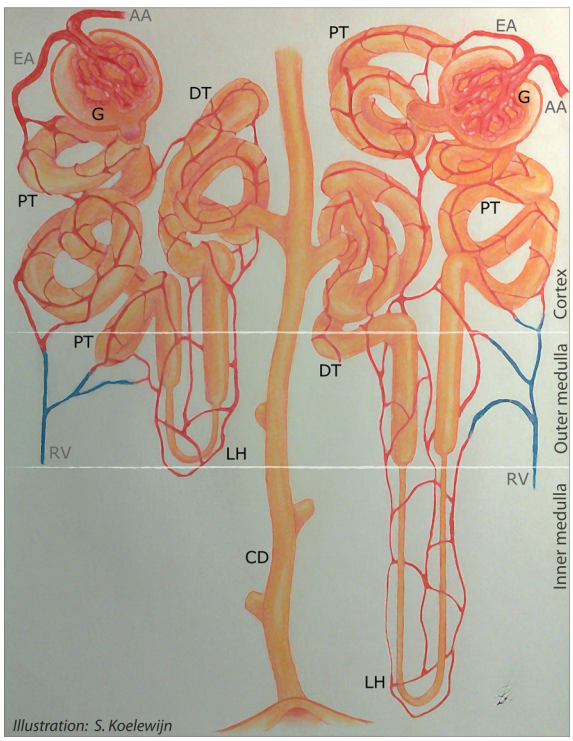


Figure 4: Schematic overview of the kidney (A) and nephrons (B), the functional units of the kidney. Abbreviations: AA/EA: afferent and efferent arteriole, G: glomerulus, PT/DT: proximal and distal tubule, LH: Lis of Henle, CD: collecting duct, RV: renal vein. (A): Adapted from www.nlm.nih.gov images, (B) Illustration made by Stuart Koelewijn.

Each kidney contains one to two million nephrons, the functional filtering units that are mainly located in the outer part of the kidney; the cortex. Two nephrons are depicted schematically in Figure 4B. Blood enters the glomeruli via an afferent arteriole (AA) of the renal artery. In the capillary network in the glomeruli (G) waste and useful molecules are filtered from the blood and transported into the proximal tubules (PT). Waste molecules are excreted into the urine via the loop of Henle (LH), the distal tubules (DT) and the collecting duct (CD) leading to the renal pelvis and the bladder. In the first, convoluted, part of the PT useful molecules like small proteins (<60 kDa) and peptides are reabsorbed to prevent loss of valuable substances.

The chelators DTPA and DOTA are charged molecules in neutral milieu, facilitating fast excretion of radiolabelled DTPA/DOTA chelated peptides via the kidneys into the urine. This is an advantage for optimal visualization of liver metastases because of the low abdominal background compared to clearance via the liver/intestines as found for iodinated peptides [17]. However, due to partial renal reabsorption and retention of administered DTPA/DOTA chelated radiopeptides, the kidneys are the activity dose limiting organs during PRRT with therapeutic β⁻-radiation emitting radionuclides [64]. Depending, among others, on the number of charged amino acid residues the renal uptake and retention of several different tumour targeting peptide analogues appeared to be highly variable [37, 65-67].

As for the mechanism of renal uptake of radiopeptides, it appeared that megalin, a receptor located at the apical side in the S1 and S2 part of the proximal tubules in the renal cortex, plays a very important role. Megalin functions, in close interaction with cubilin and amnionless, as an endocytic receptor to reabsorb glomerular filtered substances, primarily small proteins and peptides including albumin, transferrin, vitamin-carrier proteins as well as peptide hormones and drugs [68, 69]. It is a very large protein of about 600 kDa, primarily consisting of a large extracellular domain, a transmembrane segment and a small cytoplasmic domain [70]. In the extracellular part three types of repeats characteristic for members of the low-density lipoprotein (LDL)-receptor family are expressed, resulting in four ligand-binding clusters, as illustrated in Figure 5. Each of the four clusters contains both acidic and basic amino acid residues that may facilitate high-affinity, charge-dependent receptor-ligand interactions [71].

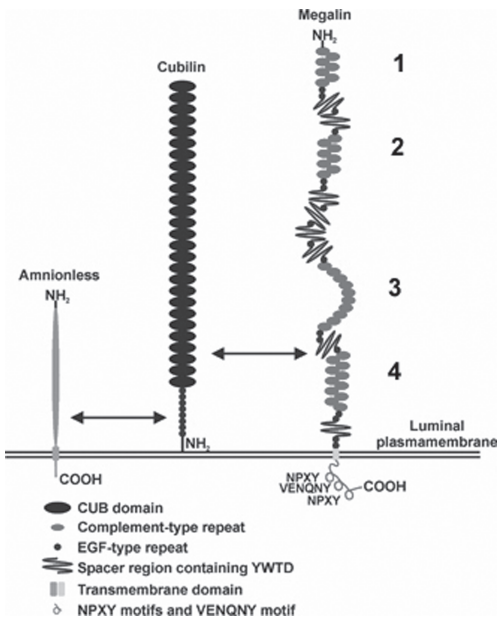


Figure 5: Schematic overview of the structure of amnionless, cubilin and megalin, adapted from [72]. The numbers 1-4 indicate the four different ligand-binding clusters of megalin.

Radiolabelled octreotide or octreotate analogues are positively charged peptides because they contain a Lys residue. There-

fore it has been hypothesized that reabsorption of somatostatin analogues involves recognition of at least one of the four ligand-binding clusters of megalin that is negatively charged [73, 74]. An important role for megalin can be postulated as well concerning the high renal uptake and retention of gastrin and GLP-1 peptide analogues [64].

The retained renal radioactivity after intravenous administration of radiopeptides is primarily localised in the cortex, because of partial reabsorption in the proximal tubules. This inhomogeneous distribution of radioactivity is demonstrated in *ex vivo* autoradiograms of kidneys, but can also be visualized in SPECT images.

4. Nephrotoxicity after PRRT

Due to partial renal uptake and retention of therapeutically radiolabelled somatostatin peptide analogues, the kidneys appeared to be the organs at risk after PRRT. In 1999 the first communication on late renal damage after ^{90}Y-DOTA,Tyr^3-octreotide PRRT was published [75]. It is known that kidneys may malfunction several months after they have been exposed to high activity doses [76]. Therefore, a safe radiation dose was accepted as threshold tolerated by kidneys on the basis of experience from external beam radiation therapy (EBRT). After EBRT the risk of development of nephropathy within 5 years after a cumulative renal absorbed dose of 23 Gy is 5%, increasing to 50% after a 28 Gy dose [77]. Although in PRRT the nature of the delivered radiation dose differs from that in EBRT, a threshold of 23-27 Gy has first been adapted for PRRT studies as well. Recently a 37-45 Gy limit of biologically equivalent dose (BED) kidney radiation dose was accepted as safe limit, taking into account a.o. characteristics of the applied radionuclide, dose fractionation, kidney mass and the non-uniform localization of renal radioactivity [78, 79]

Insights in the long-term process leading to radiation nephrotoxicity caused by PRRT has been reviewed recently [64]. Irradiation of the kidneys may lead to fibrosis, a process that takes several months. Irradiation may, dependent on the dose, induce a chronic state of oxidative stress by the generation of reactive oxygen species (ROS) [80]. These ROS a.o. inactivate mitochondrial enzymes and damage DNA which may lead to cell apoptosis [81]. In the kidneys a decreased glomerular filtration rate (GFR) and loss of protein in the urine is found, bringing about progressive kidney damage [82]. From several studies related to radiation-induced nephropathy it was clear that the renin-angiotensin-aldosterone system (RAAS) plays a crucial role [83-85]. It appeared that angiotensin II is an important mediator in the progression of renal disease. It primarily up-regulates the levels of several cytokines, e.g. interleukin-8 (IL-8), promoting monocyte/macrophage and neutrophil migration, and transforming growth factor (TGF)-β, stimulating the proliferation of fibroblasts [82, 86]. Finally, glomerulosclerosis and tubulointerstitial fibrosis ultimately lead to loss of renal function.

In rat ^{177}Lu-DOTA,Tyr^3-octreotate PRRT studies long-term nephrotoxicity has been described [87], with features also encountered in early case reports on renal insufficiency in patients after ^{90}Y-DOTA,Tyr^3-octreotide PRRT [75]. Beyond 70-80 days after a renal absorbed dose of

60 Gy excretion of protein in the urine of the rats was detected. Urinary protein content was increasing over time because of disturbed reabsorption of filtered peptides and proteins in the proximal tubules. Rats lost body weight at this stage, indicating health problems. After euthanasia histological examination of kidney sections demonstrated aberrations both in tubules and glomeruli in the renal cortex where radioactivity had been retained. Grading of the histological renal damage was performed according to the following scheme.

Grade 1	Inflammatory infiltrate in glomeruli, little dilatation of tubules
Grade 2	Grade 1 + more tubular dilatation, basal membrane thickening, few protein cylinders in tubules
Grade 3	Shrinkage of few glomeruli, flat tubule epithelium, strong tubule dilatation and basal membrane thickening, more protein cylinders
Grade 4	Increased shrinkage of glomeruli, strongly dilated tubules with massive protein cylinders and signs of fibrosis

5. Reduction of renal uptake of radiopeptides

The risk of nephrotoxicity urged for reduction of the absorbed kidney radiation dose to enable application of PRRT at high activity doses. Reduction of the uptake of the radiopeptide in the kidney will enlarge the therapeutic window; that is enabling increase of the cumulative tumour radiation dose without harmful effects on the kidney [75, 88].

Co-infusion with lysine and arginine (Lys/Arg) during PRRT partially prevents the renal reabsorption of radiopeptides resulting in less renal retention of radioactivity [88]. This method of kidney protection found its way to general clinical application during somatostatin analogue-based PRRT, resulting in approximately 40% reduction of renal radioactivity [57, 60, 89, 90].

Recently, co-infusion of the plasma expander Gelofusine was described to achieve a similar reduction of renal retention of ^{111}In-DTPA-octreotide [91]. A transient proteinuria is induced upon Gelofusine infusion, probably by disturbance of the effective megalin-mediated reabsorption processes in the kidney [92, 93]. Both in rats and in humans 40% less renal radioactivity was encountered after Gelofusine infusion [94, 95].

Moreover, as a new approach to obtain decreased levels of renal radioactivity, co-injection of fragments of the megalin ligand albumin also effectively reduced renal uptake of various radiopeptides [96, 97].

6. Mitigation of nephrotoxicity after PRRT

Besides reduction of the initial uptake and retention of radiolabelled peptides in the kidneys, interference in the process leading to kidney failure on the long-term, renal fibrosis, is another approach to accomplish less kidney damage after PRRT. This may be achieved following two pathways:

1: Application of anti-oxidants to inhibit the harmful effects of ROS

2: Inhibition of angiotensin II effects

Therefore, non-tumour-bearing and tumour-bearing rats received iv or ip amifostine or taurine, L158.809 or captopril in their drinking water starting 10 days prior PRRT with 370 or 460 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. During the follow-up of more than 140 days after PRRT body weight, levels of urinary protein, serum urea and creatinine and renal ^{99m}Tc-DMSA uptake levels were determined, supplemented with kidney histology after euthanasia, as described below.

6.1: Amifostine and taurine; antioxidants

The radical scavenger amifostine was developed to protect people from radiation effects during a nuclear war. Nowadays amifostine is FDA-approved for application as cytoprotector during radiotherapy [98, 99] and chemotherapy [100, 101] to protect healthy tissues such as kidneys, salivary glands and progenitor cells in bone marrow [102]. The pro-drug amifostine is converted into the active compound WR-1065 after dephosphorylation by the enzyme alkaline phosphatase (AP) which is 100 times more active in various healthy tissues than in tumours [103]. After EBRT in preclinical rat studies amifostine treatment resulted in kidney-protective effects without interference in tumoural therapeutic effects [104, 105], confirmed by protection from long-term toxicity by amifostine after irradiation by ^{177}Lu-DOTA,Tyr^3-octreotate PRRT in rats [106].

Taurine, 2-aminoethanesulphonic acid ($C_2H_7NSO_3$), is a small molecule acting as an anti-oxidant. Addition of taurine to drinking water of rats reduced nephrotoxicity after treatment with cisplatin or cyclosporine A, both producing free oxygen radicals [107, 108]. These results encouraged to examine taurine as kidney protector during PRRT.

6.2. L158.809 and Captopril; inhibitors of angiotensin II

As previously mentioned, angiotensin II plays a crucial role in the cascade of events leading to renal insufficiency. Therefore two ways to inhibit the harmful effects of angiotensin II leading to fibrosis were explored in PRRT.

First, the application of an angiotensin II receptor blocker (ARB) to inhibit the effects of angiotensin II from any source directly at the receptor level was tested, because a clinical case report supported successful treatment using the ARB losartan in radiation nephropathy [109]. Furthermore, preliminary studies in which the ARB L158.809 was added to the drinking water of rats after ^{177}Lu-DOTA,Tyr^3-octreotate PRRT suggested renal protection [110].

Second, an angiotensin converting enzyme inhibitor (ACEI) blocking the ACE dependent conversion of angiotensin I into angiotensin II was examined for its kidney protective effects during PRRT [111]. In a clinical trial in 55 subjects administration of the ACEI captopril after total body irradiation prior to haematopoietic stem cell transplantation suggested protection from chronic renal failure [112]. Captopril also showed favourable kidney protective results in rat studies after EBRT [113].

7. Autoradiography and small animal SPECT

In preclinical biodistribution studies with radiopeptides to quantify the uptake in various organs of (tumour-bearing) mice or rats, generally the level of radioactivity in dissected organs is determined using a γ-counter. The localization of the radioactivity within the organs or tumours is not known, however.

The techniques of autoradiography and molecular imaging both provide information on the localization of radiolabelled agents within animals and/or tissues.

7.1 Autoradiography

To obtain *ex vivo* autoradiograms, visual images of accumulated radioactivity, 10 µm cryosections are cut from freshly frozen radioactive organs or tumours. Radioactivity can originate from several radionuclides with different characteristics, such as ^{111}In, ^{177}Lu, ^{99m}Tc or ^{68}Ga. Radioactive tissue sections are exposed to sensitive phosphor imaging screens with high resolution. After the read out, detected radioactivity is depicted as black regions within the tissue section. Moreover the level of retained radioactivity can be adequately quantified. Using this technique the non-uniform distribution of radioactivity after administration of radiolabelled peptides within kidneys has been demonstrated, both in animals and humans [114, 115].

7.2 Small animal SPECT

Recent technical improvements of sensitivity and resolution of γ-cameras dedicated for small animal SPECT offer new opportunities in preclinical research in rodents [116, 117]. The resolution of SPECT is in the sub-millimeter range, allowing 3D visualization of retained radioactivity within targeted tissues after administration of radiolabelled agents. Furthermore, accurate absolute quantification of accumulated radioactivity in tissues such as tumours and kidneys is feasible [118]. Advantages of small animal molecular imaging include non-invasive *in vivo* functional imaging and following radiotracers over time within one animal. This may result in reduction of animal numbers needed to test new radiotracers. Moreover, monitoring of late side effects, development of disease or therapeutic effects after intervention, will be new features in preclinical research.

In Figure 6A the inhomogeneous distribution of radioactivity within kidneys and xenografted tumours of human origin in immunodeficient mice is visualized using multi-pinhole collimated SPECT/CT. This non-uniform pattern is confirmed by *ex vivo* autoradiography, as shown in Figures 6B and 6C [119]. With regard to rat PRRT experiments, reduction of renal retention

of radiolabelled octreotate using lysine and/or Gelofusine was nicely visualized and quantified [120], also showing that most radioactivity is retained in the renal cortex.Several clinically used radiopharmaceuticals are available to image kidney anatomy and renal function in small animals as well. The uptake of ^{99m}Tc-labelled dimercaptosuccinic acid (^{99m}Tc-DMSA) is directly related to tubular function [121]. Several hours after injection radioactivity is accumulated in the renal cortex because of peritubular extraction of ^{99m}Tc-DMSA from the blood and tubular reabsorption after glomerular filtration. Next to this static imaging the dynamic excretion processes in the kidney can be visualized and quantified using radiolabelled DTPA or ^{99m}Tc-labelled mercaptoacetyltriglycine (MAG3). Clearance of ^{111}In-DTPA can be used to monitor glomerular filtration and ^{99m}Tc-MAG3 is primarily rapidly extracted from the blood by cells lining the proximal tubules [121, 122]. Both agents are rapidly excreted via the renal pelvis into the urine. The extent of their renal uptake and excretion rate reflects the condition of the kidneys.

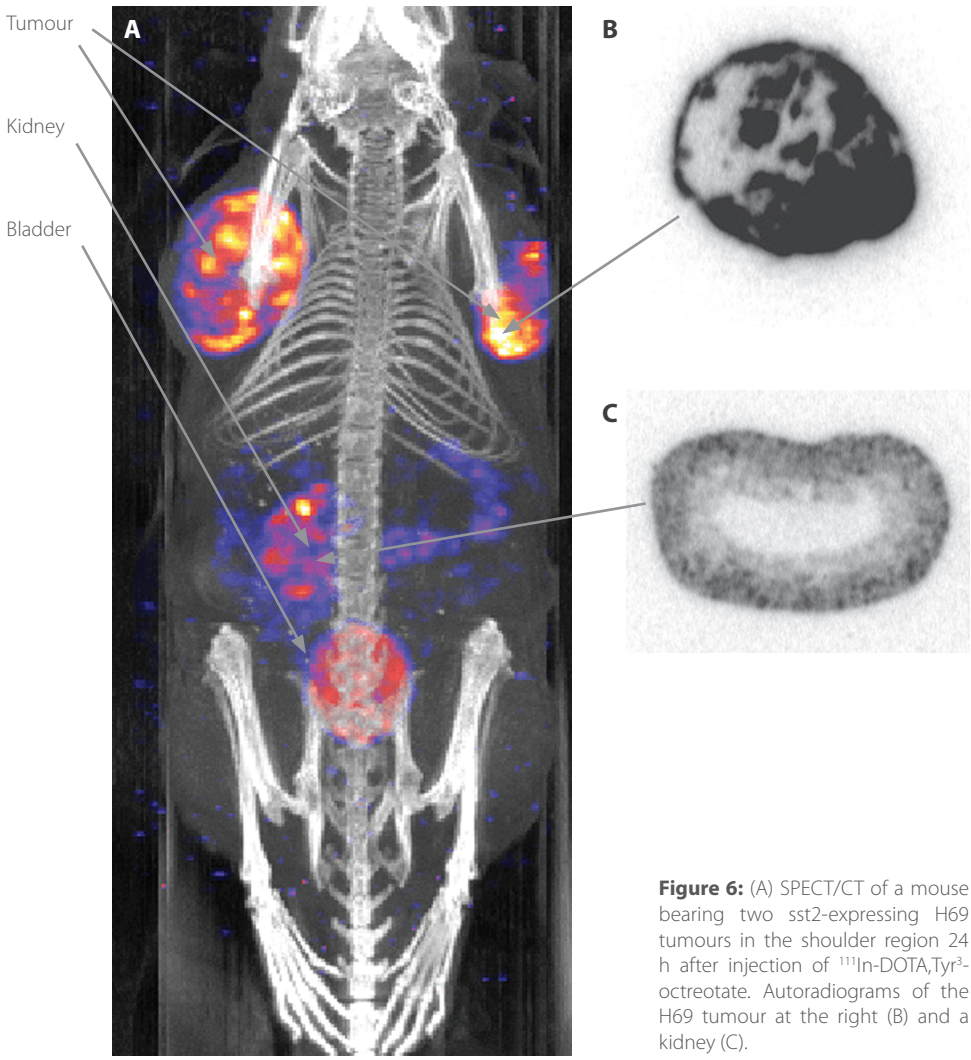


Figure 6: (A) SPECT/CT of a mouse bearing two sst2-expressing H69 tumours in the shoulder region 24 h after injection of ^{111}In-DOTA,Tyr^3-octreotate. Autoradiograms of the H69 tumour at the right (B) and a kidney (C).

Outline of the thesis

The main aims of the studies presented in this thesis are:

- To gain insight into the mechanisms of renal uptake and retention of various tumour targeting radiopeptides that are applied in PRS and PRRT
- To investigate ways to reduce the renal uptake and retention of tumour targeting radiopeptides without affecting tumour uptake
- To monitor renal function after PRRT using dedicated small animal SPECT

A general introduction of this thesis is given in **Chapter 1.1**. It consists of a short overview of the development of PRRT over the last decades, characteristics of various tumour targeting peptide analogues and their renal uptake, and a preface on a putative mechanism of renal retention of radiopeptides. Furthermore, various agents that were studied to reduce renal uptake and toxicity are introduced. In **Chapter 1.2** an overview of the status of kidney protection strategies during PRRT with radiolabelled somatostatin analogues is reported. Both clinical and preclinical data are summarized, with emphasis on co-infusion of the cationic amino acids lysine and arginine enabling a reduction of 40% of radiolabelled somatostatin peptide analogues. Suggestions for future options to reduce renal toxicity after PRRT are described.

Chapter 2 consists of four manuscripts regarding the mechanisms of renal uptake and retention of several tumour-targeting radiolabelled peptides. In **Chapter 2.1** the renal uptake of five different peptide analogues in rats and mice of both sexes is described. After renal reabsorption radiopeptides appear to be retained mainly in the proximal tubules, located in the renal cortex, as is demonstrated in **Chapter 2.2**. Administration of sodium maleate and colchicine, agents that inhibit endocytic processes, appeared to reduce the renal retention of radiopeptides. Therefore a role of the multi-ligand scavenger receptor megalin in the renal reabsorption of radiopeptides was suggested. From our studies using kidney-specific megalin deficient mice, described in **Chapters 2.3** and **2.4**, indeed it appeared that megalin plays an important role in this respect for all radiopeptides tested despite differences concerning specificity, size and charge. This conclusion was important for development of new strategies to interfere in the renal reabsorption process of radiopeptides.

Chapter 3 deals with two agents examined for reduction of renal radioactivity after administration of radiolabelled octreotate in somatostatin receptor-expressing CA20948 tumour-bearing rats. The gelatine-based plasma expander Gelofusine was one of these. Infusion of Gelofusine leads to temporary proteinuria and previous experiments demonstrated a reduction of 40 to 60% of the renal retention of radioactivity when co-administered with radiolabelled octreotate. For translation to clinical use, we investigated the dose-response effect of Gelofusine as described in **Chapter 3.1**. As second agent the anti-oxidant and cytoprotector amifostine was co-administered during PRRT because of its described long-term kidney-protective effects after EBRT and earlier PRRT studies. As described in **Chapter 3.2** we examined the effect of amifostine co-administration regarding potential interference in anti-tumour effects after PRRT, the reduction of the initial renal uptake of radiopeptides in tumour-bearing rats and of co-incubation in *in vitro* binding assays using a megalin-expressing cell line.

The development of dedicated small animal SPECT cameras opened new possibilities in preclinical research on the application of radiopeptides labelled with γ-emitters. This is because high resolution enables detailed visualization of targeted tissues as well as accurate quantification of retained radioactivity. In **Chapter 4** the application of non-invasive animal SPECT imaging to monitor the renal function of rats after ^{177}Lu-DOTA,Tyr^3-octreotate PRRT is described. Three radiopharmaceuticals were administered to visualize and quantify glomerular filtration (^{111}In-DTPA), tubular reabsorption (^{99m}Tc-DMSA) and tubular excretion (^{99m}Tc-MAG3). In **Chapter 4.1** it is described that the uptake of ^{99m}Tc-DMSA could be accurately correlated with kidney function status. The localization and quantification of retained ^{99m}Tc-DMSA was investigated using ***in vivo*** SPECT imaging and *ex vivo* autoradiography in kidney sections, as is also shown in **Chapter 4.2**. In this chapter dynamic imaging to monitor the renal clearance of ^{111}In-DTPA and ^{99m}Tc-MAG3 was performed besides static ^{99m}Tc-DMSA scintigraphy beyond 100 days after PRRT, with or without lysine kidney protection. In **Chapter 4.3** we report that nephrotoxicity was encountered in mice beyond 100 days after absorbed renal ^{111}In-radiation doses of more than 40 Gy.

A summary including indications for future directions of research into safe application of PRRT is given in **Chapter 5**.

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Kidney protection during peptide receptor radionuclide therapy with somatostatin analogues

Edgar J. Rolleman[1], Marleen Melis[1], Roelf Valkema[1], Otto C. Boerman[2], Eric P. Krenning[1] and Marion de Jong[2]

[1]Department of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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Chapter 1.2

Abstract

This review focuses on the present status of kidney protection during peptide receptor radionuclide therapy (PRRT) using radiolabelled somatostatin analogues. This treatment modality for somatostatin receptor-positive tumours is limited by renal reabsorption and retention of radiolabelled peptides resulting in dose-limiting high kidney radiation doses. Radiation nephropathy has been described in several patients. Studies on the mechanism and localization demonstrate that renal uptake of radiolabelled somatostatin analogues largely depends on the megalin/cubulin system in the proximal tubule cells. Thus methods are needed that interfere with this reabsorption pathway to achieve kidney protection. Such methods include co-administration of basic amino acids, the bovine gelatine-containing solution Gelofusine or albumin fragments. Amino acids are already commonly used in the clinical setting during PRRT. Other compounds that interfere with renal reabsorption capacity (maleic acid and colchicine) are not suitable for clinical use because of potential toxicity. The safe limit for the renal radiation dose during PRRT is not exactly known. Dosimetry studies applying the principle of the biological equivalent dose (correcting for the effect of dose fractionation) suggest that a BED of about 37 Gy is the threshold for development of kidney toxicity. This threshold is lower when risk factors for development of renal damage exist: age over 60 years, hypertension, diabetes mellitus and previous chemotherapy. A still experimental pathway for kidney protection is mitigation of radiation effects, possibly achievable by co-treatment with amifostine (Ethyol), a radiation protector, or with blockers of the renin-angiotensin-aldosterone system. Future perspectives on improving kidney protection during PRRT include combinations of agents to reduce renal retention of radiolabelled peptides, eventually together with mitigating medicines. Moreover, new somatostatin analogues with lower renal retention may be developed. Furthermore, knowledge on kidney protection from radiolabelled somatostatin analogues may be expanded to other peptides.

Introduction

Peptide receptor radionuclide therapy (PRRT) is a relatively new treatment modality for somatostatin-receptor positive tumours, such as neuroendocrine tumours that hardly respond to chemotherapy. These tumours often show a high expression of somatostatin receptors [1-3] compared to normal tissues. The basis for the use of radiolabelled somatostatin analogues in the treatment of somatostatin receptor-positive tumours is binding of the radiolabelled compound to the receptor and subsequent internalization of the receptor-ligand complex [4-6]. Receptor-mediated internalization of ^{111}In-DTPA-octreotide results in degradation to metabolites in the lysosomes [7]. These metabolites are retained in the lysosomes of tumour cells positive for somatostatin receptor subtype 2 (sst2), causing prolonged local irradiation.

The first radiolabelled somatostatin analogue used for PRRT in patients with somatostatin receptor-positive tumours was ^{111}In-DTPA-octreotide [8]. ^{111}In emits Auger electrons, internal conversion electrons and γ-radiation. Treatment of patients with metastasized neuroendocrine tumours with high-dose ^{111}In-DTPA-octreotide was encouraging, although objective responses were uncommon [9-11].

In order to improve the therapeutic efficacy, β particle-emitting radionuclides were coupled to somatostatin analogues. The chelator DTPA cannot be stably labelled with ^{90}Y (maximum β energy 2.3 MeV, half-life 64 hours). Therefore DOTA,Tyr^3-octreotide was introduced as targeting peptide which also has an improved affinity for sst_2 as compared to DTPA-octreotide (Table 1). Several groups have reported beneficial effects of ^{90}Y-DOTA,Tyr^3-octreotide in patients suffering from somatostatin receptor-positive tumours [12-16] both in terms of objective response and quality of life. Complete and partial remissions in most studies with ^{90}Y-DOTA,Tyr^3-octreotide have been in the range of 10-30%, higher than those obtained with ^{111}In-DTPA-octreotide [17].

	sst1	sst2	sst3	sst4	sst5
SS28	5.2	2.7	7.7	5.6	4.0
Octreotide	>10000	2.0	187	>1000	22
DTPA-octreotide	>10000	12	3790	>1000	299
^{111}In-DTPA-octreotide	>10000	22	182	>1000	237
DOTA,Tyr^3-octreotide	>10000	14	880	>1000	393
^{90}Y-DOTA,Tyr^3-octreotide	>10000	11	389	>10000	114
DOTA,Tyr^3-octreotate	>10000	1.5	>1000	453	547

Table 1: IC50 values (nanomoles) of somatostatin analogues for somatostatin receptor subtypes (sst) (adapted from reference [3]).

A few years ago, the radiolabelled somatostatin analogue ^{177}Lu-DOTA,Tyr^3-octreotate was introduced. It differs from DOTA,Tyr^3-octreotide in that the C-terminal threoninol is replaced with threonine. Compared to DOTA,Tyr^3-octreotide, DOTA,Tyr^3-octreotate has a ninefold higher af-

finity for sst2 [3] (Table 1). In phase 1 and 2 studies using ^{177}Lu-DOTA,Tyr^3-octreotate, partial and complete remissions were found in up to 30% of patients. Additionally, treatment resulted in a significant improvement of quality of life [18].

Overall, treatment with radiolabelled somatostatin analogues is a promising new tool in the management of patients with otherwise untreatable, metastasized neuroendocrine tumours. Tumour regression results are encouraging and compare favourably with those of the limited number of alternative treatment approaches [17]. However, soon after the start of clinical studies, concern was raised about absorbed radiation dose to the kidneys and the bone marrow. Because renal function impairment has been reported [14, 19-21], several studies have been started to reduce the risk of nephrotoxicity during PRRT.

The aim of this review is to summarize the current status on kidney protection in PRRT with radiolabelled somatostatin analogues.

Renal radiation is dose-limiting in PRRT

The radiolabelled somatostatin analogues that are being used in PRRT are predominantly cleared by the kidneys. The small peptides are filtered by the glomeruli and most of the activity is excreted via the urine. However, about 2% of the total dose is reabsorbed by the proximal tubular cells. After transport to the lysosomes, the (metabolized) radioligand is retained there [7], resulting in prolonged kidney irradiation.

In most clinical PRRT studies with ^{90}Y-DOTA,Tyr^3-octreotide it was found that the kidney absorbed radiation dose is the major dose-limiting factor and in several articles renal toxicity after PRRT was found. In a dose-escalating study [19, 20], 1 out of 30 patients had grade 2 renal toxicity. This 58 years old patient, who had suffered from hypertension for 6 years, received 3.3 GBq ^{90}Y-DOTA,Tyr^3-octreotide in three cycles, and the reported estimated kidney absorbed radiation dose was 12 Gy. One year later his serum creatinine had returned to normal, but his ^{99m}Tc-DTPA scintigraphy remained abnormal, indicating reduced glomerular filtration rate. In an intra-patient dose-escalating study [14], renal toxicity was seen in 4 out of 29 treated patients after administration of cumulative activities of 7611-8924 MBq/m^2 ^{90}Y-DOTA,Tyr^3-octreotide. None of these four patients received co-infusion of amino acids. Two of them needed haemodialysis treatment. Serum creatinine levels started to rise 2-4 months after the last treatment cycle in these patients. As the 25 patients who had no renal toxicity received cumulative activities up to 7400 MBq/m^2 (some with amino acids for kidney protection) the authors suggested this cumulative activity to be the threshold dose of ^{90}Y-DOTA,Tyr^3-octreotide for renal toxicity.

However, Cybulla et al. [21] reported a case history of a 78 years old patient who was treated with 5659 MBq/m^2 ^{90}Y-DOTA,Tyr^3-octreotide. She developed progressive deterioration of renal function within 15 months after cessation of PRRT and developed end-stage renal disease. This patient had been treated with ten chemotherapy cycles in the years preceding PRRT. Stoffel et al. [22] reported a patient who was treated with six cycles of ^{90}Y-DOTA,Tyr^3-octreotide for metastasized medullary thyroid carcinoma. She developed renal failure 10 months after the last treatment cycle, eventually in needed haemodialysis.

Based on these studies, it is generally acknowledged that the kidney is a major dose-limiting organ in PRRT with radiolabelled somatostatin analogues.

Radiation nephropathy after PRRT

Several authors have reported the clinical and histological findings in radiation nephropathy caused by PRRT. Moll et al. [23] described the clinical course of five patients that had been included in the study of Otte et al. using ^{90}Y-DOTA,Tyr^3-octreotide [14]. In these five patients the onset of renal failure ranged from 0.5-6 months after treatment with 7600-8900 MBq/m^2. All patients had normal blood pressure and serum creatinine levels before treatment; and one had received chemotherapy treatment previously. At onset of renal failure all had hypertension, proteinuria and microscopic haematuria. In one patient fragmentocytes were found. In three patients kidney biopsy showed the picture of thrombotic microangiopathy. Capillary loop collapse, focal segmental thrombi of fibrin and other proteins in capillaries, mesangiolysis and double contouring of the basement membrane were seen. Arterioles and small arteries showed transmural hyalin deposits and were partially or completely occluded by fibrin and protein thrombi. Tubular atrophy and interstitial fibrosis were prominent in all cases. Deposition of fibrin, IgM and IgA was observed in both the lumen and the wall of arterioles and arteries.

The patient described by Stoffel et al. [22] had no change in kidney function until she was admitted for progressive weakness, anaemia and arterial hypertension ten months after her last PRRT treatment. She had mild glomerular and tubular proteinuria, as well as a low creatinine clearance (CLR) of 14 ml/min. Kidney biopsy showed segmental mesangiolysis in 6/15 glomeruli with disruption and vacuolar expansion of the mesangium. No pathognomic deposits were present. There was marked tubular atrophy and compensatory interstitial fibrosis, as well as minimal arterial damage. No intra-arterial thrombosis or necrosis were observed. Unfortunately, Stoffel et al. did not include administered activity or dosimetry data. Barone et al. [24] reported two patients who suffered from hypertension, proteinuria, oedema and anaemia after ^{90}Y-DOTA,Tyr^3-octreotide treatment. At biopsy, radiation-induced microangiopathy was proven in one of these patients. The conventional calculated kidney absorbed doses were 27.5 and 27.1 Gy, whereas the biological equivalent doses (BEDs) were 54.1 and 56.1 Gy.

In our institution kidney biopsies in four patients have shown no histological damage after treatment with ^{90}Y-DOTA,Tyr^3-octreotide (kidney absorbed doses of 29.3 and 32.3 Gy) and ^{111}In-DTPA-octreotide (absorbed doses of 35 and 49 Gy) (unpublished data). The intervals between first treatment and biopsy ranged between 12 and 17 months.

Animal data on targeted radionuclide therapy-related renal toxicity are scarce. Lewis et al. [25] found no functional or histological damage up to 38 days after administration of 847-903 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (corresponding to a kidney absorbed dose of 107-114 Gy). We found that severe renal toxicity became apparent later than 100 days after injection of 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate in rats, which resulted in renal absorbed doses of about 70 Gy [26]. Although some damage was seen in the glomeruli as evidenced by glomerulus shrinkage, in this model the main site of renal damage was the tubules. Basal membrane thickening and dilatation of flat tubule epithelium with massive protein cylinders in the lumen were noticed. Signs of peripheral fibrosis were present as well.

In PRRT the site of histological damage is, amongst other factors, dependent on the particle range of the radionuclide used. In the case of ^{90}Y, radiation damage is mostly in the glomeruli, as evidenced by the picture of thrombotic microangiopathy, and tubuli. For radionuclides with shorter ranges, such as the β emitter ^{177}Lu or the α emitter in the study of Jaggi et al. [27], the damage is predominantly in the tubules with no or less damage in the glomeruli. This is in agreement with microdosimetric studies [28].

Prevention of radiation nephropathy and enlargement of the therapeutic window in PRRT

The following issues are important in the prevention of radiation nephropathy and in allowing the administration of higher doses in PRRT within safe kidney radiation limits:

- Knowing the uptake mechanism to develop renal uptake inhibitors.
- Knowing and not exceeding the maximum safe renal radiation dose and the use of dose fractionation.
- Reduction of kidney uptake of radiolabelled peptides by uptake inhibitors.
- Mitigation of radiation effects.
- Development of new peptides with a higher affinity for the receptor or lower renal uptake.

Mechanism and localisation of uptake

The proximal tubule is the site where filtered low molecular weight proteins, peptides and electrolytes are reabsorbed [29, 30] to prevent loss of valuable substrates for metabolism. Radiolabelled peptides are small and therefore filtered by the glomeruli and partly reabsorbed in the proximal tubules. This is supported by *ex vivo* autoradiography studies that have shown that part of the injected ^{111}In-DTPA-octreotide is located in the cortex of the kidney (Figure 1). Micro-autoradiography of kidneys after administration of ^{177}Lu-DOTA,Tyr^3-octreotate in rats demonstrated that radioactivity was retained in the proximal tubules [31]. In the human kidney the distribution of radioactivity was also inhomogeneously distributed [32]. Three patients that underwent nephrectomy because of renal cancer were injected with ^{111}In-DTPA-octreotide prior to the surgery. *Ex vivo* autoradiography of healthy kidney tissue showed that the radioactivity was localized predominantly in the cortex in a striped pattern, with the highest radioactivity in the inner cortical zone [32].

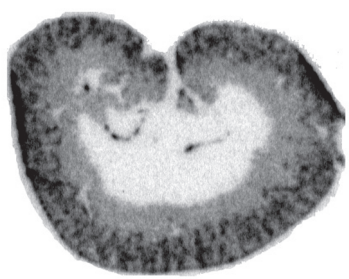

Figure 1. *Ex vivo* autoradiogram of a rat kidney 24 h after injection of ^{111}In-DTPA-octreotide showing the radioactivity distribution.

The megalin/cubilin system plays a significant role in the reabsorption of many low molecular weight proteins and peptides via receptor-mediated endocytosis. Barone et al. showed in studies with wild opossum kidney cells that uptake of ^{111}In-DTPA-octreotide was inhibited by different megalin ligands [33]. In kidney-specific megalin-deficient mice it was shown that the uptake of ^{111}In-DTPA-octreotide was only 15-30 % of uptake in normal mice [34]. These studies indicate that megalin plays an essential role in renal uptake of radiolabelled somatostatin analogues.

Although most data point to proximal tubular reabsorption as the main site of uptake, other factors like sst2-mediated or peritubular uptake may play a role as well. Glomeruli, tubule cells and vasa recta in the human kidney express sst2 [35, 36]. In a study of 10 patients the kidney uptake of ^{111}In-DTPA-octreotide was reduced by 18% due to treatment with high doses of unlabelled octreotide as compared to no octreotide treatment [37].

Stahl et al. reported a 20% reduction of renal uptake of ^{111}In-DOTA,Tyr^3-octreotide by probenecid, which is a blocker of various organic anion transporters [38], suggesting that also peritubular uptake of somatostatin analogues can occur. We were not able to confirm this finding in rats (unpublished data) however.

Maximum safe renal radiation dose: dosimetry and effects of dose fractionation

In the recent decade, several data have been reported that can help to estimate the maximum radiation dose that can be safely delivered to the kidney in PRRT.

In fractionated external beam radiation therapy, the risk to develop radiation nephropathy within 5 years after renal absorbed doses of 23 Gy is 5%, rising to 50% after renal absorbed doses of 28 Gy [39]. However, it should be kept in mind that a radiation dose delivered during radionuclide therapy differs in many respects from a dose delivered by external beam irradiation. In radionuclide therapy: (a) the dose rate is lower, (b) the penetration range of the radiation is shorter and (c) the radionuclide has a specific intra-organ distribution, while in external beam radiation therapy the radiation is delivered homogeneously to the organ that is in the field of irradiation [40-42] .

So, in PRRT dosimetry is needed for establishment of the maximum safe dose to the kidneys and for patient-tailored therapy planning. Several authors reported large interindividual uptake and radiation doses [43-46] stressing the need for such an approach. For optimal dosimetry time-consuming methods are required, that include pharmacokinetic, biodistribution and washout studies, preferably with the same peptide and radionuclide used for therapy [43]. This is feasible with radionuclides that have both β^-- and γ-emissions like ^{111}In and ^{177}Lu, but not with the pure β^--emitter ^{90}Y.

To determine absorbed doses of ^{90}Y-DOTA,Tyr^3-octreotide, strategies using ^{111}In-DTPA-octreotide [44], ^{86}Y-DOTA,Tyr^3-octreotide [24, 47] and ^{111}In-DOTA,Tyr-octreotide [45, 48-50] have been reported. It seems reasonable to consider ^{86}Y-DOTA,Tyr^3-octreotide the most reliable because ^{86}Y is chemically identical to ^{90}Y. However, the use of the positron-emitter ^{86}Y is limited by its poor availability, the positron abundance of 33% and the relatively short half-life of about 14 hours. ^{111}In is more convenient in use, but minor changes in the peptide and differences in radionuclide can significantly influence sst2-mediated uptake and therefore the reliability of dosimetry data [3]. As the major part of kidney uptake is not sst2-mediated, this might not play a dominant role for the kidney. So, both ^{86}Y-labelled and ^{111}In-labelled DOTA,Tyr^3-octreotide can probably reliably predict the renal absorbed radiation dose during treatment with ^{90}Y-DOTA,Tyr^3-octreotide. For the use of ^{111}In-DTPA-octreotide, comparison studies have shown that dosimetry values obtained using ^{86}Y-DOTA,Tyr^3-octreotide can be either underestimated [51] or overestimated [44].

Another method to estimate absorbed doses is the use of ^{68}Ga-labelled analogues. The level of somatostatin receptor expression of primary and/or metastasized tumours may be quantified (expressed in SUV) using ^{68}Ga PET imaging, as described for ^{68}Ga-DOTA,Tyr^3-octreotide [52, 53] and ^{68}Ga-DOTA-NOC (^{68}Ga-DOTA,1-Nal^3-octreotide) [46, 54, 55]. It has been shown that, especially combined with CT scanning, this imaging modality has a very high specificity and sensitivity [53]. ^{68}Ga PET/CT imaging is safe, reproducible and relatively cheap and is therefore attractive to select and monitor patients during PRRT. The normal physiologic distribution of ^{68}Ga-DOTA-NOC shows a SUV_{max} of approximately 1 as background level for muscle, up to 125 for tumour whereas the mean kidney uptake has a SUV_{max} of 12.6, although with a range of 4-22 [56]. This means that optimization of PRRT for each individual might be realizable using ^{68}Ga PET/CT scintigraphy [57], although dosimetry is hampered by the short half life of ^{68}Ga. Again, it must be considered when the radiopeptide used for imaging is different from that used for PRRT, the reliability of dosimetry might be adversely affected. Dosimetry performed in PRRT is therefore only valuable, when its outcome correlates with radiation effects and when it predicts toxicity. Ultimately, factors that may influence the response of the kidney to a radiation dose are: the nature of the peptide, the type of radionuclide used in combination with its intrarenal distribution, fractionation and the existence of risk factors for renal damage. These aspects are discussed in the following sections.

Choice of peptide

The choice of peptide might slightly influence the kidney absorbed dose. Forrer et al. [50] found a (not statistically significant) higher tumour-to-kidney for ^{111}In-DOTA,Tyr^3-octreotide as compared to ^{111}In-DOTA,Tyr^3-octreotate. Data from our institution show that tumour-to-kidney ratio was significantly higher for ^{177}Lu-DOTA,Tyr^3-octreotate than for ^{177}Lu-DOTA,Tyr^3-octreotide [58].

Choice of the radionuclide and its intrarenal distribution

In a study of 21 patients with renal absorbed doses up to 45 Gy following treatment with ^{111}In-DTPA-octreotide, no renal toxicity was seen during a follow-up of 3 years [9]. ^{111}In emits two γ-rays and in addition Auger electrons with a tissue penetration of 0.02 - 10 μm and conversion electrons with a penetration of 200-500 μm. Using a microdosimetry model, Konijnenberg et al. [59] found that ^{111}In- and ^{177}Lu-labelled somatostatin analogues are likely to have higher renal toxicity thresholds than ^{90}Y, whose emitted electrons have a path length of 12 mm. Consequently, the inhomogeneous, mostly cortical, intrarenal distribution of radiolabelled somatostatin analogues is only of importance for ^{177}Lu- and ^{111}In-labelled peptides.

In studies with ^{177}Lu-DOTA,Tyr^3-octreotate in our institution, the maximum kidney absorbed dose was limited to 23 Gy. In over 500 patients treated so far only 2 patients have had a reduction in renal function, both probably unrelated to the therapy. One patient had a CRL of 41 ml/min at baseline and renal function further deteriorated during therapy. The other patient had heart failure due to increasing tricuspid insufficiency [60]. In another study, we evaluated the loss of renal function in 28 patients treated with ^{90}Y-DOTA,Tyr^3-octreotide and 37 patients treated with ^{177}Lu-DOTA,Tyr^3-octreotate over a period of at least 18 months after the last therapy cycle [61]. All patients received amino acid solutions and the total kidney absorbed radiation dose, was limited to 27 Gy. In the ^{90}Y-DOTA,Tyr^3-octreotide group nine patients had a CRL

loss per year of more than 15% versus two patients in the ^{177}Lu-DOTA,Tyr^3-octreotate group. The mean yearly decline in CRL was not significantly different between the two groups (7.3% per year in the ^{90}Y-DOTA,Tyr^3-octreotide group versus 3,8% per year in the ^{177}Lu-DOTA,Tyr^3-octreotate group) but the cumulative renal absorbed doses was significantly higher in the ^{90}Y-DOTA,Tyr^3-octreotide group (26.9 versus 19.7 Gy).

Bodei et al. [62] analysed the renal function loss in 23 patients treated with ^{90}Y-DOTA,Tyr^3-octreotide and in 5 patients treated with ^{177}Lu-DOTA,Tyr^3-octreotate. In nine patients kidney toxicity occurred (according to NCI criteria), all from the ^{90}Y-DOTA,Tyr^3-octreotide group. However, all five patients in the ^{177}Lu-DOTA,Tyr^3]-octreotate had some CRL function loss at 1-year follow-up.

In general, it seems safe to treat with ^{177}Lu-labelled peptides. In this respect the study of Forrer et al. [63] is of particular relevance. These authors administered one cycle of 7400 MBq ^{177}Lu-DOTA,Tyr^3-octreotide to 27 patients who had been treated with ^{90}Y-DOTA,Tyr^3-octreotide. Serum creatinine did not rise in the course of this treatment, offering new, safe treatment options.

Effects of fractionation

Experimental studies show that the kidney has an extensive capacity for repair of sublethal radiation damage. The size per fraction markedly influenced the total dose tolerated [64, 65]. So, there is reason to take advantage of the effects of fractionation in the current dosimetry protocols.

Barone et al. [24] reported dosimetric data of 18 patients treated with ^{90}Y-DOTA,Tyr^3-octreotide and correlated these data with changes in CRL loss. The injected activities were individualized so that the renal absorbed dose did not exceed 27 Gy. Despite this limit, several patients developed renal toxicity as notified by significant CRL loss, proteinuria, hypertension, oedema and anaemia. The CRL loss did not correlate with the renal absorbed dose, even after correction for actual kidney volume using CT. In contrast, the BED (corrected for CT-assessed actual kidney volume) that was estimated according to the linear quadratic (LQ) model correlated well with the yearly loss of CRL. The LQ model allows evaluation of the effects of fractionation on biological effects of radiation. The effects are a linear and quadratic function of the dose per fraction and a function of the fraction number [66]. The study of Barone et al. [24] indicates that a calculated BED between 27 and 42 Gy correlates with CRL loss rates of up to 10% per year, whereas a BED higher than 45 Gy would result in a yearly CRL loss of 26-56%.

For comparison, when the available data of Otte et al. [14] are put in the LQ model (assuming that the absorbed dose was 3.2 mGy/MBq [45], and the $T1/2_{EFF}$ was 31 as calculated from Barone et al. [24]) the BEDs uncorrected for actual kidney volume ranged from 56 to 64 Gy. The patient reported by Cybulla et al. [21], who had had chemotherapy in the past, would have had a BED of 39 Gy. These numbers underline the proposed safe BED by Valkema et al. [61] and Barone et al. [24] of about 37 Gy. A recent study of Bodei et al. [62] suggests a similar maximum BED of 40 Gy in patients without risk factors.

Effects of previous toxic insults on radiation effects

The clinical observation of kidney damage after PRRT in patients with hypertension ([19, 20], or after chemotherapy [21] indicates that other medical factors influence the radiation effects to the kidney. In the study of Valkema et al., five risk factors were identified for more rapid kidney function loss: cumulative renal absorbed doses more than 25 Gy, diabetes, hypertension, age>60 years and renal radiation dose >14 Gy per cycle [61]. This was confirmed in the study of Barone et al., who found that hypertension, diabetes mellitus or previous chemotherapy negatively influenced the response of kidney to radiation [24]. Bodei et al. [62] recently recommended a BED of 28 Gy as the maximum safe BED in patients with risk factor such as diabetes and hypertension.

Classical dosimetry is performed with standard reference kidney volumes, based on planar scintigraphy and is not corrected for fractionation. Therefore, new methods that include the actual kidney size and that take into account the intrarenal activity distribution and the effects of fractionation may be better tools for clinical practise in PRRT. Additional studies need to be performed to evaluate the value of such methods.

Reduction of uptake of radiolabelled somatostatin analogues

Methods to reduce the uptake of radiolabelled somatostatin analogues are shown schematically in Figure 2.

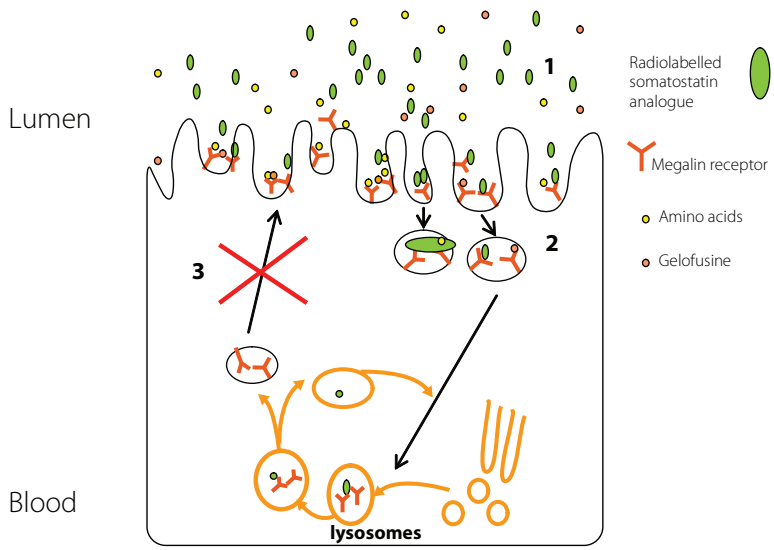

Figure 2: Schematic drawing of two methods to reduce the uptake of radiolabelled somatostatin analogues in a proximal tubule cell (adapted from Behr et al. [67]). Infused molecules of Gelofusine or positively charged amino acids are freely filtered and enter the tubular fluid (step 1). This results in megalin-mediated reabsorption of these two compounds, preventing reabsorption of freely filtered radiolabelled somatostatin analogues (step 2). Consequently, a lower amount of radiolabelled metabolites is retained in the lysosomes. Colchicine inhibits the return of the megalin-receptor to the cell surface (step 3).

Mogensen and Solling [68] found that proximal tubular reabsorption of low molecular weight proteins and peptides can be reduced by positively charged basic amino acids. This finding led many to investigate whether these amino acids might reduce renal reabsorption of radiolabelled somatostatin analogues. Hammond et al. [69] were the first to publish a semiquantitative study in humans showing that the renal uptake of [^{111}In-DPTA^{0}]octreotide could be reduced by a commercially available mixture of amino acids. A total of 35.2 g arginine and 9.8 g lysine was infused in each of these 16 patients. Behr et al. [70] showed that the renal uptake of radiolabelled monoclonal antibody fragments could be significantly reduced by about 80% in mice. De Jong et al. [71] showed that co-injection of 400 mg/kg lysine reduced rat kidney uptake of [^{111}In-DPTA^{0}]octreotide by about 40%. Arginine reduced the kidney uptake by 18% [71]. Also, D-lysine co-injection lowered kidney uptake of [^{111}In-DPTA^{0}]octreotide [72]. Based on these (pre)clinical data various amino acid infusion schedules have been tested in patients.

We [73] tested five different amino acid solutions in patients injected with ^{111}In-DTPA-octreotide. Infusion of a commercially available amino acid solution (1500 ml, containing 10.3 g lysine and 16 g arginine) reduced the kidney uptake by 21%. Infusion of 25, 50 and 75 g of lysine resulted in reductions of 17%, 15% and 44%, respectively. A combination of 25 g of lysine and 25 g arginine (combination named Lys/Arg) resulted in a reduction of 33%. Figure 3 shows the results of abdominal scintigraphy in an example patient in this study. Two signs of toxicity were noted in this study. Due to high osmolarity, patients often suffer from nausea and vomiting. In this study the frequency of nausea and vomiting was the highest (50% of patients) during infusion of the commercially available amino acid solution. Vomiting and nausea were less frequent when the other solutions tested were infused. The second toxicity concern was hyperkalaemia. This might be caused by an extracellularly directed shift of potassium secondary to an increased production of ketonic bodies in an acidic environment, as lysine is a ketogenic amino acid [74-78]. In our study, infusion of 75 g lysine resulted in

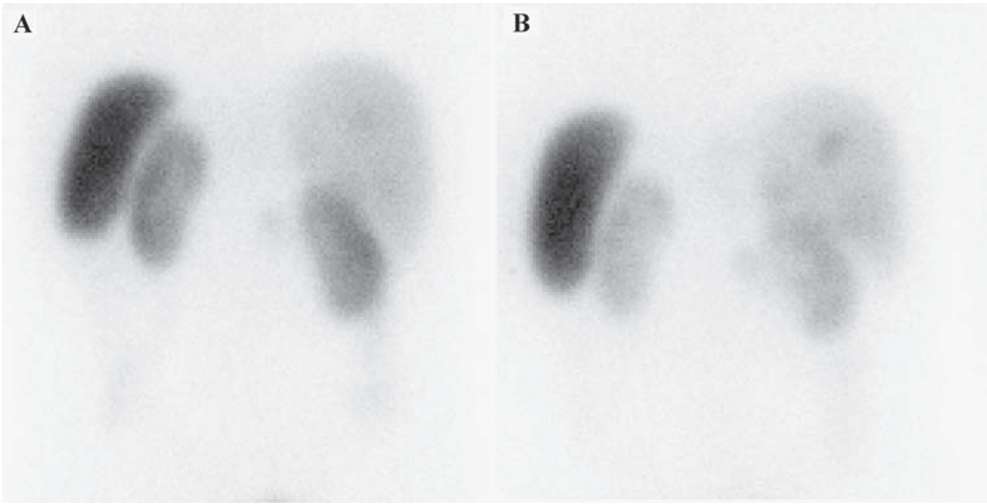

Figure 3. Abdominal scintigraphy in the same patient after administration of 220 MBq ^{111}In-DTPA-octreotide. Images were obtained without (A) and with (B) co-infusion of Lys/Arg. Renal radioactivity was 52% of control when Lys/Arg was infused. From reference [73].

elevated potassium concentrations in three patients (6.3, 6.7 and 6.8 mmol/l) out of eight. The highest potassium concentration among the nine patients infused with 25 g lysine plus 25 g arginine was 6.0 mmol/l [73]. Other metabolic changes caused by infusion of amino acids were mild hypophosphataemia (with both lysine solutions and mixed amino acids solutions) and elevated urea levels (with mixed amino acid solutions) [79].

Jamar et al. [47] also compared various amino acid solutions as well. They showed that a mixed amino acid solution (1,800 ml, containing 240 g mixed amino acids including 10.3 g lysine and 16 g arginine) reduced the renal uptake of ^{86}Y-DOTA,Tyr^3-octreotide by 21 % with concurrent reductions in estimated kidney radiation doses when applied to ^{90}Y-DOTA,Tyr^3-octreotide therapy. Prolongation of the infusion further enhanced the reduction. The maximum allowed dose of ^{90}Y-DOTA,Tyr^3-octreotide that would result in a 23 Gy kidney absorbed dose was calculated. The maximum allowed dose was 46% and 62% higher for mixed amino acids and the combination of 25 g lysine and 25 g arginine, respectively. Chinol et al. [80] reported the absence of renal toxicity after cumulative injected activities of 7.4-11 GBq ^{90}Y-DOTA,Tyr^3-octreotide. The administered activity could safely be enlarged to 12-18 GBq with co-infusion of amino acids in a new cohort of patients, suggesting that co-infusion of amino acids allows dose-escalation. Only reversible grade 1 renal toxicity was seen in two patients.

Taken together it can be concluded that co-administration of amino acid solutions can indeed be used to administer higher doses of radiolabelled somatostatin analogues without risk of nephrotoxicity. The side effects are moderate and mostly easy to handle. In our institution, we have not encountered any serious problems with the routine infusion of Lys/Arg administrations in hundreds of patients. Some studies in rats have shown renal damage after high-dose lysine administration [81-83], whereas others show beneficial effects of amino acid solutions in acute tubular necrosis in patients [84]. The long-term toxicity of amino acid solutions remains to be determined in humans, but no cases of nephrotoxicity induced by lysine or arginine have been reported.

As already discussed, the renal uptake of radiolabelled somatostatin analogues is at least partly associated with the megalin/cubilin system. Triggered by this finding, Van Eerd et al. [85] tested the potential of the plasma expander Gelofusine in mice and rats to reduce kidney uptake of radiolabelled somatostatin analogues (Figure 4). Gelofusine consists of succinylated bovine gelatine molecules and is used in clinical emergencies as a plasma expander. Infusion of Gelofusine has been reported to result in enhanced excretion of megalin ligands [86, 87]. Van Eerd et al. found that co-injection of Gelofusine reduced kidney uptake of ^{111}In-DTPA-octreotide by about 45 %, comparable to the reductions found following co-injection of lysine [85]. This preclinical study was followed by a study in five healthy subjects, which showed comparable results [88]. Recently, we [89] and Gotthardt et al. [90] showed that the combination of Gelofusine and lysine can even further reduce the renal uptake of radiolabelled somatostatin analogues in rats as an additive effect.

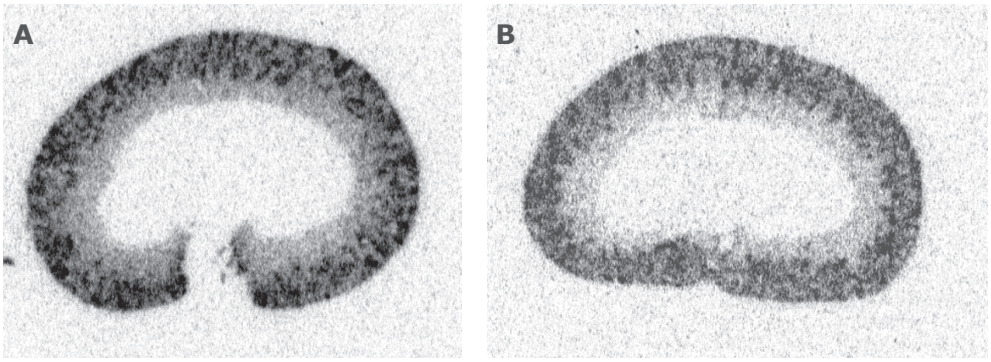

Figure 4. *Ex vivo* autoradiography of kidney sections 24 h after injection of 40 MBq ^{111}In-DOTA,Tyr^3-octreotate with co-injection of saline (A) or 80 mg/kg Gelofusine (B).

There have been some safety concerns because of a relatively high incidence of allergic reactions, however [91, 92]. Patients infused with Gelofusine during PRRT in Bad Berka showed promising results concerning the reduction of renal retention of either ^{90}Y- or ^{177}Lu-labelled somatostatin analogues [93]. A randomized study to compare renal uptake of ^{177}Lu-DOTA,Tyr^3-octreotate in patients either receiving Lys/Arg alone or a combination of amino acids and Gelofusine, is planned to be performed in our institution. In a preclinical study tumour uptake was unaffected when the combination of Gelofusine plus lysine was administered to sst_2 receptor-expressing tumour-bearing rats [94].

The anti-gout drug colchicine is known to inhibit microtubule function in cells, especially those in proximal tubule cells. Microtubules are essential in the intracellular trafficking of all kinds of organelles, including endocytosed receptor-ligand complexes. After injection of colchicine, it has been shown that intracellular distribution of megalin is greatly changed: it moves from the brush membrane to other cellular compartments [95]. In line with this, we [96] have shown that administration of colchicine significantly lowers the kidney uptake of ^{111}In-DTPA-octreotide in rats. The combination of colchicine with lysine results in an additive reduction of renal uptake [96]. The use of colchicine in the clinical setting is hindered by the small therapeutic index of the compound.

Sodium maleate, has been shown to reduce renal uptake of radiolabelled somatostatin analogues in rats. The sodium maleate was administered either as a co-injection or several hours prior to administration of radiolabelled peptide and reduced renal uptake to 15-30% of the uptake in controls [31, 71]. Maleate impairs megalin-mediated endocytosis by reducing energy metabolism in the cells. Maleate administration did not affect localization of ^{111}In-DTPA-octreotide in *ex vivo* autoradiography. Also megalin expression was unchanged in immunohistochemistry studies [31]. Although an effective tool to lower renal uptake or radiolabelled peptides, this drug is not an option for clinical use because serious toxic effects have been described [71, 97, 98].

Recently, a new method to reduce the renal retention of several radiolabelled peptides, including somatostatin analogues, has been introduced: co-administration of albumin frag-

ments with a molecular weight between 3 and 50 kDa, in both *in vitro* and *in vivo* studies [99, 100]. Albumin (molecular weight 67 kDa), which is a megalin ligand, is only marginally reabsorbed in the renal tubular cells, whereas the smaller fragments are reabsorbed to a high extent. Therefore this method probably also interferes with the megalin-mediated reabsorption pathway, as the albumin fragments may inhibit renal absorption of radiopeptides in a competitive way which can be different from the mechanism of interference when basic amino acids are co-administered. Combination of these two methods to reduce renal retention would give more insight into this issue.

Mitigating the effects of radiation

Another renoprotective strategy is to reverse or interfere with the cascade of events after radiation. As radiation causes a chronic state of oxidative stress with continuous production of reactive oxygen species, the radioprotective drug amifostine (Ethyol, WR-2721) has successfully been used in the protection of healthy tissues in the case of radiation therapy of throat tumours [101] and during chemotherapy with cisplatinum [102]. After injection, this drug is activated by alkaline phosphatases and subsequently taken up by healthy cells. The kidney concentrations of the active metabolite WR-1065 are about 100 times higher than tumour concentrations [102], which forms the basis for selective protection of healthy tissue. We have recently shown that amifostine can ameliorate functional renal damage in rat kidneys [103].

The renin-angiotensin-aldosterone system (RAAS) plays a key role in the development of radiation damage to the kidney. There are several reports of Cohen et al. indicating that treatment with angiotensine converting enzyme (ACE) inhibitors or angiotensin II-receptor blockers (ARB's) prevents the development of radiation damage, and in bone marrow transplantation experiments in rats after total body irradiation could even successfully correct established radiation nephropathy [104-108]. A randomized controlled study in patients on the protective effect of the ACE inhibitor captopril on nephropathy after bone marrow transplantation showed increased survival in the experimental group compared to the placebo controls [109]. Jaggi et al. [110] tested the effects of RAAS block in a mouse model of nephropathy induced by internal radiation using radioimmunotherapy with an antibody labelled with an α-emitter. They showed, however, that treatment with captopril enhanced renal functional and histological damage. This might point to species differences in renal radiation sensitivity, or to a compensatory mechanism of increased angiotensine II production by a non-ACE-dependent pathway which is not activated in the external radiation-induced nephropathy [111]. But treatment with the aldosterone receptor antagonist spironolactone and, to a lesser extent, treatment with an angiotensine II receptor blocker was able to prevent the development of histopathological and functional changes caused by α emitter internal radiation. Preliminary data from our institution show that addition of an ARB to lysine (in order to interfere with the RAAS system) ameliorates ^{177}Lu-DOTA,Tyr^{3}-octreotate-induced nephropathy in rats [112].

Development of new peptides with a higher affinity for the somatostatin receptor or with lower renal uptake

Enlarging the therapeutic window in PRRT can be achieved either by increasing the tumour uptake or by reducing the renal uptake, as has recently been reviewed [113]. Development of new somatostatin analogues with better targeting properties in PRRT is a possible approach

to increasing the therapeutic tumour dose. New, stable somatostatin analogues with a higher affinity for somatostatin receptors compared to the commonly used somatostatin analogues might improve the therapeutic effect [114]. A nice example is DOTA-NOC ([DOTA^0,1-Nal^3]octreotide), which has a high affinity for sst2, 3 and 5, and in preclinical models has shown increased tumour uptake together with lower renal uptake compared to other somatostatin analogues [115]. This resulted in a more than twofold increase in tumour to kidney ratio. However, a recent study in 95 patients, revealed that uptake by normal organs, but not the kidney, and radiation doses were higher with ^{177}Lu-DOTA-NOC than with ^{177}Lu-DOTA,Tyr^3-octreotide, whereas tumour uptake was higher with ^{177}Lu-DOTA,Tyr^3-octreotate [46]. Besides DOTA-NOC, the Thr^8 counterpart DOTA-NOC-ATE (DOTA^0,1-Nal^3,Thr^8]octreotide) has also been developed, as well as DOTA-BOC-ATE ([DOTA^0,BzThi^3,Thr^8]octreotide). Overviews of IC-50 values of these peptides, labelled with ^{68}Ga, ^{90}Y, ^{111}In or ^{177}Lu, have been published [93, 116]. Their application in imaging and PRRT of neuroendocrine tumours has not yet been established.

Recently pansomatostatin receptor-targeting molecules have been developed that recognise all sst subtypes, offering broad application in patients suffering from several cancer types, either nonlabelled or radiolabelled [117]. The renal uptake of these radiopeptides is relatively high however.

Almost all somatostatin analogues used in diagnostic and therapeutic settings are agonists. These molecules are internalized into the cells after receptor binding, resulting in deposition and retention of the radionuclide in the lysosome close to the nucleus. It has recently been demonstrated that somatostatin antagonist analogues show much higher binding capacity to tumour cells than agonists, leading to convincingly higher tumour to kidney ratios [118]. The renal retention of both antagonists and agonists was comparable. Because also the retention over time of antagonists on tumour cells is longer than that of agonists, antagonizing radiopharmaceuticals may be very useful for diagnostic imaging. The therapeutic efficacy of non-internalized radiolabelled antagonistic peptides will depend on the range and energy of the radionuclide used, because the distance to the cell nucleus to induce fatal DNA damage is larger than for internalized agonistic radiopeptides. Depending on the therapeutic dose administered, reduction of renal uptake may be applied to prevent a harmful kidney dose [117].

Conclusions and future outlook

PRRT using radiolabelled somatostatin analogues is a promising new treatment modality for otherwise incurable sst2-positive tumours. The renal uptake of the radiolabelled peptides is, however, dose-limiting as indicated by clinically observed renal toxicity and the rapidly reached kidney threshold of 23 Gy, adopted from external beam radiation therapy. Histological damage in PRRT is not different from that seen in external beam radiation therapy. Various factors play a key role in the development of renal injury, amongst which are angiotensine II and TGF-β. Much progress has been made in recent years in improving PRRT, i.e. achieving the highest tumour radiation dose possible within safe margins for the kidney,. Although the maximum safe radiation dose to the kidneys is not completely established, recent work indicates that the LQ model can describe the dose-effect relationship seen in PRRT. The critical safe renal dose may be different for the different radionuclides and radiopharmaceuticals used

in PRRT. Factors that lower one patient's individual maximum safe kidney dose are hypertension, diabetes, age and a history of chemotherapy.

When searching for methods that lower kidney uptake of radiolabelled peptides, knowledge of the uptake mechanism is required. Convincing evidence shows that the proximal tubule cell is the site of uptake, with the scavenger molecule megalin being the mediator of uptake. Methods that interfere with this receptor-mediated endocytosis process have been extensively studied. Infusion of positively charged amino acids is now routinely used to significantly reduce kidney radioactivity uptake, empowering PRRT substantially. To further enhance this reduction, combining amino acids with the expander Gelofusine or albumin fragments might be considered.

Other strategies to protect the kidneys during PRRT comprise treatment with radioprotective drugs (amifostine) and interference with the RAAS-system. Renal function in rats treated with amifostine during PRRT has been found to be less affected than in non-protected rats, but more studies are needed to address the effects on the tumour. Preliminary results have not shown an effect of amifostine on tumour uptake of radiolabelled somatostatin analogues [119]. Interference with the RAAS showed some benefits in one animal study [112], but more studies are needed to broaden our knowledge on the renoprotective effect of ARB and ACE inhibitors in PRRT.

The combination of reducing initial renal uptake during PRRT (by administration of lysine, Gelofusine or albumin fragments) with agents to interfere in the development of nephropathy (such as amifostine or RAAS interfering drugs), offers new opportunities to increase the therapeutic dose to tumours without risk of late renal failure, leading to improved survival of patients.

The risk of bone marrow toxicity increases with increasing therapeutic dose. Studies into how to increase the therapeutic dose (more cycles, higher dose per cycle, improvement of specific activity of the compound) to the tumour in a renoprotective (and bone marrow-protective) regimen are needed to further improve PRRT with somatostatin analogues in the future.

Studies on renal protection during PRRT have mainly focused on radiolabelled somatostatin analogues, but should be expanded to other peptide analogues. Radiolabelled gastrin analogues targeting CCK-2 receptors are expected to be valuable in imaging and treatment of patients with medullary thyroid carcinoma or small-cell lung cancer. However, these analogues show a higher renal retention than somatostatin analogues though [90, 120]. The same holds true for the peptide analogue exendin which binds to the GLP-1 receptor, and is expressed on insulinomas. As the charge of these peptides is different from that of somatostatin derivatives, lysine and arginine co-infusion might not be the first choice to lower renal retention. Gelofusine or albumin fragments might be better choices for reduction of renal retention in these cases as has been confirmed in several preclinical studies [90, 99]. In future studies when patients are imaged for diagnostic purposes with radiolabelled gastrin or exendin peptide analogues, dosimetry will reveal the need for methods to reduce kidney uptake.

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Renal retention of radiolabelled peptides; role of megalin

Chapter 2

Renal uptake and retention of radiolabelled somatostatin, bombesin, neurotensin, minigastrin and CCK analogues: species and gender differences

Marleen Melis, Eric P. Krenning, Bert F. Bernard, Monique de Visser, Edgar Rolleman, Marion de Jong

Dept. of Nuclear Medicine, Erasmus MC Rotterdam, Rotterdam, The Netherlands

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Chapter 2.1

Abstract

Introduction: During therapy with radiolabelled peptides, the kidney is most often the critical organ. Newly developed peptides are evaluated preclinically in different animal models before their application in humans. In this study, the renal retention of several radiolabelled peptides was compared in male and female rats and mice.

Methods: After intravenous injection of radiolabelled peptides [somatostatin, cholecystokinin (CCK), minigastrin, bombesin and neurotensin analogues], renal uptake was determined in both male and female Lewis rats and C57Bl mice. In addition, *ex vivo* autoradiography of renal sections was performed to localize accumulated radioactivity.

Results: An equal distribution pattern of renal radioactivity was found for all peptides: high accumulation in the cortex, lower accumulation in the outer medulla and no radioactivity in the inner medulla of the kidneys. In both male rats and mice, an increasing renal uptake was found: ^{111}In-DTPA-CCK8 < ^{111}In-DTPA-Pro^{1},Tyr^{4}-bombesin >> ^{111}In-DTPA-neurotensin < ^{111}In-DTPA-octreotide << ^{111}In-DTP-MG0. Renal uptake of ^{111}In-DTPA-octreotide in rats showed no gender difference, and renal radioactivity was about constant over time. In mice, however, renal uptake in females was significantly higher than that in males and decreased rapidly over time in both genders. Moreover, renal radioactivity in female mice injected with ^{111}In-DTPA-octreotide showed a different localization pattern.

Conclusions: Regarding the renal uptake of different radiolabelled peptides, both species showed the same ranking order. Similar to findings in patients, rats showed comparable and constant renal retention of radioactivity in both genders, in contrast to mice. Therefore, rats appear to be the more favourable species for the study of the renal retention of radioactivity.

Introduction

Various tumours frequently overexpress receptors for regulatory peptides. In nuclear medicine, analogues of these regulatory molecules are used as peptide-based radiopharmaceuticals, specifically targeting these receptors for diagnostic and therapeutic purposes [1]. Visualization of (metastasized) neuroendocrine tumours using ^{111}In-DTPA-octreotide (Octreoscan®) targeting somatostatin receptors (sst2, 3 and 5) is a successful example of this strategy, which has been performed routinely since the early 1990s [2, 3]. Peptide receptor radionuclide therapy (PRRT) using ^{90}Y-labelled or ^{177}Lu-labelled somatostatin analogues has been accepted as a clinical application in tumour treatment [1, 4]. The development of novel radiopharmaceuticals specifically targeting other overexpressed receptors is an ongoing process [5, 6]. Among others, bombesin analogues have been developed for, for example, breast and prostate cancers [7-10]. Cholecystokinin (CCK)/gastrin analogues can be applied for the diagnosis (and therapy) of medullary thyroid carcinoma and small cell lung cancer [11, 12]. Neuroendocrine tumours sometimes overexpress CCK-B (CCK2) receptors and can be diagnosed by these analogues as well [13]. Finally, neurotensin analogues are candidates for tumour imaging of patients with pancreatic adenocarcinoma [14, 15].

Conjugation of small peptides with an appropriate chelator is needed for the complexation of radioactive metal ions to the peptide with a high stability. Whereas non-chelated peptides are cleared via the liver and intestines, peptides chelated by diethylene triamine pentaacetate (DTPA) or dodecanetetraacetic acid (DOTA) are mainly cleared via the urinary tract. This relatively fast renal clearance route is favourable in patients and results in low abdominal background [16]. However, due to the renal clearance and partial reabsorption of radiolabelled DTPA/DOTA-chelated peptides, the uptake and retention of radioactivity in the kidneys can be substantial. Therefore the kidneys are the main dose-limiting organs during peptide-based radiotherapy of cancer patients using high doses of therapeutic radionuclides.

Recently, we demonstrated that renal radioactivity after ^{111}In-DTPA-octreotide administration does not show a uniform distribution. Moreover, different patterns were found in human and rodent kidneys [17, 18]. The highest amount of radioactivity was found in the renal cortex, located in proximal tubular cells. In mice, megalin, an endocytic receptor in proximal tubules, was shown to be essential for the renal retention of ^{111}In-DTPA-octreotide [19].

Research on new radioligands includes modifying the design of peptides while preserving binding affinity for the target receptor. The receptor specificity of newly developed radioligands has to be characterized in *in vitro* experiments using tumour cell cultures or tumour sections, followed by *in vivo* biodistribution studies in (tumour-bearing) animals [20]. For biodistribution studies, several species of rodents, of which rats and mice are the most common ones, are being used. The tumour specificity of peptide analogues is usually tested in athymic nude mice or severe combined immuno-deficiency (SCID) mice bearing xenografted tumours. However, the choice of animal species and the gender might influence the results of these studies.

The aim of this study was to investigate renal uptake and retention of different ^{111}In-labelled analogues such as CCK, gastrin, bombesin, neurotensin and somatostatin in rats and mice. Furthermore, a comparison of the renal retention of ^{111}In-DTPA-octreotide in male and female rats and mice was studied at several time points to investigate gender differences.

Materials and Methods

Radiopharmaceuticals

$^{111}InCl_3$ was obtained from Tyco Health Care (Petten, The Netherlands). Radiolabelling was performed according to previously published procedures [21, 22]. The peptide analogues used are listed in table 1.

Peptide	Structure	Company	Ref.
Somatostatin	DTPA-D-Phe-octreotide	Tyco Health Care, Petten, The Netherlands.	[2]
Cholecystokinin	DTPA-D-Asp^{1}, Nle^{3,6}-CCK-8	BioSynthema, St Louis Mo, USA	[23, 24]
Bombesin	DTPA-Pro^{1},Tyr^{4}-bombesin	BioSynthema, St Louis Mo, USA	[25]
Bombesin	DTPA-ACMpip^{5},Tha^{6},βAla^{11}, Tha^{13},Nle^{14}-bombesin$^{5-14}$ = MP2653	BioSynthema, St Louis Mo, USA	[26]
Neurotensin	DTPA-G(Pip)^{6},G(Pam)^{8}, tBuG^{12}-neurotensin$^{6-13}$	BioSynthema, St Louis Mo, USA	[15]
Minigastrin 0	DTPA-D-Glu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2)	kindly provided by Dr. M. Behe, Marburg, Germany	[27]

Table 1: Structure of investigated peptide analogues and their manufacturers.

Biodistribution experiments

Animal studies were conducted in agreement with the Animal Welfare Committee, using generally accepted guidelines.

Lewis rats and C57Bl6 mice or NMRI nude mice (both from Harlan, The Netherlands) were used. For biodistribution studies comparing the renal uptake of several radiolabelled peptides, male animals were anesthetized with isoflurane and injected intravenously into the dorsal vein of the penis. Rats received 0.5 µg of peptide (bombesin analogues, 0.1 µg) radiolabelled with 3 MBq of ^{111}In in 500 µl of saline (n=3-6 per group); mice received 0.1 µg of peptide radiolabelled with 4-10 MBq of ^{111}In in 200 µl of saline (n=4 per group). In the experiment using both genders, ^{111}In-DTPA-octreotide was administered intraperitoneally to unanesthetized aminals, 6 MBq per rat (n=2 per group) and 10 MBq per mouse (n=4 per group). The route of administration does not influence renal uptake [28].

After euthanasia at indicated time points post injection (p.i.), organs were isolated and blood samples were taken. Organs were weighed, and radioactivity was measured using a gamma counter (1480 Wizard 3", Perkin Elmer/Wallac, Turku, Finland). Uptake of radioactivity was expressed as the percentage of injected activity per gram of tissue (%IA/g). One kidney of each animal was frozen embedded in Tissue Tek (OCT compound; Sakura, Zoeterwoude, The Netherlands) using isopentane cooled in liquid nitrogen for autoradiography and immunohistochemistry.

Ex vivo autoradiography

Frozen kidneys were cut into sections of 10 µm (Cryo-Star HM 560 M; Microm, Walldorf, Germany) and mounted on Superfrost plus slides (Menzel, Braunschweig, Germany) for autoradiography. Adjacent slides were used for immunohistochemistry.

Autoradiography was performed with renal sections exposed to SR phosphor imaging screens (Packard Instruments Co., Meriden, USA) in X-ray cassettes. After 24-72 h, screens were read by a Cyclone phosphor imager and analysed with the OptiQuant 03.00 image processing system (Packard Instruments Co., Groningen, The Netherlands). Quantification of radioactivity was performed in five regions of interest in the cortex and in the outer medulla in five different sections of each tested kidney and radioactivity was expressed in digital light units per millimetre square (DLU/mm^2).

Immunohistochemistry

Anti-megalin immunostaining was performed as described previously [18, 19], using the primary antibody goat-anti-megalin SC-16478 P-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), directed against both rat megalin and mouse megalin.

Results

Renal retention of different radiolabelled peptides was determined in male rats and mice. Table 2 shows accumulated radioactivity 4 and 24 h after the intravenous injection of ^{111}In-labelled peptides in kidneys, ranking from the lowest to the highest values. The renal uptake of radioactivity was lowest for the CCK analogue, followed by that for the bombesin analogue and that for the neurotensin analogue. In rats, renal retention of the neurotensin analogue was comparable with that of the somatostatin analogue octreotide, whereas in mice, the neurotensin analogue showed an evidently lower renal uptake than octreotide. Minigastrin 0 (MG0) showed renal retention remarkably higher than those of the other peptides, both in rats and mice. The rank order of the renal uptake of all analogues included was the same for rats and mice. It is noteworthy that renal radioactivity declined readily over time in mice, whereas this was not the case in rat kidneys.

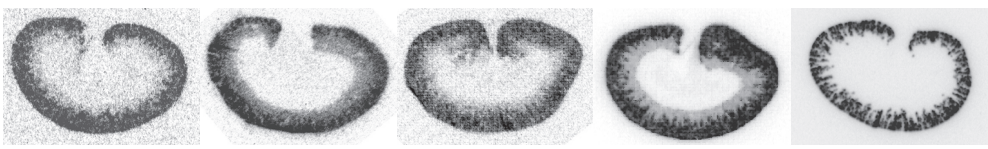
Despite quantitative differences in renal uptake, *ex vivo* autoradiography showed comparable localization patterns of radioactivity in kidneys from both rats and mice after the injection of different radiolabelled compounds. Figure 1 shows *ex vivo* autoradiograms of these com-

Peptide	^{111}In-labelled peptide analogue	Rat		Mouse	
		4 h p.i.	24 h p.i.	4 h p.i.	24h p.i.
Cholecystokinin	DTPA-D-Asp^{1}, Nle^{3,6}-CCK-8	0.2 ± 0.0	0.2 ± 0.1	1.3 ± 0.3	0.9 ± 0.2
Bombesin	DTPA-Pro^{1},Tyr^{4}-bombesin	1.0 ± 0.1	0.6 ± 0.1	4.7 ± 0.7	1.6 ± 0.8
Neurotensin	DTPA-G(Pip)^{6},G(Pam)^{8}, tBuG^{12}-neurotensin$^{6-13}$	1.0 ± 0.0	1.4 ± 0.2	5.3 ± 2.7	2.1 ± 0.7
Somatostatin	DTPA-D-Phe-octreotide	1.6 ± 0.1	1.5 ± 0.2	19.6 ± 1.5	3.9 ± 0.6
Minigastrin 0	DTPA-D-Glu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH$_2$)	17.6 ± 1.5	16.3 ± 1.7	105.6 ± 7.0	72.1 ± 16.2

Table 2: Uptake (%IA/g) of several ^{111}In-labelled peptide analogues in the kidneys of male rats and mice 4 and 24 h after the administration of 3 MBq/0.5 µg peptide (0.1 µg of bombesin) in rats (n=3-6 per group) or the administration of 4 MBq/0.1 µg in mice (n=4 per group).

pounds in rat kidneys. We found high radioactivity in the cortex, less radioactivity in the outer medulla, and no radioactivity in the inner medulla.

The influence of the gender on the uptake and retention of ^{111}In-DTPA-octreotide in the kidneys was investigated over time (4 h, 24 h, 96 h and 168 h p.i.), both in rats and mice.

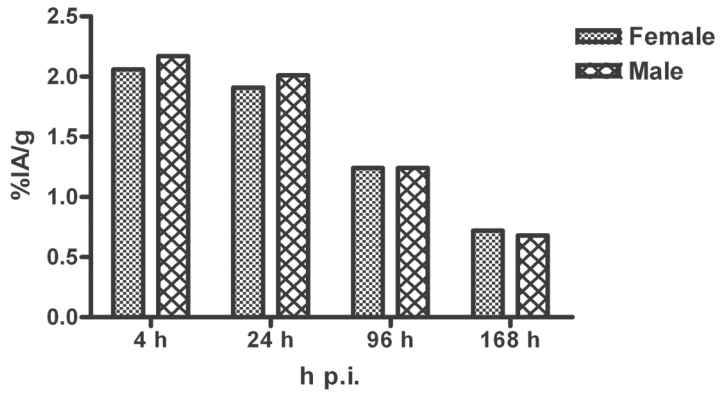


Cholecystokinin	**Bombesin**	**Neurotensin**	**Octreotide**	**Minigastrin 0**
Range 3-100	Range 3-500	Range 3-750	Range 3-1000	Range 3-7500

Figure 1: *Ex vivo* autoradiograms of frozen sections of rat kidneys 24 h after the administration of 0.5 µg (0.1 µg of bombesin) of radiolabelled DTPA peptides with 3 MBq of ^{111}In.

In rats, the decline in the renal radioactivity of ^{111}In-labelled DTPA-octreotide over time was low, ranging from 2.2 %IA/g at 4 h p.i. to 1.2 %IA/g at 168 h p.i., as demonstrated in Figure 2A. Moreover, the gender of rats did not influence kidney uptake: the ratio of uptake in the kidneys of females to uptake in the kidneys of males (F/M ratio) was close to 1 at all time points (Table 3). The distribution pattern of radioactivity in the kidneys of male and female rats, determined by *ex vivo* autoradiography, was comparable at all time points post injection (Figure 3A), and the ratios of accumulated radioactivity in the cortex to accumulated radioactivity in the outer medulla (C/OM ratio) were equal in both genders as well (Table 4).

A:



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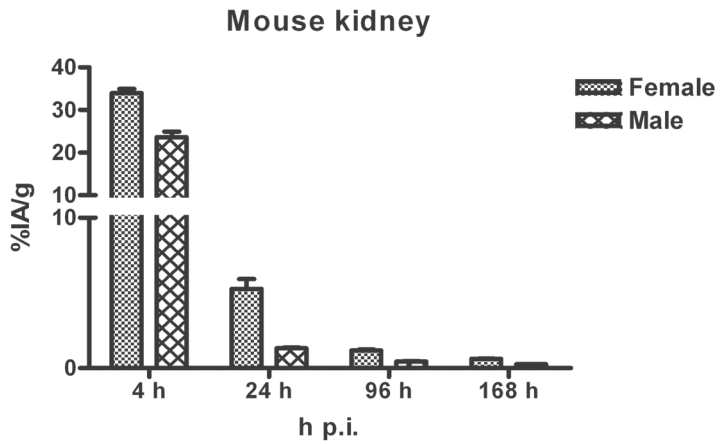

Figure 2: Mean uptake (%IA/g) of ^{111}In-DTPA-octreotide in the kidneys of female and male rats (**A**) and mice (**B**) at 4, 24, 96 and 168 h p.i. Rats (n=2 per group) received 6 MBq/0.5 µg radiolabelled peptide, and mice (n=4 per group) received 10 MBq/0.1 µg radiolabelled peptide. The difference in renal uptake between female and male mice was significant ($p<0.001$) at all time points.

In contrast, the same experiment performed in mice clearly showed a marked decrease in renal radioactivity over time, from >20% IA/g at 4 h p.i. to <1% IA/g at 168 h p.i. (Figure 2B). Furthermore, the retained radioactivity in female kidneys was significantly higher than the retained radioactivity in male kidneys. The F/M ratio of renal uptake varied and reached a maximum value of 4.7 at 24 h p.i. (Table 3).

hours post injection	Rat	Mouse
4	0.95	1.4
24	0.95	4.7
96	1.01	2.7
168	1.05	2.6

Table 3: Rats (n=2 per group) received 6 MBq/0.5 µg radiolabelled peptide, and mice (n=4 per group) received 10 MBq/0.1 µg radiolabelled peptide.

	hours post injection	Female	Male
Rat	4	2.4	2.3
	24	3.1	3.1
	96	5.4	5.0
	168	5.2	5.0
Mouse	4	0.3	0.9
	24	0.7	1.2
	96	0.6	1.9
	168	0.6	2.0

Table 4: C/OM ratio determined in *ex vivo* autoradiography studies, using Optiquant software, of the kidneys of female and male rats and mice 4, 24, 96 and 168 h after the administration of ^{111}In-DTPA-octreotide.

Gender difference in the renal retention of radiolabelled peptide analogues in mice was confirmed in the biodistribution experiment with the bombesin analogue MP2653 (Figure 4). In the female kidneys, 2.4 times more radioactivity was retained compared to that in the male kidneys, determined 4 h p.i. The localization of the renal radioactivity of ^{111}In-labelled DTPA-octreotide in mice showed a clear difference between female and male kidneys. In male kidneys, we found the pattern described above, whereas in female kidneys, the highest amount of radioactivity was found in the outer medulla and less radioactivity was seen in the cortex (Figure 3B). This different pattern was found at all time points in female kidneys and, to a lesser extent, at 4 h p.i. in male mouse kidneys. Therefore the C/OM ratios showed marked differences between male and female mouse kidneys (Table 4). The C/OM ratio in females was always <1, whereas it resulted in a ratio of >1 in males, as was also shown in rats after the administration of ^{111}In-DTPA-octreotide.

Immunohistochemistry using anti-megalin antibodies showed no difference in megalin expression between male and female mice (Figure 5).

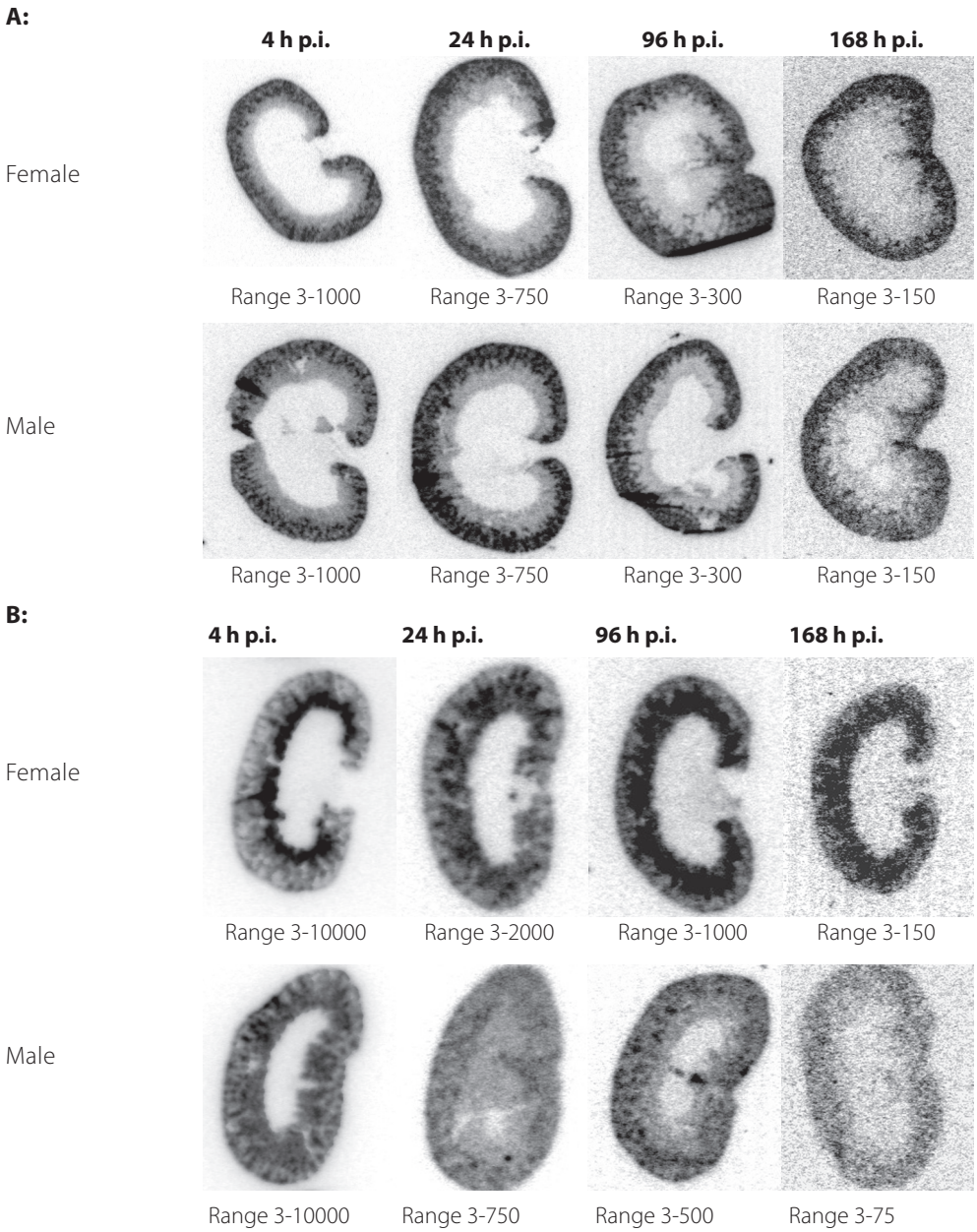

Figure 3: *Ex vivo* autoradiograms of frozen sections of rat (**A**) and mouse (**B**) kidneys from female and male animals 4, 24, 96 and 168 h after the administration of 6 MBq/0.5 µg ^{111}In-DTPA-octreotide in rats and the administration of 10 MBq/0.1 µg ^{111}In-DTPA-octreotide in mice.

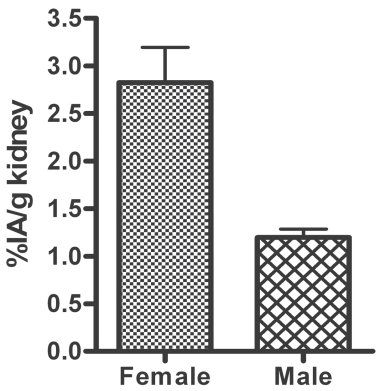

Figure 4: Uptake (%IA/g) of ^{111}In-MP2653 in the kidneys of female and male mice at 4 h p.i. Mice received 10 MBq/0.1 µg radiolabelled peptide (n=4 per group). p=0.0015; F/M ratio=2.4.

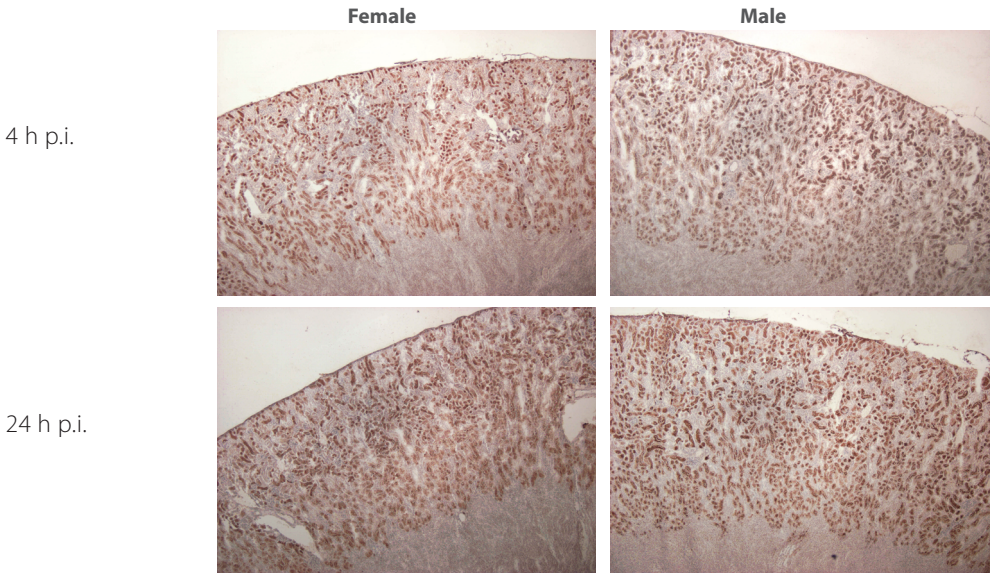

Figure 5: Immunohistochemical staining on frozen sections of female and male mouse kidneys 4 and 24 h after the administration of 10 MBq/0.1 µg ^{111}In-DTPA-octreotide using anti-megalin antibody, visualized with DAB using peroxidise-labelled second-stage antibody. Haematoxylin counterstaining was performed. Original magnification, x40.

Discussion

High receptor affinity is a crucial issue in the development of new radiopeptides, mediating the ability to target tumour cells expressing the specific receptor. On the other hand, it is also essential that unspecific binding to healthy cells and organs be low. While unspecific uptake of radioactivity in, for example, the bowel, liver or perirenal region during scintigraphy can cause difficulties in the interpretation of scintigraphic images, it could induce severe side effects due to high radiation absorbed doses during PRRT. As side effects of radiation absorbed

doses on the bone marrow (myelodysplastic syndrome) or kidneys (nephrotoxicity) have been described after PRRT [4, 29].

Before newly developed radiopharmaceuticals can be introduced in clinics, their pharmacokinetics, including specific (e.g., tumour) and non-specific (e.g., kidney, liver) uptakes, has to be studied in animal models. Besides mice, rats are also frequently used for biodistribution studies due to lower costs compared to nude or SCID mice. No study investigating the influence of the species and gender of laboratory animals on the *in vivo* biodistribution of radiolabelled peptides has been published so far.

In this study, we demonstrated that the extent of renal uptake and retention is different for various ^{111}In-labelled compounds. The same rank order of increasing renal uptake of radioactivity was found in male rats and mice. Renal uptake increased with the charge of the peptide analogue. The CCK8 analogue does not contain charged amino acids, whereas bombesin contains an arginine (Arg) residue, and both neurotensin and somatostatin analogues contain a lysine (Lys) residue, with Arg and Lys being positively charged amino acids. With six negatively charged glutamic acid (Glu) residues and one aspartic acid (Asp) residue, MG0 had the highest renal uptake and retention. Newly designed minigastrin analogues that contained less Glu residues showed lower renal uptake, pointing to a direct relation of charged amino acids with renal retention [30].

The localization pattern of renal radioactivity was comparable for the different analogues, suggesting a similar pathway of renal uptake and retention. All peptides showed high radioactivity in the cortex and lower amounts of radioactivity in the outer medulla. We have shown in mice that the negatively charged multi-ligand scavenger receptor megalin [31] is essential for the renal uptake of ^{111}In-DTPA-octreotide [19, 31]. It is present at the apical side of cells in proximal tubules, with high expression in the S1 and S2 segments of the tubules located in the cortex and with lower expression in the S3 segment located in the outer medulla [32], which correlates with the localization of retained renal radioactivity [18]. An essential contribution of megalin to the reabsorption of radiolabelled octreotide was also demonstrated by *in vitro* experiments with opossum kidney cells [33]. However, since megalin expression is equal in female and male mouse kidneys (Figure 5), the aberrant distribution pattern of radioactivity in *ex vivo* autoradiograms of the female kidney after ^{111}In-DTPA-octreotide administration cannot be explained by differences in megalin-mediated uptake alone.

The ratio of retained radioactivity in the renal cortex to retained radioactivity in the outer medulla (C/OM ratio) for ^{111}In-DTPA-octreotide in male rats 24 h p.i is 3 (Table 3), whereas this ratio is 12 for the radioactive MG0 peptide analogue, indicating that the renal localization of the latter is mainly found in the cortex. This high ratio could be due to the strong negative charge of the MG0 analogue. It is unclear if the negatively charged megalin is the scavenging receptor for the MG0 analogue. In renal proximal tubules, several transporter families are present. Organic anion transporters (OATs) and organic cation transporters, both at the apical and basolateral sides of tubular cells, are two of them. These transporters are responsible for the elimination of many compounds, such as drugs and metabolites, from endogenous and exogenous origins [34]. The (high) renal retention of MG0 might be due to binding to an

OAT transporter molecule. Reduction of the renal uptake of MG0 has been achieved after the co-administration of a chain of at least five consecutive polyglutamic acids [27]. Cationic amino acids Lys and Arg) interfere with the mechanism of the renal uptake of cationic peptides (megalin in the case of ^{111}In-DTPA-octreotide), and anionic amino acids may reduce the renal uptake of anionic peptides such as MG0 by interfering with another mechanism that might be an OAT transporter system. Gotthardt et al. [35] demonstrated that Gelofusine, a gelatine-based plasma expander, effectively reduced the renal retention of both MG0 and octreotide, in agreement with the involvement of reabsorption mechanisms other than megalin in the renal retention of radiolabelled peptides.

We have previously shown in rats that the amount of radioactivity retained in the outer medulla slowly decreased over time between 1 and 24 h p.i., whereas cortical radioactivity remained quite constant, resulting in a rising C/OM ratio over time [18]. In the present study we expanded these results until 168 h p.i., demonstrating an increased C/OM ratio until 96 h p.i., after which it remained constant until 168 h p.i.. Thus, retention of radioactivity appears to be more stable in cortical proximal tubuli (S1 and S2) than in proximal tubuli located in the outer medulla (S2 and S3). The reason for these differences in renal retention is the subject of further studies.

From patient studies, it has been concluded that the renal retention of radiolabelled somatostatin analogues over time was fairly stable [36]. These authors also showed that after correction for actual kidney volume based on CT studies, the renal uptake of radioactivity was not different between male and female patients [36]. In the present study, rat renal retention of radiolabelled somatostatin analogues was found to be fairly constant, without a clear gender difference. In contrast, renal uptake of radiolabelled somatostatin analogues in mice was readily declining over time, and a marked gender difference was found. Therefore, rats appear to be a better choice than mice for the investigation of renal uptake in a preclinical animal model. Consequently, when using xenografted mice to study renal uptake versus tumour uptake, the gender of mice and the time point after injection have to be considered.

The observation made - that renal radioactivity after the injection of radiolabelled octreotide analogues in mice is higher in females than in males - has been previously described by our group [19, 37]. This high kidney uptake of ^{111}In-DTPA-octreotide in females was also found by Froidevaux et al. [38]. However, a satisfying explanation for this phenomenon is still lacking. In biodistribution studies using a bombesin analogue, we also found higher renal uptake in females compared to males. Thus, gender difference in the renal retention of radiolabelled peptides in mice is not restricted to somatostatin analogues.

Some notions on the possible mechanism of this sex difference may be a guide for future investigations unravelling the role of gender in the renal retention of radiolabelled peptides.

An explanation of the gender difference in renal retention could be the sex-dependent excretion of proteins in the urine of rats and mice. Both females and males excrete protein but the amount is fourfold to six fold higher in males in mice (ranging from 10 to 30 mg/ml) [39]. The synthesis is sex dependent under the influence of androgens [40]. In rats, the major urinary

protein is α_2-microglobulin, whereas it is called major urinary protein in mice. These two proteins have a strong homology, especially for specific domains [40]. However, the amino acid sequence of the binding pocket is different, leading to differences in ligand profile and affinities. Another major difference is that major urinary proteins are glycosylated in rats, but not in mice. The high amount of low-molecular-weight proteins (19 kDa) in the primary urine of males may interfere with the clearance/reabsorption of other small proteins and peptides, as it is known from positively charged amino acids [41].

Another possible explanation for the difference in renal radioactivity retention between mice and rats might be kidney-androgen-regulated protein (KAP). This is a 20 kDa protein that is abundantly expressed in mouse kidney proximal tubular cells and that has been described by Cebrian et al. [42]. The function of KAP is still unknown. The expression of KAP in the cortical S1 and S2 segments is both androgen receptor mediated and testosterone dependent, whereas no androgen is required for the expression in the medullar S3 segment. Thus, in male kidneys, KAP is expressed on all proximal tubular cells, whereas KAP expression is only found in the medullary proximal tubules in females. KAP expression in the female kidney shows a pattern [42] similar to the aberrant localisation of radioactivity found in female mice in the present study. This might indicate an involvement of KAP in the renal uptake of radiolabelled analogues. In rats mRNA for KAP has been found as well, but exhibits only a 53% homology with the mouse counterpart. The low homology of the molecule in rats could be the reason for the molecule having a different function in these animals.

Further investigations are needed to fully understand mouse gender differences in renal retention.

Conclusions

Both rats and mice can be used to compare the renal retention of different radiolabelled peptides since the same rank order in renal uptake was found. Renal uptake increases with the charge of the peptide analogue.

Similar to findings in patients, rats showed constant retention and equal distribution of renal radioactivity in both genders, whereas a fast decline of renal radioactivity over time and marked gender differences with aberrant radioactivity localization pattern were found in mice. Thus, compared to mice, the rat appears to be the more favourable animal model for the study of the renal retention of radiolabelled analogues.

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Localization and mechanism of renal retention of radiolabelled somatostatin analogues

Marleen Melis[1], Eric P. Krenning[1], Bert F. Bernard[1], Raffaella Barone[2], Theo J. Visser[3], and Marion de Jong[1]

[1] Dept. of Nuclear Medicine, Erasmus MC Rotterdam, The Netherlands
[2] Centre of Nuclear Medicine and Laboratory of PET, UCL, Brussels, Belgium
[3] Dept. of Internal Medicine, Erasmus MC Rotterdam, The Netherlands

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Chapter 2.2

Abstract

Introduction: Radiolabelled somatostatin analogues, such as octreotide and octreotate, are used for tumour scintigraphy and radionuclide therapy. The kidney is the most important critical organ during such therapy owing to the reabsorption and retention of radiolabelled peptides. The aim of this study was to investigate in a rat model both the localization and the mechanism of renal uptake after intravenous injection of radiolabelled somatostatin analogues. The multi-ligand megalin/cubilin receptor complex, responsible for reabsorption of many peptides and proteins in the kidney, is an interesting candidate for renal endocytosis of theses peptide analogues.

Methods: For localization studies, *ex vivo* autoradiography and micro-autoradiography of rat kidneys were performed 1-24 h after injection of radiolabelled somatostatin analogues and compared with renal anti-megalin immunohistochemical staining pattern. To confirm a role of megalin in the mechanism of renal retention of ^{111}In-DTPA-octreotide, the effects of three inhibitory substances were explored in rats.

Results: Renal *ex vivo* autoradiography showed high cortical radioactivity and lower radioactivity in the outer medulla. The distribution of cortical radioactivity was inhomogeneous. Micro-autoradiography indicated that radioactivity was only retained in the proximal tubules. The anti-megalin immunohistochemical staining pattern showed a strong similarity with the renal ^{111}In-DTPA-octreotide *ex vivo* autoradiograms. Biodistribution showed that co-injection of positively charged D-lysine reduced renal uptake to 60% of control. Sodium maleate reduced renal ^{111}In-DTPA-octreotide uptake to 15% of control. Finally, cisplatin pre-treatment of rats reduced kidney uptake to 70% of control.

Conclusion: Renal retention of ^{111}In-DTPA-octreotide is confined to proximal tubules in the rat kidney, in which megalin-mediated endocytosis may play an important part.

Introduction

Tumours of neuroendocrine origin frequently show an over-expression of receptors with high affinity for regulatory peptides, such as somatostatin. Tumour targeting using the radiolabelled somatostatin analogue ^{111}In-DTPA-octreotide (Octreoscan®) [1, 2] is commonly performed for diagnostic purposes. For therapeutic applications, somatostatin analogues labelled β⁻-particle-emitting radionuclides, such as ^{90}Y-DOTA,Tyr^3-octreotide or ^{177}Lu-DOTA,Tyr^3-octreotate, have shown promising anti-tumour effects [3, 4]. One of the topics in the field of peptide receptor radionuclide therapy (PRRT) concerns the radiation dose to tumours versus critical organs, such as kidney and bone marrow.

Although the vast majority of radiolabelled peptides is excreted into the urine after intravenous injection into the patient, a small percentage is retained in the kidneys after glomerular filtration and reabsorption from the primary urine. Late irreversible radiation damage in kidneys has been described [5, 6] when high activities are administered during PRRT. In many PRRT clinical trials, individual dosimetry calculations and renal protection using amino acid infusions have been performed to avoid renal radiation doses exceeding 23-27 Gy. External beam radiation studies have shown that higher renal radiation doses may lead to nephrotoxicity in 5-50% of the patients after 5 years of follow-up [7]. Dosimetric estimation is usually performed assuming a homogeneous distribution of radioactivity in renal parenchyma. However, a recent study [8] in three patients showed that after i.v. ^{111}In-DTPA-octreotide administration, radioactivity is confined to certain areas in the kidney.

The aim of this study was therefore to gain more insight into both the localization and the mechanism of uptake and retention of radiolabelled somatostatin analogues in the rat kidney. A potential mechanism for renal retention of these somatostatin analogues has not previously been revealed. Megalin, a 600 kDa protein and a member of the LDL protein family, might be a candidate for receptor-mediated retention of radiolabelled peptides in the kidney. Besides expression in lungs, oviducts, thyroid, parathyroid, eyes and ears, the most abundant expression of megalin is found in the proximal tubules in the kidney [9]. The megalin (gp330)/cubilin complex is a scavenger protein receptor which is involved in reabsorption of a large variety of ligands such as (binding) proteins, hormones, drugs, toxins and enzymes [10, 11]. It is probably the most important receptor in the active receptor-mediated endocytosis of proteins in the proximal tubule. Megalin is negatively charged, showing preferential affinity for positively charged molecules, such as the somatostatin analogues, in primary urine. The expression of megalin in rat kidney varies between the segments of the proximal tubules. Expression increases in the first part (S1) of the proximal convoluted tubule (PCT), is strongest in the S2 part of the PCT and is weaker in the proximal straight tubule (S3) [12]. S1 and S2 are located in the renal cortex, whereas S3 is located in the outer zone of the medulla (Figure 1).

We hypothesised that this megalin/cubilin complex is responsible for renal uptake and retention of radiolabelled somatostatin analogues after PRS and PRRT. Therefore, we performed biodistribution studies in rats to unravel the possible part of megalin in renal uptake of radiolabelled peptides, using whole kidney counts, *ex vivo* autoradiography and anti-megalin immunohistochemistry.

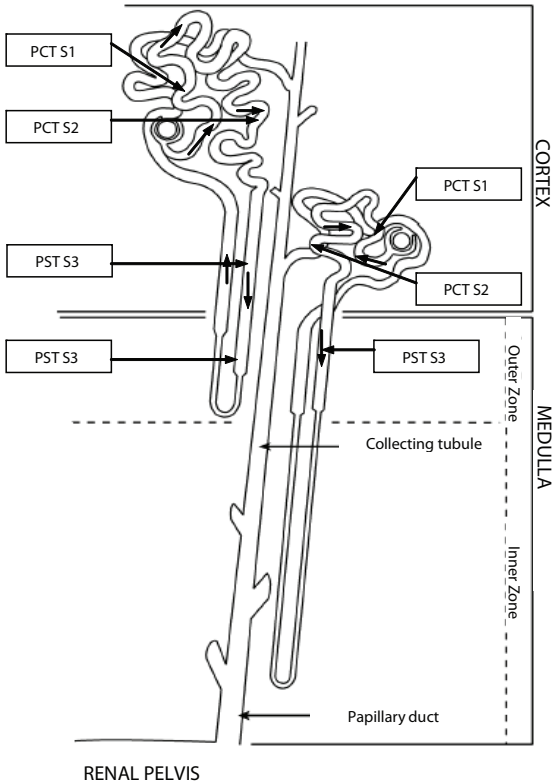


Figure 1: Illustration of a nephron in the kidney. The localization of S1, S2 and S3 segments in the proximal tubules is depicted. PCT = proximal convoluted tubule, PST = proximal straight tubule.

Materials and Methods

Radionuclides and peptides

Commercially available kits of DTPA-octreotide and $^{111}InCl_3$ were obtained from Tyco Health Care (Petten, The Netherlands). The radiolabelling procedure was performed in accordance with standard procedures [13].

$^{177}LuCl_3$ was from IDB, Baarle Nassau, The Netherlands. DOTA,Tyr^3-octreotate was supplied by BioSynthema (St Louis Mo, USA). ^{177}Lu-and ^{111}In labelling of DOTA,Tyr^3-octreotate was performed as described [14, 15]. Specific activities for ^{111}In-labelled peptides ranged from 6 to 100 MBq/µg: normally 6 MBq/µg was used; the higher specific activity of 100 MBq/µg was used when micro-autoradiography was to be performed. For ^{177}Lu-DOTA,Tyr^3-octreotate, specific activity was 50 MBq/µg. Labelling efficiency of all analogues exceeded 99%, as confirmed by instant thin-layer chromatography.

Biodistribution experiments

Animal studies were conducted in agreement with the Animal Welfare Committee requirements of our institution using generally accepted guidelines. Male Lewis rats (Harlan, The Netherlands, 150-180 g) were used in all experiments, except for those studying gender differences. For biodistribution studies, rats were ether anesthetized and injected intravenously with 0.5 µg peptide, labelled with at least 3 MBq radionuclide, in 500 µl saline into the dorsal vein of the penis. In gender studies, radiolabelled peptide was administered in 200 µl into the sublingual vein in both male and female rats.

After euthanasia at the indicated time points post injection (p.i.), several organs and blood samples were taken. Organs were weighed and radioactivity was measured using a gamma counter (Perkin Elmer, Wallac, 1480 Wizard 3", Turku, Finland). Uptake of radioactivity was expressed as percentage of injected activity per gram tissue (%IA/g).

One of the kidneys of each animal was quickly frozen and embedded in Tissue Tek (OCT compound, Sakura, Zoeterwoude, The Netherlands) using liquid nitrogen cooled isopentane for autoradiography and immunohistochemistry.

To block renal uptake, D-lysine (400 mg/kg, LD50 not available; LD50 L-lysine: 4.000 mg/kg i.p. and 10.000 mg/kg oral), maleate (di-sodium salt) (400 mg/kg, LD50: 700 mg/kg oral) or cisplatinum (cis-diamineplatinum dichloride) (5 mg/kg, LD50: 7.4 mg/kg i.v.) were used. All these compounds were obtained from Sigma, Steinheim, Germany.

D-lysine (400 mg/kg) was co-injected with the radiolabelled somatostatin analogue. Maleate (400 mg/kg) was either co-injected or administered 4 h before the radiolabelled somatostatin analogue. Cisplatin (5 mg/kg) was given i.p. daily 5 days before the analogue injection. In these experiments organs were collected 4 or 24 h post tracer administration.

Ex vivo autoradiography

After isolation and freezing of the radioactive rat kidney, 10 µm frozen sections (Microm CryoStar HM 560 M, Walldorf, Germany) were mounted on Superfrost plus slides (Menzel, Braunschweig, Germany). Several slides were used for autoradiography, whereas adjacent slides were used for immunohistochemistry. For autoradiography, renal sections were exposed to phosphor imaging screens (Packard Instruments Co., Meriden, USA) in X-ray cassettes. After 24-72 h screens were read by a Cyclone phosphor imager and analysed with an OptiQuant 03.00 image processing system (Packard Instruments Co., Groningen, The Netherlands). Conventional haematoxylin and eosin (HE) or periodic acid-Schiff staining was performed on the same or adjacent sections.

Quantification of radioactivity was performed using OptiQuant in five regions of interest of approximately 5 mm^2 each drawn over the cortex and the outer medulla, on five different sections of each tested kidney. Radioactivity was expressed in digital light units (DLU) per square millimetre.

Statistics

Uptake data from biodistribution studies (%IA/g) and quantified radioactivity data from *ex vivo* autoradiograms (DLU/mm^2) were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t test or one-way ANOVA.

Immunohistochemistry

Frozen renal sections adjacent to those used in *ex vivo* autoradiography were fixed with 10% formalin and used for indirect immunohistochemical staining for megalin. Primary antibody goat-anti-rat megalin SC-16478 P-20 (Santa Cruz Biotechnology, Santa Cruz, USA) was incubated in optimal dilution (1:25) overnight at 4°C. P-20 is an affinity purified polyclonal antibody raised against a peptide mapping near the carboxy terminus of megalin. Horseradish peroxidise-conjugated secondary antibody rabbit-anti-goat-Ig (DAKOcytomation, Glostrup, Denmark) was incubated for 1 h at room temperature. Visualisation of the staining was achieved with H_2O_2 activated diaminobenzidin (DAB) substrate (DAKOcytomation, Glostrup, Denmark). Nuclei were counterstained using haematoxylin.

Micro-autoradiography

Kidneys from rats injected ^{177}Lu-DOTA,Tyr^3-octreotate were removed at 24 h p.i., fixed in 10% formalin for 3 days, dehydrated and embedded in paraffin. Sections, 4 µm thick, were mounted on Superfrost slides, rehydrated and dried in a 60°C incubator for 30 min. Slides were coated in dark room conditions with LM-1 Hypercoat emulsion RPN40 (Amersham Biosciences, Freiburg, Germany) and dried overnight. Each gram of the photographic emulsion contained 0.09 g of silver grains with an average crystal diameter of 0.2 µm. After 3 weeks of exposure at 4°C, slides were developed with Kodak D-19 (Kodak, Rochester, USA) for 5 min and fixed with 250 mM $Na_2S_2O_3.5H_2O$ and 25 mM $K_2S_2O_5$ for 10 min. Counterstaining was performed using conventional HE staining.

Results

Localization studies

The amount and distribution of radioactivity in organs of rats after i.v. administration of radiolabelled somatostatin analogues was examined by counting radioactivity in several intact organs and by *ex vivo* autoradiography of frozen renal sections prepared after isolation of the radioactive kidney. From the renal autoradiograms, the distribution of various somatostatin analogues appeared to be inhomogeneous, showing a clearly localised pattern, as demonstrated in Figure 2. The strongest signal was found in the cortex, showing a striped pattern, whereas a more homogeneous and weaker signal was demonstrated in the outer medulla (about 50-60% of cortical activity, expressed as DLU/mm^2). No radioactivity was found in the inner medulla or the renal pelvis.

No difference in localization of radioactivity was found in ex-vivo autoradiograms of the somatostatin analogues ^{111}In-DTPA-octreotide, ^{177}Lu-DOTA,Tyr^3-octreotate or ^{111}In-DOTA,Tyr^3-octreotate (Figure 2), whereas absolute uptake ranged from about 1.5 to 2.5 % IA/g kidney.

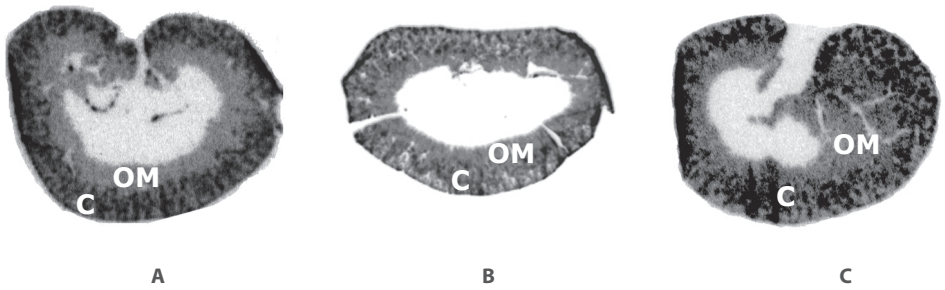


Figure 2: *Ex vivo* autoradiograms of frozen sections of rat kidney. A: 24 h p.i. ^{111}In-DTPA-octreotide. B: 24 h p.i. ^{177}Lu-DOTA,Tyr^3-octreotate. C: 24 h p.i. ^{111}In-DOTA,Tyr^3-octreotate.
C = cortex, OM = outer medulla

No gender variation was detected in renal retention of radiolabelled peptides; renal uptake of male and female rats was not significantly different and in both genders a similar localization pattern of retained renal radioactivity was found (Figure 3).

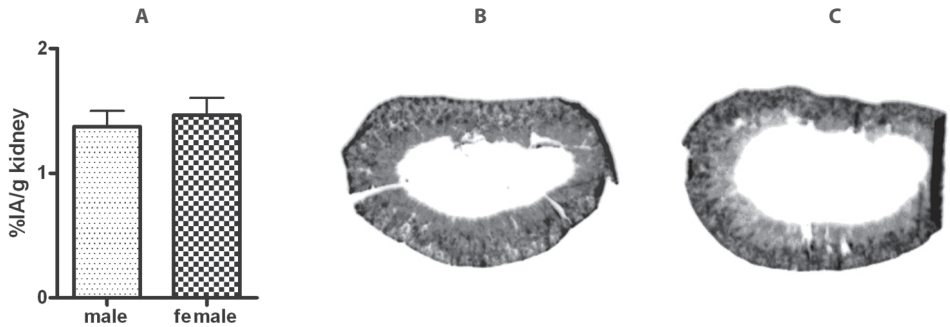


Figure 3: A: Uptake (%IA/g) of ^{177}Lu-DOTA,Tyr^3-octreotate in kidney of male and female Lewis rats, 24 h p.i. Mean ± SD. n = 2. B, C: *Ex vivo* autoradiograms of frozen sections of rat kidney, 24 h p.i. ^{177}Lu-DOTA,Tyr^3-octreotate: B male rat, C female rat

Micro-autoradiography was performed to show the microscopic localization of the renal radioactivity. Black silver grains, revealing the presence of radioactivity, were mostly localized in the proximal tubules of the kidney whereas much less radioactivity was detected in distal tubules and glomeruli (Figure 4).

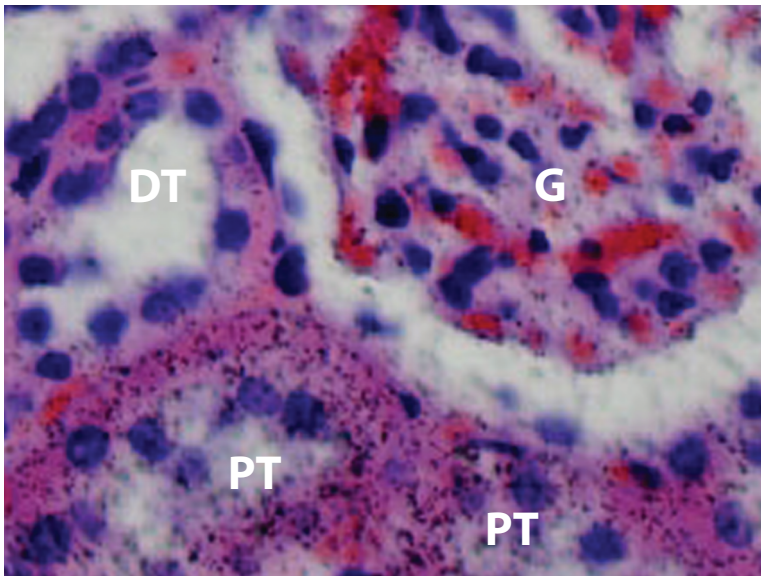


Figure 4: Micro-autoradiography of a paraffin section of rat kidney 24 h p.i. of ^{177}Lu-DOTA,Tyr^3-octreotate counterstained with conventional HE staining. Black silver grains indicate high radioactivity concentration. G: glomerulus, PT: proximal tubule, DT: distal tubule. Magnification x 1.000.

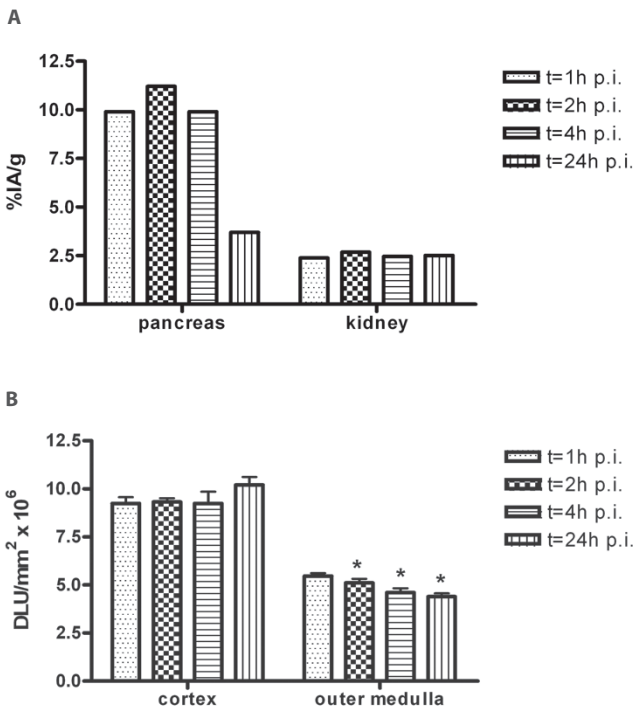


Figure 5: A: Uptake (%IA/g) of ^{177}Lu-DOTA,Tyr^3-octreotate in pancreas and kidney of rats at 1, 2, 4 and 24 h p.i. B: Radioactivity (DLU/mm^2) in cortex and outer medulla of rat renal sections at 1, 2, 4 and 24 h p.i. of ^{177}Lu-DOTA,Tyr^3-octreotate. Mean ± SD; n = 25. * $p < 0.005$ versus t = 1 h p.i.

In agreement with previous reports [16], we showed renal retention of radioactivity after injection of radiolabelled somatostatin analogues. No changes in total renal uptake of ^{177}Lu-DOTA,Tyr^3-octreotate were observed when measurements were performed at different time points (1, 2, 4, and 24 h p.i.) (Figure 5A). Quantification of autoradiogram data in cortex and outer medulla revealed that the amount of radioactivity present in the outer medulla decreased slightly with time, whereas cortical radioactivity remained at the same level (Figure 5B).

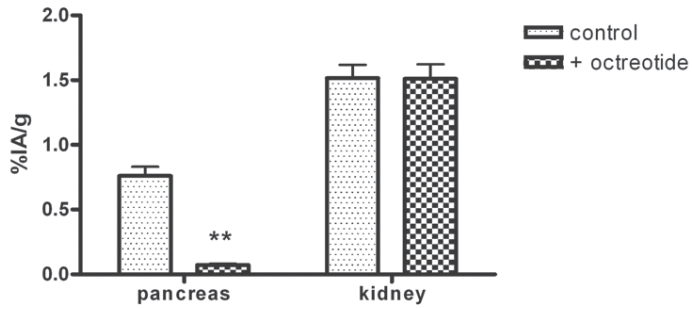


Figure 6: Uptake (%IA/g) of ^{111}In-DTPA-octreotide in rat pancreas and kidney at 24 h p.i. with or without co-injection of excess (0.5 mg) unlabelled octreotide. Mean ± SD; n = 3. ** p < 0.001 versus control.

Mechanism studies

We investigated the mechanism(s) responsible for the renal retention of these radiolabelled peptides. Excess, 1.000-fold molar of unlabelled octreotide almost abolished ^{111}In-DTPA-octreotide uptake in somatostatin receptor-positive organs, such as pancreas, but was not effective in blocking renal uptake (Figure 6), thus suggesting that the latter is not a process mediated by somatostatin receptors.

Immunohistochemical localization of megalin in rat kidneys showed a strong similarity to renal *ex vivo* autoradiograms after administration of radiolabelled peptides. Strong cortical expression and weaker expression in the outer medulla on the proximal tubules were found (Figure 7).

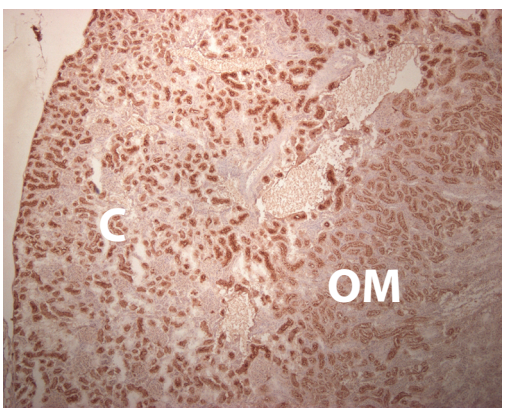
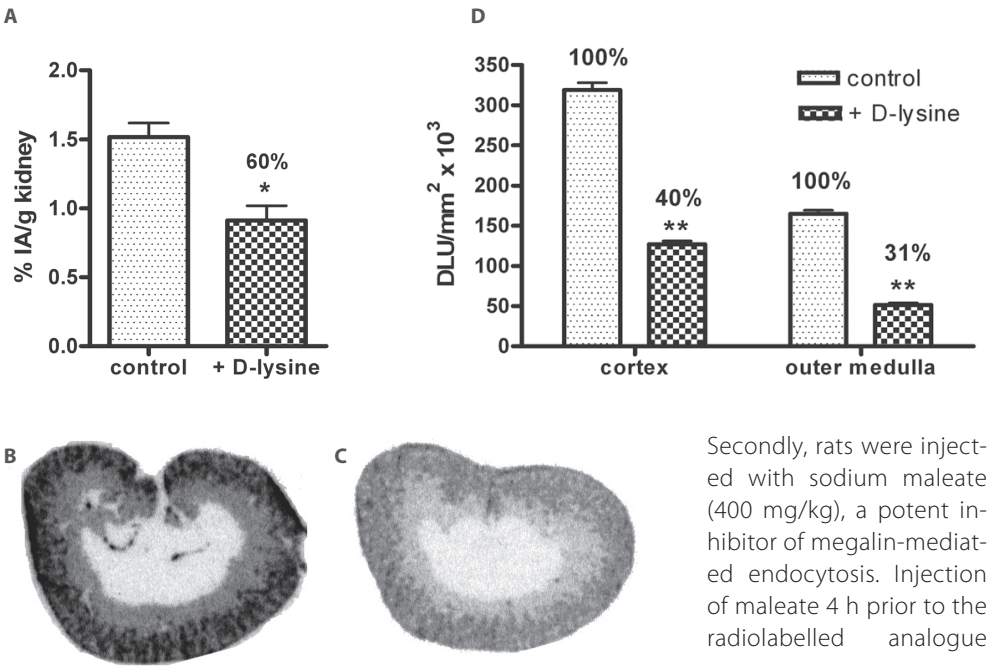


Figure 7: Immunohistochemical staining on a frozen section of rat kidney using anti-megalin antibody, visualised with DAB using peroxidase-labelled second stage antibody. Haematoxylin counterstaining was performed. C: cortex, OM: outer medulla. Magnification x 20.

In light of these findings, we studied the role of megalin as scavenging receptor for radiolabelled somatostatin analogues in the kidneys in several biodistribution studies in rats.

Firstly, we investigated the effect of i.v. co-injection of the positively charged D-lysine (400 mg/kg) with ^{111}In-DTPA-octreotide. A significant reduction of retained radiolabelled peptide (60% compared with controls) was found in the intact kidney and in the renal autoradiograms for both cortical and outer medullary radioactivity (Figure 8).



Secondly, rats were injected with sodium maleate (400 mg/kg), a potent inhibitor of megalin-mediated endocytosis. Injection of maleate 4 h prior to the radiolabelled analogue

Figure 8: Renal radioactivity in rat kidneys 24 h p.i. of ^{111}In-DTPA-octreotide with or without co-injection of D-lysine (400 mg/kg). A: Uptake (%IA/g) of ^{111}In-DTPA-octreotide. n = 3. * $p < 0.05$ versus control. B, C: *Ex vivo* autoradiograms of frozen sections of rat kidney, 24 h p.i.. ^{111}In-DTPA-octreotide: B: control, C: co-injection with D-lysine. D: Radioactivity (DLU/mm^2) in cortex and outer medulla of renal autoradiograms. Mean ± SD; n = 25. ** $p < 0.001$ versus control.

^{111}In-DOTA,Tyr^3-octreotate or by co-injection resulted in an impressive reduction of renal uptake to 15% of control (Figure 9).

Finally, after cisplatin (5 mg/kg) pre-treatment a significant reduction to 70% of control renal uptake was found. Renal autoradiograms of cisplatin-treated rats revealed strongly decreased and disturbed localization of radioactivity in the outer medulla with scattering of radioactivity throughout the renal medulla, whereas the cortical autoradiogram was not affected (Figure 10).

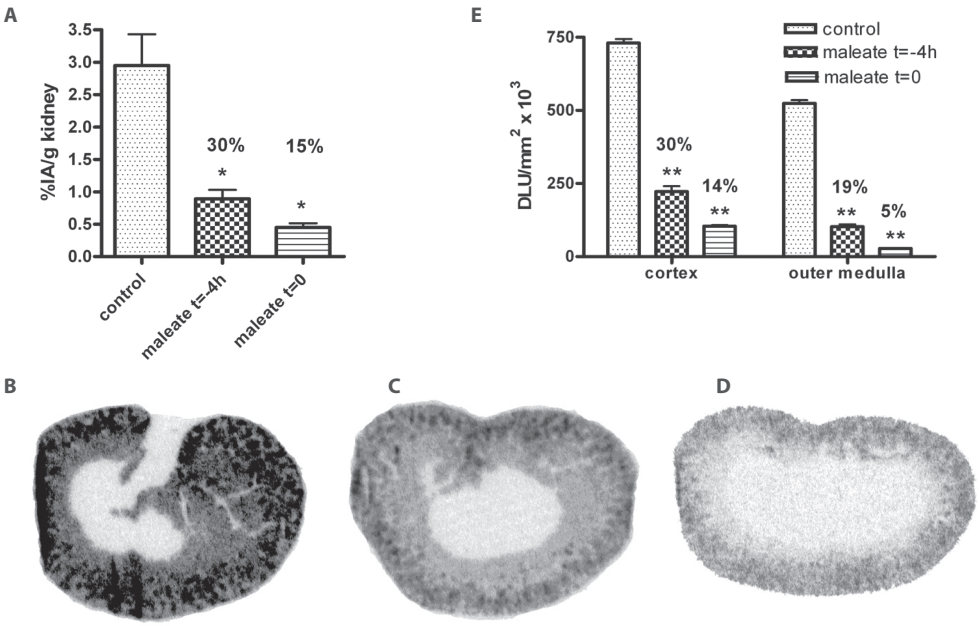


Figure 9: Renal radioactivity in rat kidneys 24 h p.i. of ^{111}In-DOTA,Tyr^3-octreotate with or without co- or pre-injection of sodium maleate (400 mg/kg). A: Uptake (%IA/g) of ^{111}In-DOTA,Tyr^3-octreotate. n = 2. * $p < 0.05$ versus control. B-D: *Ex vivo* autoradiograms of frozen sections of rat kidney, 24 h p.i.. ^{111}In-DOTA,Tyr^3-octreotate: B: control, C: maleate, t=-4 h, D: co-injection with maleate. E: Radioactivity (DLU/mm^2) in cortex and outer medulla of renal autoradiograms. Mean ± SD; n = 25. ** $p < 0.001$ versus control.

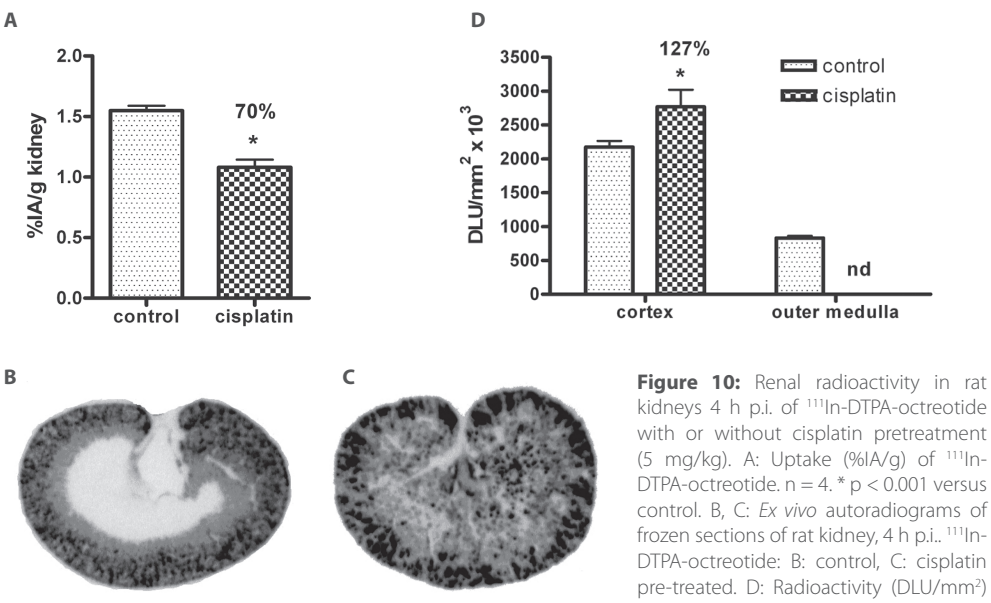


Figure 10: Renal radioactivity in rat kidneys 4 h p.i. of ^{111}In-DTPA-octreotide with or without cisplatin pretreatment (5 mg/kg). A: Uptake (%IA/g) of ^{111}In-DTPA-octreotide. n = 4. * $p < 0.001$ versus control. B, C: *Ex vivo* autoradiograms of frozen sections of rat kidney, 4 h p.i.. ^{111}In-DTPA-octreotide: B: control, C: cisplatin pre-treated. D: Radioactivity (DLU/mm^2) in cortex and outer medulla of renal autoradiograms. Mean ± SD; n = 25. * $p < 0.001$ versus control. nd: could not be determined.

Discussion

After intravenous administration of radiolabelled somatostatin analogues, there is fast accumulation in receptor-dense organs and/or tumours and rapid clearance from the blood. Metabolic studies have shown that the majority of radiolabelled analogues are excreted in the urine, mostly as the intact analogue [17]. Due to partial reabsorption of radiolabelled peptides after glomerular filtration, the retention of radioactivity in the radiosensitive kidney is, however, substantial.

The first aim of this study was to reveal the localization of retained radioactivity in the rat kidney after intravenous injection of various radiolabelled somatostatin analogues. Most radioactivity appeared to be retained in the proximal tubules (i.e. the S1 and S2 segments) in the renal cortex, while less uptake was found in the outer medulla (the S3 segment of the proximal tubules). No radioactivity was found in cortical glomeruli, distal tubules, inner medulla or renal pelvis (Figures 2, 4). This inhomogeneous distribution of radioactivity in rat kidney after the injection of radiolabelled somatostatin analogues is in agreement with recent reports in human kidneys [8].

In this study in rats, we observed no gender-determined differences in renal uptake and localization of ^{111}In-DTPA-octreotide (Figure 3), whereas in a study in Balb/c mice we found significantly different renal uptake of ^{111}In-DTPA-octreotide in males and females [18]. It seems that this sex-dependent distinct renal handling of the analogue only occurs in mice, since in humans, too, no differences have been described. No satisfactory explanation for this observation can be given at present.

The renal handling of proteins and peptides is a complex process, consisting of glomerular filtration followed by tubular reabsorption. As early as 1977 it was assumed that negatively charged "receptors" on the proximal tubules play a part in the mechanism of renal clearance of proteins and peptides, which was confirmed later for radiolabelled peptides and antibody fragments [19, 20]. However, these receptors have not yet been identified. Therefore, the second aim of the current study was to gain more insight into the mechanism of renal retention of radiolabelled somatostatin analogues.

Co-injection of excess of unlabelled octreotide did not reduce renal uptake of radiolabelled somatostatin analogues. Furthermore, in an earlier study in somatostatin receptor subtype 2 (sst2) knockout mice we found an equal or even higher renal retention of the analogue compared with wild type mice after administration of ^{111}In-DTPA-octreotide. *Ex vivo* autoradiograms showed the same localization of renal radioactivity in both mouse strains [21]. From these observations it can be concluded that the somatostatin receptor is not responsible for renal radioactivity retention.

In the kidney, megalin is present on the microvilli and apical membrane of proximal tubules and acts as a scavenger receptor for many ligands, predominantly proteins and peptides [11]. Therefore, we hypothesised that megalin plays a part in the renal retention of positively charged radiolabelled somatostatin analogues, particularly because megalin is a negatively

charged molecule. It is known that the molecular charge of the labelled peptide influences the renal uptake and retention. In 1983, Christensen et al. [22] reported that cationic proteins showed an enhanced proximal tubular uptake compared with anionic proteins. A higher isoelectric point enhanced renal reabsorption. Akizawa et al. concluded that an increase in negatively charged amino acids in peptide molecules reduced renal uptake of radiolabelled peptides [23].

The comparable localization pattern of renal radioactivity of the various radiolabelled somatostatin analogues used, suggesting a common transporter, is probably due to the nearly identical amino acid sequence of the peptide analogues. Most importantly, all somatostatin analogues examined contain a positively charged lysine residue at position 5.

The similar immunohistochemical staining pattern in renal sections using anti-megalin antibody compared with renal ^{111}In-DTPA-octreotide *ex vivo* autoradiogram patterns also points to a role for megalin in the renal retention of radiolabelled analogues.

It is interesting to study the interference in this putative megalin-mediated mechanism of retention in animal models because development of strategies to reduce renal uptake and retention of radioactivity is a field of great interest. Nowadays, infusion or injection of cationic amino acids, lysine and arginine, is widely used in patients undergoing PRRT [20, 24, 25]. Renal uptake of radiolabelled peptides is significantly reduced in patients receiving amino acids compared with controls. This allows the administration of higher activities during therapy in order to increase the therapeutic effect. The mechanism of this renal protection by amino acids has not been fully elucidated, although the positively charged amino group in the amino acids seems to be essential for achievement of the protective effect in the kidney.

In our rat biodistribution experiments, co-injection of D-lysine with ^{111}In-DTPA-octreotide resulted in reduction of renal uptake, in both the cortex and the outer medulla, without affecting megalin expression in the kidney. Competition between D-lysine and octreotide molecules for binding to megalin can explain this phenomenon. Probably because megalin is a receptor with a very high capacity, renal uptake of radiolabelled octreotide cannot be blocked completely by lysine; 50-60% reduction is the maximum achievable. Another possibility is that fluid phase endocytosis partly contributes to renal uptake of the radiolabelled analogues.

Maleate has often been used in rats to induce an experimental model of Fanconi syndrome by a generalised renal transport defect in the proximal tubules [26]; increased urinary excretion of glucose, amino acids, peptides and proteins is found after maleate administration. Maleate inhibits membrane recycling during endocytosis, and decreases energy metabolism by reduction of coenzyme A levels, thus inhibiting citric acid cycle and reduction of cellular ATP. Megalin has been proposed as a target molecule for the effect of maleate on renal proximal tubules [27]. In this study we examined the effect of sodium maleate treatment on the renal uptake of ^{111}In-DTPA-octreotide, expanding our previous results [28]. The renal uptake of ^{111}In-DTPA-octreotide was strikingly affected when maleate was given prior to or co-injected with the radiolabelled peptide. The kidney uptake in the maleate groups, both in whole organ counts and in *ex vivo* autoradiograms, was dramatically reduced compared with controls. The

observation that the reduction of renal uptake was less impressive when maleate was given several hours prior to administration of radiolabelled peptide pointed to a temporary effect of maleate on the endocytic processes in the kidney. Immunolabelling in the affected kidneys revealed that megalin expression was not affected by maleate, indicating that impairment of the energy metabolism in the endocytosis process is most likely the reason for the reduction in renal uptake. Recent experiments using colchicine [29, 30], an inhibitor of microtubule function, confirm the observation that interference in the process of endocytosis reduces renal uptake of radiolabelled somatostatin analogues.

The use of the anti-cancer drug cisplatin (cis-diamine-platinum dichloride) is limited because of the high risk of nephrotoxicity [31]. In rats, polyuria with increased excretion of proteins, including albumin and vitamin D binding protein, is induced [32]. Pathologically, damage in S3 segment (straight part) of the proximal tubule is found [33]. We examined the effect of cisplatin pre-treatment of rats on the uptake and localization of ^{111}In-DTPA-octreotide in the kidney. The 30% reduction in renal uptake achieved by cisplatin pre-treatment was low compared with the results using D-lysine or maleate. The first parts (S1 and S2) of the proximal tubules were not affected by cisplatin, whereas S3 in the outer medulla was clearly affected, as shown by both *ex vivo* autoradiograms and anti-megalin immunohistochemistry. Therefore, the reabsorption process of the radiolabelled peptides was impaired only in PST (S3).

These results point to an important role of megalin in the renal retention of radiolabelled analogues. This may be crucial for further research into renal uptake-reducing strategies during PRRT. Competition for megalin using irrelevant cationic peptides or amino acids or use of known megalin inhibitors can be further explored.

Conclusion

Renal radioactivity after intravenous injection of various radiolabelled somatostatin analogues in rats was localised predominantly in the proximal convoluted tubules in the renal cortex and to a lesser extent in the proximal straight tubules located in the outer medulla, but not in glomeruli or distal tubules.

Several lines of evidence point to megalin being the receptor responsible for renal uptake of radiolabelled somatostatin analogues. In animal models, renal uptake of radiolabelled somatostatin analogues could be reduced by competing with megalin or by interfering in the endocytosis process or energy metabolism of megalin-mediated reabsorption.

Identification of megalin as the receptor for tubular uptake of radiolabelled somatostatin analogues may be essential for the development of new strategies to further reduce renal uptake when using PRRT in patients.

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Megalin is essential for renal proximal tubule reabsorption of ^{111}In-DTPA-octreotide

Marion de Jong[1], Raffaella Barone[2], MD; Eric Krenning[1], Bert Bernard[1], Marleen Melis[1], Theo Visser[3], Michael Gekle[4], Thomas E. Willnow[5], Stephan Walrand[2], François Jamar[2], and Stany Pauwels[2]

[1]Department of Nuclear Medicine, Erasmus MC Rotterdam, The Netherlands
[2]Department of Nuclear Medicine, Cliniques Universitaires Saint-Luc, Brussels, Belgium
[3]Department of Internal Medicine, Erasmus MC Rotterdam, The Netherlands
[4]Physiological Institute, Würzburg, Germany
[5]ReceptIcon ApS, Aarhus, Denmark

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Chapter 2.3

Abstract

Introduction: Radiolabelled somatostatin analogues have been shown to be important radiopharmaceuticals for tumour diagnosis and radionuclide therapy. The kidney has appeared to be the critical organ during radionuclide therapy because of peptide reabsorption and retention in the proximal tubules after glomerular filtration. The molecular mechanism of renal reabsorption of these analogues has not been clarified. A possible receptor candidate is megalin, a multi-ligand scavenger receptor in the renal proximal tubules. The objective of this study was to investigate the role of megalin in tubular reabsorption of radiolabelled somatostatin analogues by using kidney-specific megalin-deficient mice versus mice with normal renal megalin expression. ^{111}In-DTPA-octreotide was used as a practical model of peptide.

Methods: Renal uptake of ^{111}In-DTPA-octreotide was determined by animal SPECT scintigraphy at different time points after injection of the tracer and by measurement of radioactivity after isolation of the organs. Furthermore, *ex vivo* autoradiography of renal sections revealed the zonal distribution of radioactivity in the megalin-deficient and megalin-expressing kidneys.

Results: SPECT scintigraphy of ^{111}In-DTPA-octreotide at 3 and 24 h after injection clearly showed lower renal radioactivity in megalin-deficient kidneys than in megalin-expressing kidneys, both in male and female mice, in accordance with counts obtained after isolation of the organ (70%-85% reduction of uptake in the megalin-deficient kidneys; $p<0.001$). Renal uptake of ^{111}In-DTPA-octreotide was significantly higher in female than in male kidneys ($p<0.001$). *Ex vivo* autoradiograms clearly showed that renal radioactivity was not homogeneously distributed in the megalin-expressing kidneys, but localised in the renal cortex. Quantification of the autoradiogram data confirmed the reduced radioactivity in the renal cortex of megalin-deficient kidneys.

Conclusion: This study revealed the molecular mechanism of ^{111}In-DTPA-octreotide uptake in renal proximal tubules involving the receptor megalin. Identification of megalin may be crucial for further research into strategies to reduce renal uptake.

Introduction

Radiolabelled receptor-binding peptides have been shown to be important radiopharmaceuticals for tumour diagnosis and therapy. Radiolabelled somatostatin analogues are successfully applied for visualization of somatostatin receptor-positive tumours, and scintigraphy with ^{111}In-DTPA-octreotide (Octreoscan®) has proven to be a sensitive and specific method to localize somatostatin receptor-positive tumours and their metastases [1]. Continuing research is aimed at developing a therapeutic analogue taking advantage of the specificity of the receptor binding and the localised radiation dose from the radionuclide linked to the peptide. Apart from short-ranged therapeutic Auger electrons, ^{111}In also emits two lonγ-range γ-rays and therefore it is not optimal for therapeutic usage. ^{90}Y-DOTA,Tyr^3-octreotide, with the high-energy β$^-$-emitter ^{90}Y (mean energy, 0.93 MeV; half-life, 64 h) stably linked in the DOTA-cage, is now clinically being evaluated for peptide receptor radionuclide therapy (PRRT) by various groups [2-8]. The promising rate of complete plus partial responses seen in the various ^{90}Y-DOTA,Tyr^3-octreotide studies consistently exceeds those obtained with ^{111}In-DTPA-octreotide for radionuclide therapy.

Another β$^-$-emitter used for PRRT is ^{177}Lu (half-life, 6.7 d). It emits β$^-$-particles with a lower energy (mean energy, 0.13 MeV) than ^{90}Y and γ-rays of 113 keV at 6% per decay and 208 keV at 10% per decay. ^{177}Lu complexed to the somatostatin analogue Tyr^3-octreotate via the chelator DOTA forms a promising therapeutic compound with considerably enhanced uptake in receptor-positive tumours and excellent therapeutic results in animal models and patient studies [9-11].

During PRRT using somatostatin analogues labelled with the β$^-$-emitters ^{90}Y and ^{177}Lu, the kidney is the critical organ because it excretes and partially retains radioactivity, leading to a high renal radiation dose. Lowering renal uptake of radioactivity will allow higher activities to be administered and higher tumour radiation doses to be obtained. Studies on animals and patients have shown that renal uptake of radiolabelled octreotide can be inhibited by administration of the positively charged amino acids lysine and arginine [4, 12-15]. The effects of these positively charged amino acids can be explained by competition for negatively charged binding sites at the proximal tubule cell surface. We also found that tubular reabsorption of radiolabelled somatostatin analogues requires energy. Injection of 400 mg/kg maleate, which inhibits the citric acid cycle, reduced renal uptake of ^{111}In-DTPA-octreotide by about 74% in rats [12].

The molecular mechanism of uptake of radiolabelled somatostatin analogues in the kidney has not been clarified. Previous *in vitro* studies using opossum kidney cells and *in vivo* studies in rats pointed to a role of megalin in reabsorption of radiolabelled octreotide [16, 17]. Megalin serves as a scavenger receptor for endocytosis of multiple ligands in the kidneys. It is expressed in the proximal tubules, consistent with the localisation of renal radioactivity in rat kidneys after injection of radiolabelled somatostatin analogues [18, 19].

The aim of this study was to reveal the radioactivity distribution in mouse kidneys and the role of megalin therein by the use of renal megalin-deficient mice [20]. These mice have kidney-

specific inactivation of the megalin gene. ^{111}In-DTPA-octreotide, a practical model of peptide for the somatostatin analogues currently used for scintigraphy and radionuclide therapy, was injected in kidney-specific megalin-deficient versus megalin-expressing control mice. Renal uptake of radioactivity was determined by animal SPECT scintigraphy at different time points after injection and by measurement of radioactivity using a γ-counter after isolation of the organs. Furthermore, *ex vivo* autoradiography revealed the zonal distribution of radioactivity in the megalin-deficient and megalin-expressing kidneys.

Materials and methods

Radiolabelling

Commercially available Octreoscan® kits of DTPA-octreotide (MW 1,500) and $^{111}InCl_3$ were obtained from Tyco Health Care (Petten, The Netherlands). The radiolabelling procedure was performed in accordance with the standard procedure listed on the package insert. Labelling efficiency was >99%, as confirmed by instant thin-layer chromatography.

Tissue distribution of ^{111}In-DTPA-octreotide

Animal experiments were performed in compliance with the regulations of the institutions and with generally accepted guidelines governing such work.

Generation of the mouse model used has been described by Leheste et al. [20]. In short, Lox P recombination sites were introduced into the murine megalin gene, and mice carrying the modified receptor gene through their germ line were generated. Animals homozygous for the floxed megalin gene (megalin$^{lox/lox}$) exhibit normal development and unimpaired viability. In parallel, a mouse model was established with renal expression of Cre recombinase (Cre) using a fragment of the human apolipoprotein (apo) E promoter to drive the Cre transgene (apoE^{Cre}). Mice doubly transgenic for the floxed megalin gene and the Cre gene (megalin$^{lox/lox}$;apoE^{Cre}) were produced by breeding of the individual lines and used in these experiments. Cre-mediated inactivation of the megalin gene in (megalin$^{lox/lox}$;apoE^{Cre}) mice resulted in significant reduction of renal megalin expression. No decrease in megalin levels was observed in other tissues expressing the receptor.

In various experiments, male and female kidney-specific megalin-deficient and megalin-expressing mice were injected with ^{111}In-DTPA-octreotide (10-100 MBq, 1-2 μg). Injections were performed intravenously or intraperitoneally. At indicated time points, SPECT scintigrams were made. After euthanasia of the animals at 3 and 24 h after injection, organs were isolated. Radioactivity was measured in isolated organs using a γ-counter. Groups consisted of 2-6 animals. Statistical analysis was performed using the Student t test.

Animal SPECT

SPECT Device. The animal SPECT (LinoView Systems) is based on four γ-detectors (5.1 x 12.7 cm [2 x 5 inches]) based on pixelated CsI(Na) scintillators (5-mm thickness, 2.44 x 2.44 mm pixel size). The intrinsic detector energy resolution at 140 keV is 35%, and the intrinsic sensi-

tivity in an energy window of 35% width centred on the photopeak is 42%. Detectors were equipped with rake collimator with a tuneable slit aperture, composed of two iridium square rods (2 x 2 x 60 mm).

Acquisition. Mice were scanned 3 or 24 h after injection with a collimator width of 0.2-0.4 mm. Two acquisitions of 15 min each were performed, the bed being shifted by 1.22 mm between the two acquisitions, resulting in reconstructed transverse slices spaced every 1.22 mm. The orbit ranges of the detectors were set in such a way that the four slit apertures would draw the narrowest rectangle possible around the object to be imaged. The distance between the slit aperture and the phantom or the animal boundary was typically about 3 mm. The acquired data were stored in list mode and included the following: detector number, (x, y) event position, event time and event energy. This linear orbit acquisition generates linograms forming a complete set of tomographic data, that is, sufficient to exactly reconstruct the activity map [21].

Reconstruction. Events from the list mode file, with an energy within a 50% window centred on the photopeaks, were rebinned to provide linograms. All reconstructions were performed using the maximum likelihood expectation maximization algorithm without attenuation, scatter correction or spatial resolution recovery [22, 23].

Autoradiography

One of the kidneys of each animal was frozen quickly after isolation at 3 or 24 h after injection and processed for cryosectioning and autoradiography. Tissue sections (10 mmm) were mounted on glass slides. Several slides were used to make autoradiograms, and adjacent sections were haematoxylin-eosin or periodic acid Schiff stained. The sections were exposed overnight to phosphor imaging screens (Packard Instruments Co., Meriden, USA) in radiographic cassettes. The screens were analysed using a Cyclone phosphor imager and a computer-assisted OptiQuant 03.00 image processing system (Packard Instruments Co, Groningen, The Netherlands).

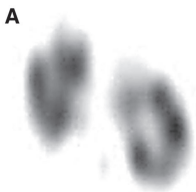
Immunohistochemistry

Frozen sections were fixed with 10% formalin and used for indirect immunohistochemical staining for megalin. Primary goat-anti-rat megalin antibody SC-16478 (Santa Cruz Biotechnology, USA) was incubated at optimal dilution overnight at 4°C. Horseradish peroxidase-conjugated secondary rabbit-anti-goat-Ig antibody (DakoCytomation, Glostrup, Denmark) was incubated for 1 h at room temperature. Staining was achieved with H_2O_2 activated diaminobenzidine substrate. Nuclei were counterstained using haematoxylin.

Results

Renal uptake in male mice

SPECT scintigraphy at 3 h after injection of ^{111}In-DTPA-octreotide in renal megalin-deficient and renal megalin–expressing male mice (Figure 1) showed that radioactivity was not homogeneously distributed over the kidney but concentrated in the cortical area. Furthermore, renal radioactivity was lower in the megalin-deficient kidneys than in control kidneys. This is in accordance with biodistribution data obtained after isolation and counting of the kidneys at 3 and 24 h after injection (Figure 2), indicating that injected ^{111}In-DTPA-octreotide was partially reabsorbed after glomerular filtration and retained in megalin-expressing kidneys, whereas uptake there in the megalin-deficient kidneys was much lower ($p<0.001$ at both time points). In the other organs, both somatostatin receptor positive and somatostatin receptor negative, uptake was not significantly different between mice with megalin-deficient kidneys and mice with megalin-expressing kidneys (data not shown).



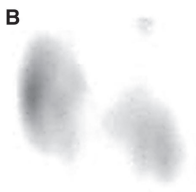


Figure 1: Male megalin-expressing (A) and megalin-deficient (B) kidneys. Images were obtained in a similar way and with the same acquisition time on an animal SPECT device (LinoView Systems) 3 h after injection of ^{111}In-DTPA-octreotide. Collimator width was 0.2 mm. Two acquisitions of 15 min each were performed, the bed being shifted by 1.22 mm between the two acquisitions. Reconstructions were performed using the maximum-likelihood expectation maximization algorithm without attenuation correction, scatter correction or spatial resolution recovery.

Figure 3 shows *ex vivo* autoradiograms of megalin-deficient and megalin-expressing kidneys at 3 h after injection of ^{111}In-DTPA-octreotide in male mice. Radioactivity was not homogeneously distributed in the megalin-expressing kidneys, as was shown also on the scintigrams, but localised in the cortex and outer medulla of the kidney. Quantification of the autoradiography data revealed strongly reduced radioactivity in the megalin-deficient kidneys, likely reflecting the residual renal megalin activity in this tissue-specific deficient line (Figure 4).

Comparison of renal uptake in male and female mice

SPECT scintigraphy at 24 h after injection of ^{111}In-DTPA-octreotide is shown for female renal megalin-deficient and female control mice in Figure 5. Renal radioactivity was lower in megalin-deficient kidneys than in megalin-expressing kidneys, in accordance with the data obtained in the male mice. The scintigrams are in agreement with the autoradi-

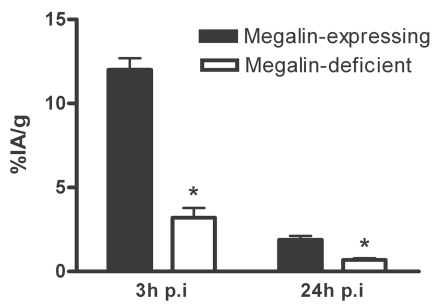


Figure 2: Renal uptake of ^{111}In-DTPA-octreotide in renal megalin-deficient and megalin-expressing male mice at 3 and 24 h after intravenous injection. Uptake is expressed as percentage injected activity per gram of kidney (%IA/g) (mean and SD, n=2-6 for each group). *: $p<0.001$ vs. megalin-expressing kidneys.

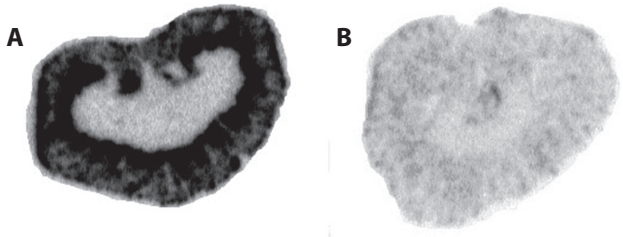


Figure 3: Autoradiograms of longitudinal renal sections from megalin-expressing (A) and megalin-deficient (B) kidneys from male mice 3 h after injection of ^{111}In-DTPA-octreotide. Both sections had same (overnight) period of exposure.

ography data (Figure 6) and with biodistribution data obtained after isolation of the organs at 24 h after injection (Figure 7), indicating significantly lower uptake of ^{111}In-DTPA-octreotide in megalin-deficient kidneys than in megalin-expressing kidneys in both male and female mice ($p<0.001$). Renal uptake of ^{111}In-DTPA-octreotide was significantly higher in female than in male kidneys, in both megalin-expressing and megalin-deficient kidneys.

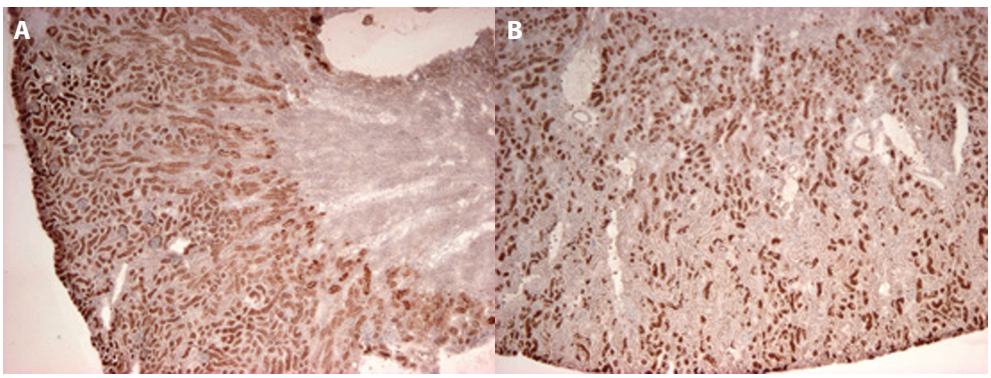


Figure 4: Immunohistology (using primary goat-anti-rat megalin antibody SC-16478) showing megalin distribution in sections from megalin-expressing (A) and megalin-deficient (B) kidneys from male mice.

Discussion

After intravenous administration to patients, radiolabelled DTPA- or DOTA-chelated somatostatin analogues accumulate in receptor-positive tumours, and radioactivity clears rapidly from the blood via the kidneys. Because part of the radiolabelled analogues is reabsorbed after glomerular filtration, retention in the kidney is substantial [3, 24, 25]. This renal uptake and retention is the major dose-limiting factor in PRRT using these somatostatin analogues, and lowering the renal accumulation will allow larger activities to be administered, thereby enlarging the therapeutic window of PRRT using somatostatin analogues. It has been shown that renal uptake of radiolabelled octreotide in rats is reduced by positively charged amino acids such as lysine and arginine; about a 50 % reduction is obtained by a single intravenous administration of 400 mg/kg of body weight L- or D-lysine in rats [12]. Therefore, during PRRT using somatostatin analogues, an infusion containing the positively charged amino acids L-lysine

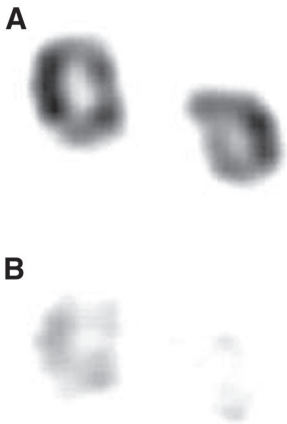


Figure 5: Animal SPECT images of female megalin-expressing (A) and megalin-deficient (B) kidneys 24 h after injection of ^{111}In-DTPA-octreotide. Images were obtained in a similar way and with the same acquisition time. Collimator width was 0.4 mm. Two acquisitions of 15 min each were performed, the bed being shifted by 1.22 mm between the two acquisitions. Reconstructions were performed using the maximum-likelihood expectation maximization algorithm without attenuation correction, scatter correction or spatial resolution recovery.

and L-arginine can be given to patients during and after the infusion of the radiopharmaceutical to reduce renal uptake. Various protocols have been described, resulting in up to a 55% reduction in renal uptake of radioactivity, thereby allowing a higher administered dose [4, 13-15]. The mechanism of this renal protection by amino acids has not been fully elucidated. The positively charged amino group in the amino acids seems to be essential to achieve the protective effect in the kidney.

With regard to localisation of renal radioactivity in rats, we recently found that most renal radioactivity after injection of radiolabelled somatostatin analogues appeared to be retained in the proximal tubules of the renal cortex, whereas no radioactivity was present in the glomeruli or distal tubules [17]. Less radioactivity than in the cortex was present in the outer medulla, whereas no radioactivity was found in the inner medulla and pelvis of the kidney. Our conclusion that radioactivity is not homogeneously distributed in rat kidneys is consistent with the results of a recent study on human kidneys [26] and with the results from the present study on mice. Here, we also encountered a significant difference in renal uptake between male and female mice, in accordance with earlier findings [27].

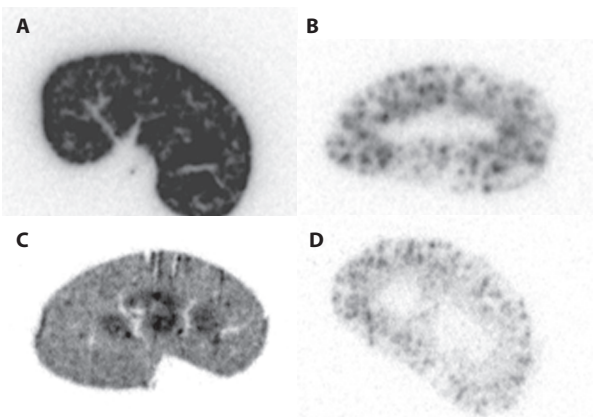


Figure 6: Autoradiograms of longitudinal renal sections from female (A and B) and male (C and D) mice having megalin-expressing (A and C) or megalin-deficient (B and D) kidneys. Images were obtained 24 h after injection of ^{111}In-DTPA-octreotide. All sections had the same (overnight) period of exposure.

The molecular mechanism of the uptake of radiolabelled octreotide analogues in renal proximal tubules has not been clarified. Megalin is a negatively charged 600-kDa type I transmembrane glycoprotein belonging to the low-density-lipoprotein receptor family. It is present on the microvilli and apical membrane of proximal tubules and is a scavenger receptor for tubular reabsorption of many ligands, predominantly proteins and peptides [18, 19]. Therefore, we hypothesised that megalin is the receptor responsible for the renal retention of radiolabelled soma-

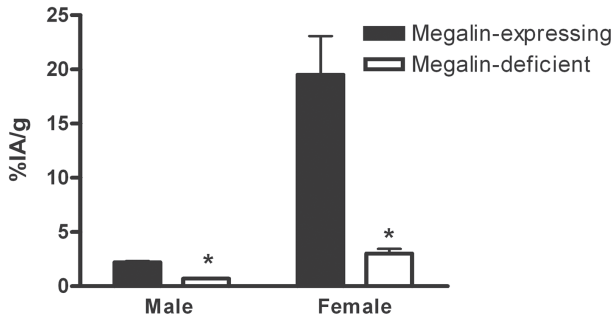


Figure 7: Renal uptake of ^{111}In-DTPA-octreotide in renal megalin-deficient and megalin-expressing male and female mice 24 h after intraperitoneal injection. Uptake is expressed as percentage injected activity per gram of kidney (%IA/g) (mean and SD, n=2-6 for each group). *: p<0.001 vs. megalin-expressing kidneys.

tostatin analogues, and previous *in vitro* studies using opossum kidney cells and *in vivo* studies in rats were consistent in finding a role for megalin in reabsorption of radiolabelled octreotide [16, 17].

The present study demonstrated by the use of megalin-deficient mice that the endocytic receptor megalin is indeed essential for the renal reabsorption of ^{111}In-DTPA-octreotide, because megalin-deficient mice showed severely impaired renal tubular uptake of this analogue (70%-85% reduction of uptake). The Cre-mediated inactivation of the megalin gene in these (megalin$^{lox/lox}$;apoE^{Cre}) mice did not result in complete cessation of renal megalin expression (Figure 4), possibly accounting for the residual renal uptake of ^{111}In-DTPA-octreotide found in the megalin-deficient mice.

The vast extracellular domains of megalin can accommodate a variety of ligands. Therefore, it seems plausible that megalin, together with the multi-ligand receptor cubilin, facilitates uptake of many proteins and peptides, including radiolabelled somatostatin analogues, from the primary filtrate in the kidney. This possibility is supported by the finding of low-molecular-weight proteinuria in megalin-deficient mice [20].

Conclusion

This study revealed the molecular mechanism of ^{111}In-DTPA-octreotide uptake in the renal proximal tubule cells involving the endocytic receptor megalin. Identification of the receptors for tubular uptake of ^{111}In-DTPA-octreotide may be essential for development of new strategies to reduce renal uptake of these radiolabelled peptides.

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Renal uptake of different radiolabelled peptides is mediated by megalin – SPECT and biodistribution studies in megalin-deficient mice

Erik Vegt[1,3]*; Marleen Melis[2]*; Annemarie Eek[1]; Monique de Visser[2]; Maarten Brom[1]; Wim J.G. Oyen[1]; Martin Gotthardt[1]; Marion de Jong[2]; Otto C. Boerman[1]

[1] Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
[2] Department of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands.
[3] Department of Nuclear Medicine, GROW School for Oncology & Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

*these authors contributed equally to the work

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Chapter 2.4

Abstract

Introduction: Radiolabelled peptides used for peptide receptor radionuclide therapy are excreted mainly via the kidneys and are partly reabsorbed and retained in the proximal tubular cells. The resulting high renal radiation dose can cause nephrotoxicity, limiting the maximum activity dose and the effectiveness of peptide receptor radionuclide therapy. The mechanisms of kidney reabsorption of these peptides are incompletely understood, but the scavenger receptor megalin has been shown to play a role in the reabsorption of ^{111}In-octreotide. In this study the role of megalin in the renal reabsorption of various relevant radiolabelled peptides is investigated.

Methods: Groups of kidney-specific megalin-deficient mice and wild-type mice were injected with ^{111}In-labelled somatostatin, exendin, neurotensin or minigastrin analogues. Single photon emission computed tomographic (SPECT) images of the kidneys were acquired and analysed quantitatively, or animals were euthanized 3 h post injection and the activity concentration in the kidneys was measured.

Results: Megalin-deficient mice showed significantly lower uptake of all studied radiolabelled peptides in the kidneys, ranging from 22% (^{111}In-DTPA-octreotide) to 65% (^{111}In-DTPA-exendin) of uptake in wild-type kidneys. Quantitative analysis of renal uptake by SPECT and *ex vivo* measurements showed very good correlation.

Conclusions: Megalin is involved in the renal reabsorption of radiolabelled octreotide, octreotate, exendin, neurotensin and minigastrin. This knowledge can help in the design of strategies to reduce this reabsorption and the resulting nephrotoxicity in peptide receptor radionuclide therapy, enabling more effective therapy. Small animal SPECT is an accurate tool, allowing *in vivo* quantification of renal uptake and serial measurements in individual mice.

Introduction

In peptide receptor radionuclide therapy (PRRT), radiolabelled peptide analogues are used to target tumours expressing particular receptors, such as the somatostatin receptor. Most radiolabelled peptides are predominantly cleared from the body via the kidneys. Rapid clearance of the radiolabelled peptides from the blood and low retention in the kidneys minimise the radiation dose to normal tissues. However, part of the filtered load of these small proteins and peptides is reabsorbed from the ultrafiltrate in the proximal tubules. Evidence suggests that, after glomerular filtration, proteins and peptides in the ultrafiltrate bind to endocytic receptors at the luminal surface of proximal tubular cells and are internalised. Subsequently, the compounds are transferred to the lysosomes, where they are proteolytically degraded into amino acids [1]. These are transported back into the bloodstream. However, residualising radiolabels (e.g. N-terminal amino acid chelate conjugates or lysine chelate conjugates) are trapped in the tubular cell lysosomes and can deliver high radiation doses to the kidney tubules and glomeruli [2]. Nephrotoxicity is dose limiting in PRRT with somatostatin analogues such as ^{90}Y-DOTA,Tyr^3-octreotide [3, 4].

The receptors involved in the tubular reabsorption of peptides have not yet been completely characterised, but for various non-radiolabelled peptides involvement of megalin has been shown [5]. Megalin is a multiligand receptor belonging to the LDL receptor family. The receptor contains four large cysteine rich ligand binding domains and is a high-capacity pathway for the reabsorption of different structurally non-related peptides and proteins such as albumin, vitamin D binding protein, β_2-microglobulin and aprotinin [5, 6]. De Jong et al. recently described that the renal uptake of ^{111}In-octreotide was significantly lower in kidney-specific megalin-deficient mice than in their wild-type counterparts, implicating involvement of megalin in the renal reabsorption of radiolabelled somatostatin analogues [7]. Many other radiolabelled peptides that are being studied for their potential in tumour imaging and PRRT display a renal retention several fold higher than octreotide. Examples include exendin and minigastrin, targeting glucagon-like peptide-1 and cholecystokinin$_2$ receptors, respectively [8, 9]. It is unknown whether megalin is also involved in the renal uptake of these peptides.

In clinical PRRT with somatostatin analogues, the standard renoprotective regimen nowadays consists of co-infusion of basic amino acids, which are thought to interfere with the binding of somatostatin analogues to megalin or other endocytic receptors on the proximal tubular cells [10, 11]. However, Gotthardt et al. showed that the renal uptake of ^{111}In-minigastrin was not reduced significantly by co-infusion of basic amino acids [9], suggesting that the reabsorption of this peptide is mediated by other receptors. Knowledge about the molecular mechanisms of proximal tubular reabsorption of different radiolabelled peptides is important to devise new methods to reduce their renal retention, for example by selecting or designing more efficient inhibitors of their renal reabsorption, or by structurally modifying the peptides to reduce their binding to renal receptors.

In this study the role of megalin in the renal retention of ^{111}In-DTPA,D-Phe^1-octreotide (^{111}In-octreotide), ^{111}In-DTPA,D-Phe^1,Tyr^3-octreotate (^{111}In-octreotate), Lys^{40}(^{111}In-DTPA)-exendin-3 (^{111}In-exendin), ^{111}In-DOTA,Glu^1-minigastrin (^{111}In-minigastrin) and ^{111}In-DTPA^0-neurotensin$^{[6-13]}$

(^{111}In-neurotensin) was studied in mice. The renal uptake of the peptides was measured by single photon emission computed tomography (SPECT) imaging and by *ex vivo* measurements of kidney-specific megalin-deficient mice (megalin$^{lox/lox}$;apoE^{Cre} [12]) and wild-type mice. Since the level of renal uptake of radiopeptides may differ between female and male mice [13], both genders were imaged by SPECT.

Materials and Methods

Animals

The megalin-deficient mice used were megalin$^{lox/lox}$;apoE^{Cre} mice [12]. The principles of creating tissue-specific gene knockout models are described in detail elsewhere [14, 15]. In short, in megalin$^{lox/lox}$;apoE^{Cre} mice, expression of the enzyme Cre recombinase is controlled by the apolipoprotein E (apoE) promotor, and thus produced only in tissues where the apoE gene is transcribed. In these tissues, Cre recombinase excises sequences from the genome that are flanked by two loxP sequences, in this case the megalin gene [14]. Animals were bred locally using males heterozygous in the apoE^{Cre} gene. Offspring expressing the apoE^{Cre} gene were identified by means of polymerase chain reaction (PCR) analysis as described below. Animals not expressing the apoE^{Cre} gene (megalin$^{lox/lox}$ mice) were used as wild-types in the biodistribution studies. In the SPECT studies, C57Bl/6 mice were used as wild-types.

PCR analysis

For PCR the primers CCCAAGAAGAGGAAGGTG (forward) and GCTGGCCCAAATGTTGCTG (reverse) were used. The reaction mixture consisted of approximately 50 ng mouse DNA in a total of 25 µl colourless PCR-buffer (Go Taq Flexi Reaction buffer, Promega) with 5 mM $MgCl_2$ (Promega), 0.5 mM deoxyribonucleotide triphosphate mix (dNTP, Promega), 12.5 pmol forward primer, 12.5 pmol reverse primer and 2.5 IU Taq polymerase (Promega). This mixture was heated for 4 min at 95^{0}C, followed by 32 cycles of 30 s at 95^{0}C, 30 s at 56^{0}C and 30 s at 72^{0}C. After these cycles, the temperature was maintained at 72^{0}C for 10 min and subsequently lowered to 10^{0}C. The formed DNA was analysed by agarose gel electrophoresis.

Radiolabelled compounds

The following peptide derivatives were studied: ^{111}In-DTPA,D-Phe^1-octreotide (Covidien) [16], ^{111}In-DTPA,D-Phe^1,Tyr^3-octreotate (Biosynthema) [17], DTPA^0-neurotensin$^{[6-13]}$ (Biosynthema) [18], Lys^{40}-DTPA-exendin-3 (exendin, Peptide Specialty Laboratories) [19], DOTA-Glu^1-minigastrin (Peptide Specialty Laboratories) [20]. Relevant properties of the peptides are summarized in Table 1.

Peptides were labelled with $^{111}InCl_3$ (20 MBq/nmol for SPECT studies, 10-80 MBq/nmol for *ex vivo* biodistribution studies) after addition of 1.25 M sodium acetate, pH 4.0-4.5 and a mixture of gentisic acid, ascorbic acid and ethanol. Methionine-containing peptides were labelled in the presence of 50 mM L-methionine instead of ethanol. The mixtures were incubated for 20-30 min at room temperature (DTPA-peptides) or at 95^{0}C (DOTA-minigastrin).

Characteristics of studied peptide analogs

Peptide analogue	Target receptor	Molecular weight (kDa)	Number of AA residues (positive / negative)	Charge at pH 7
^{111}In-octreotide	Somatostatin R_2	1.5	8 (1+ / 0-)	+1
^{111}In-octreotate	Somatostatin R_2	1.5	8 (1+ / 0-)	+1
^{111}In-exendin	Glucagon-like peptide R-1	4.8	39 (4+ / 6-)	-2
^{111}In-minigastrin	Cholecystokinin R_2 and Gastrin R	2.1	13 (0+ / 6-)	-6
^{111}In-neurotensin	Neurotensin R	1.3	8 (1+ / 0-)	+1

Table 1: R = receptor, AA = amino acid.

Labelling efficiency and radiochemical purity of the labelled peptides and proteins were determined by silica gel instant thin layer chromatography (SG-ITLC) and reverse phase high performance liquid chromatography (RP-HPLC). Radiochemical purity was >95% for all compounds.

SPECT studies

Megalin-deficient and wild-type mice (male and female, n = 4-6 per group) received an intravenous (i.v.) injection of 40 MBq (0.2 ml, 2 nmol) ^{111}In-octreotide. The exact injected activity was determined by measuring the syringe in a dose calibrator before and after injection. Three and 24 h after injection, a 24 min SPECT scan of the kidney region was acquired with the four-headed helical NanoSPECT/CT system (Bioscan) using Nucline software (v2.01, Mediso). Multi-pinhole mouse collimators with 9 pinholes (1.4 mm diameter) per head were used, with a matrix of 256x256 and 24 projections (2 min per projection). During the scan the animals were isoflurane/O_2 anaesthetized and body temperature was maintained. This procedure was repeated in the same mice with ^{111}In-octreotate, ^{111}In-exendin, ^{111}In-neurotensin and ^{111}In-minigastrin consecutively, after intervals of at least three weeks. Some of the animals developed signs of kidney damage at the end of this series of experiments, probably caused by the relatively high renal uptake of ^{111}In-exendin, resulting in a high radiation dose to the kidneys [21]. Therefore, the ^{111}In-exendin, ^{111}In-neurotensin and ^{111}In-minigastrin data were discarded and an extra experiment with these peptides was carried out in a new set of animals. To reduce the risk of kidney damage, the injected activity of ^{111}In-minigastrin and ^{111}In-exendin was reduced to 10 MBq, while the peptide dose was kept constant at 2 nmol. In this second series, SPECT scans were acquired only at 3 h post injection (p.i.).

SPECT scans were reconstructed iteratively, using InVivoScope software (v1.32, Bioscan) with medium noise reduction, a voxel size of 0.3 mm^3 and standard reconstruction settings. The amount of radioactivity in a volume of interest drawn around the kidneys was quantified and expressed as % of injected activity per gram tissue (%IA/g). To achieve accurate quantification,

the camera was calibrated by scanning a 20 ml polypropylene tube mouse phantom filled with a known amount of ^{111}In activity.

After the final SPECT scan, the animals were euthanized and biodistribution of ^{111}In-minigastrin and ^{111}In-exendin was studied as described in the next section, to confirm the accuracy of the SPECT measurements.

Ex vivo biodistribution studies

Animals (n = 4-6 per group) received an i.v. injection of 0.4 MBq (0.2 ml, 5-40 pmol) ^{111}In-octreotide, ^{111}In-exendin, ^{111}In-neurotensin or ^{111}In-minigastrin. For ^{111}In-octreotide the experiment was performed with male and female mice, for the other peptides only female mice were used. Three hours after injection, the animals were euthanized and organs were dissected. Biodistribution of the ^{111}In-labelled peptides was assessed by weighing the organs and measuring radioactivity in a gamma counter. Measured activity was expressed as %IA/g. The right kidney of each animal was cut in half. One half was snap-frozen in liquid nitrogen and processed for cryosectioning, the other half was processed for paraffin sectioning. Frozen 10-µm sections were mounted on glass slides to perform autoradiography. A phosphor imaging screen was exposed to the sections for two days and scanned using a BAS 1800-II phosphor imager (Fujifilm).

Immunohistochemistry

Frozen 5 µm kidney sections were fixed in 4% formalin for 10 min. After rinsing with 0.05% polysorbate 80 in PBS, the sections were incubated with goat anti-rat megalin polyclonal antibody (SC-16478, Santa Cruz) 10 µg/ml in PBS, 5% BSA for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated donkey anti-goat IgG, $F(ab')_2$ (SC-3851, Santa Cruz) 1/100 for 30 min at room temperature. Peroxidase activity was visualized with diaminobenzidine (Powervision) and nuclei were counterstained using haematoxylin. Slides were dehydrated with ethanol and xylene and embedded in slide mounting fluid (Permount), after which they were studied microscopically. Megalin expression was scored visually by an independent, blinded observer on an arbitrary scale of 0 (negative) to 4 (all tubules positive).

Statistical analysis

Data are presented as mean values ± standard deviation. Comparison of renal uptake values was performed using Student t test. For the SPECT studies of ^{111}In-minigastrin and ^{111}In-exendin after which the mice were sacrificed, the correlation between renal uptake measured on the SPECT images and uptake in the same mice measured *ex vivo* was determined. Spearman's rank correlation coefficient was calculated with SPSS 16.0 (SPSS Inc).

Results

Immunohistochemistry

Immunostaining revealed lower expression of megalin in the kidney cortex of the megalin-deficient mice as compared to the wild-type mice. The expression of megalin in the megalin-

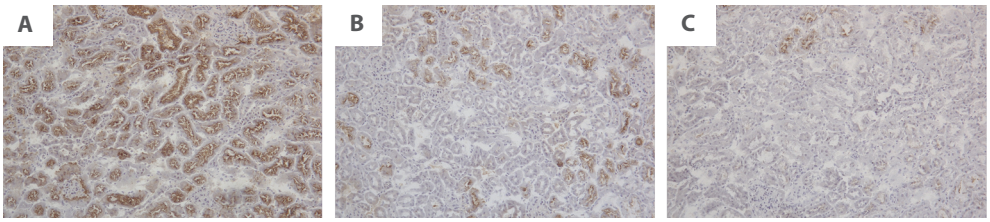

Figure 1: Anti-megalin immunostaining of kidney cortex from a wild-type mouse (A) and two megalin-deficient mice, exhibiting relatively high (B) and relatively low (C) residual expression of megalin

deficient kidneys varied considerably, ranging from almost absent (score 0) to moderate expression (score 2). Examples are shown in Figure 1.

SPECT measurement of renal peptide uptake

As shown in Figure 2, the kidneys were visualized very well on the SPECT scans acquired at 3 h p.i. The images indicated that the radioactivity mainly accumulated in the renal cortex.

The measured renal uptake values of the ^{111}In-labelled peptides as derived from the SPECT images at 3 h p.i. and the ratios of uptake between megalin-deficient mice and wild-type mice are presented in Table 2A. The data are summarised together with the *ex vivo* biodistribution data in Figure 3.

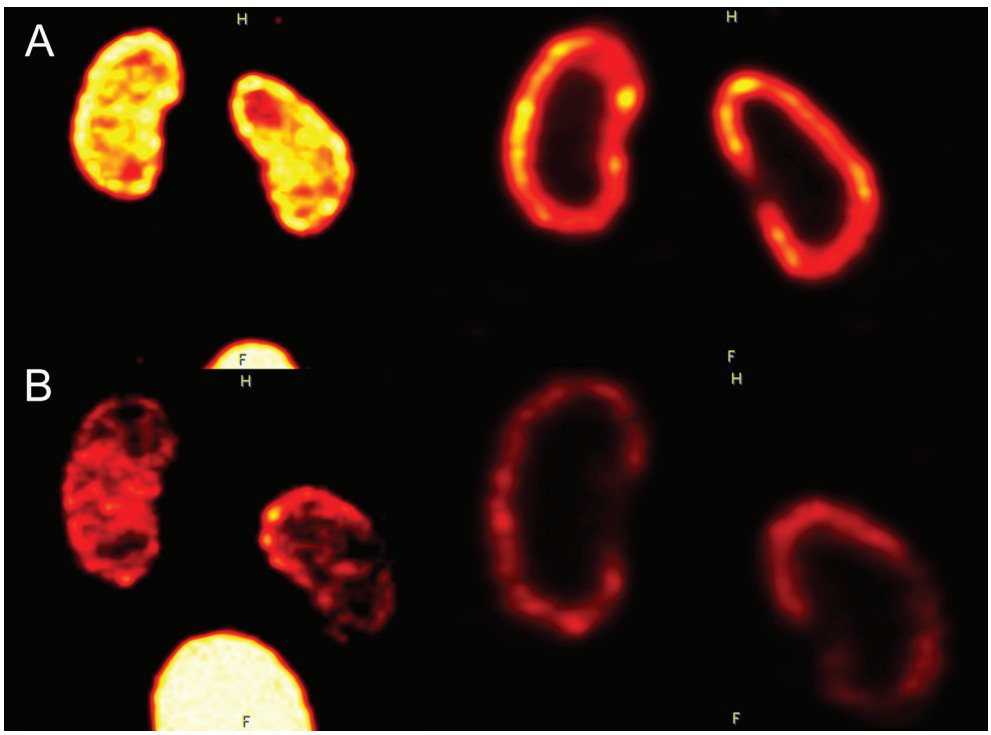

Figure 2: SPECT images of the kidneys of wild-type (A) and megalin-deficient (B) mice, 3 h p.i. of ^{111}In-minigastrin. Maximum intensity projections on the left, coronal slices on the right.

Renal uptake of radiolabelled peptides on SPECT, 3 h p.i.

		Wild-type (%IA/g±SD)	Megalin-deficient (%IA/g±SD)	Ratio megalin-deficient/ wild-type (%)
^{111}In-octreotide	female	17 ± 2.4	8.5 ± 2.6	49% (p=0.001)
	male	22 ± 5.6	5.7 ± 2.0	26% (p=0.0003)
^{111}In-octreotate	female	16 ± 4.6	7.6 ± 2.4	46% (p=0.007)
	male	16 ± 2.8	5.9 ± 1.9	36% (p=0.0001)
^{111}In-exendin	female	371 ± 35	230 ± 58	62% (p=0.003)
	male	328 ± 51	171 ± 34	52% (p=0.001)
^{111}In-minigastrin	female	89 ± 10	33 ± 8.9	37% (p<0.0001)
	male	104 ± 13	52 ± 14	49% (p=0.0003)
^{111}In-neurotensin	female	15 ± 2.7	3.6 ± 1.18	23% (p<0.0001)
	male	18 ± 9.0	4.6 ± 1.1	25% (p=0.02)

Table 2A: Renal uptake of radiolabelled peptides in wild-type and megalin-deficient mice. %IA/g = % injected activity per gram kidney; SD = standard deviation.

^{111}In-exendin expressed the highest renal uptake: 371 ± 35 %IA/g in female wild-type mice. The peptide with the lowest renal uptake (15 ± 2.7 %IA/g in female wild-type mice) was ^{111}In-neurotensin. In the SPECT studies, the renal retention of all ^{111}In-labelled peptides was significantly lower in megalin-deficient mice than in wild-type mice, both in males and females. The effect was most prominent for ^{111}In-neurotensin, for which the renal uptake in female megalin-deficient mice was only 23% of the uptake in wild-type mice (p<0.0001). The least effect was observed with ^{111}In-exendin, for which the uptake in female megalin-deficient mice was 62% of the uptake in wild-type mice (p=0.003).

Renal uptake of radiolabelled peptides on SPECT, 24 h p.i.

		Wild-type (%IA/g±SD)	Megalin-deficient (%IA/g±SD)	Ratio megalin-deficient/ wild-type (%)
^{111}In-octreotide	female	2.2 ± 0.25	1.4 ± 0.47	64% (p=0.0001)
	male	not measurable	not measurable	-
^{111}In-octreotate	female	5.3 ± 0.97	2.5 ± 0.71	47% (p=0.001)
	male	1.6 ± 0.37	0.44 ± 0.20	28% (p=0.0003)
^{111}In-exendin	female	148 ± 11	82 ± 24	55% (p=0.0005)
	male	142 ± 10	69 ± 19	49% (p=0.0001)

Table 2B: Renal uptake of radiolabelled peptides in wild-type and megalin-deficient mice. %IA/g = % injected activity per gram kidney; SD = standard deviation.

Renal uptake of radiolabelled peptides measured *ex vivo*, 3 h p.i.

		Wild-type (%IA/g±SD)	Megalin-deficient (%IA/g±SD)	Ratio megalin-deficient/ wild-type (%)
^{111}In-octreotide	female	38 ± 9.8	8.4 ± 3.5	22% (p=0.0007)
	male	25 ± 12	7.9 ± 2.7	32% (p=0.03)
^{111}In-exendin 2 nmol**	female	331 ± 33	215 ± 34	65% (p=0.001)
^{111}In-exendin 5 pmol**	female	225 ± 35	183 ± 39	81% (ns)
^{111}In-minigastrin	female	66 ± 5.9	17 ± 9.1	26% (p<0.0001)
^{111}In-neurotensin	female	5.1 ± 0.94	1.7 ± 0.18	34% (p<0.0001)

Table 3: Renal uptake of radiolabelled peptides in wild-type and megalin-deficient mice. %IA/g = % injected activity per gram kidney; SD = standard deviation; ns = not significant.
** ^{111}In-exendin was measured *ex vivo* in two experiments: after the final SPECT scan using 2 nmol peptide, and in a separate biodistribution experiment using 5 pmol peptide.

The data measured 24 h p.i. are presented in Table 2B. The renal uptake of the studied radiolabelled peptides remained significantly lower in the megalin-deficient mice, both in females and in males. Overall, the uptake of ^{111}In-octreotide and ^{111}In-octreotate was significantly lower in male mice than in female mice: the retention of ^{111}In-octreotide in males was too low to delineate the kidneys and the uptake of ^{111}In-octreotate was more than threefold lower in males than in females. For ^{111}In-exendin, no difference between the genders was observed.

Ex vivo measurement of renal uptake

The *ex vivo* measured renal uptake of the ^{111}In-labelled peptides in megalin-deficient and wild-type mice is presented in Table 3 and summarized in Figure 3. The renal uptake of ^{111}In-octreotide, ^{111}In-octreotate, ^{111}In-minigastrin and ^{111}In-neurotensin was significantly lower in the megalin-deficient mice than in the wild-type mice. The effect was most prominent for ^{111}In-octreotide, for which the renal uptake in female megalin-deficient mice was only 22% of the uptake in wild-type mice (p=0.0007). For ^{111}In-exendin, no significant difference in renal uptake between both groups was observed when a peptide dose of 5 pmol was used. However, when a higher peptide dose of 2 nmol was administered for SPECT imaging, a significant difference was measured, both on SPECT and *ex vivo* (see Table 3).

Autoradiography of the kidneys revealed a patchy distribution of the ^{111}In-labelled peptides, mainly in the renal cortex (Figure 4) and confirmed lower uptake in the kidneys of the megalin-deficient mice.

Biodistribution of ^{111}In-labelled octreotide, octreotate, neurotensin and minigastrin in organs other than the kidneys did not differ significantly between the wild-type and megalin-deficient mice (data not shown). For the high dose of ^{111}In-exendin (2 nmol), lung uptake in the wild-type animals was significantly higher than in the megalin-deficient mice (1.2 ± 0.18 %IA/g vs. 0.23 ± 0.037 %IA/g in females and 0.98 ± 0.039 %IA/g vs. 0.26 ± 0.034 %IA/g in males, both p<0.0001). However, in the animals that received the low dose of 5 pmol ^{111}In-exendin, no

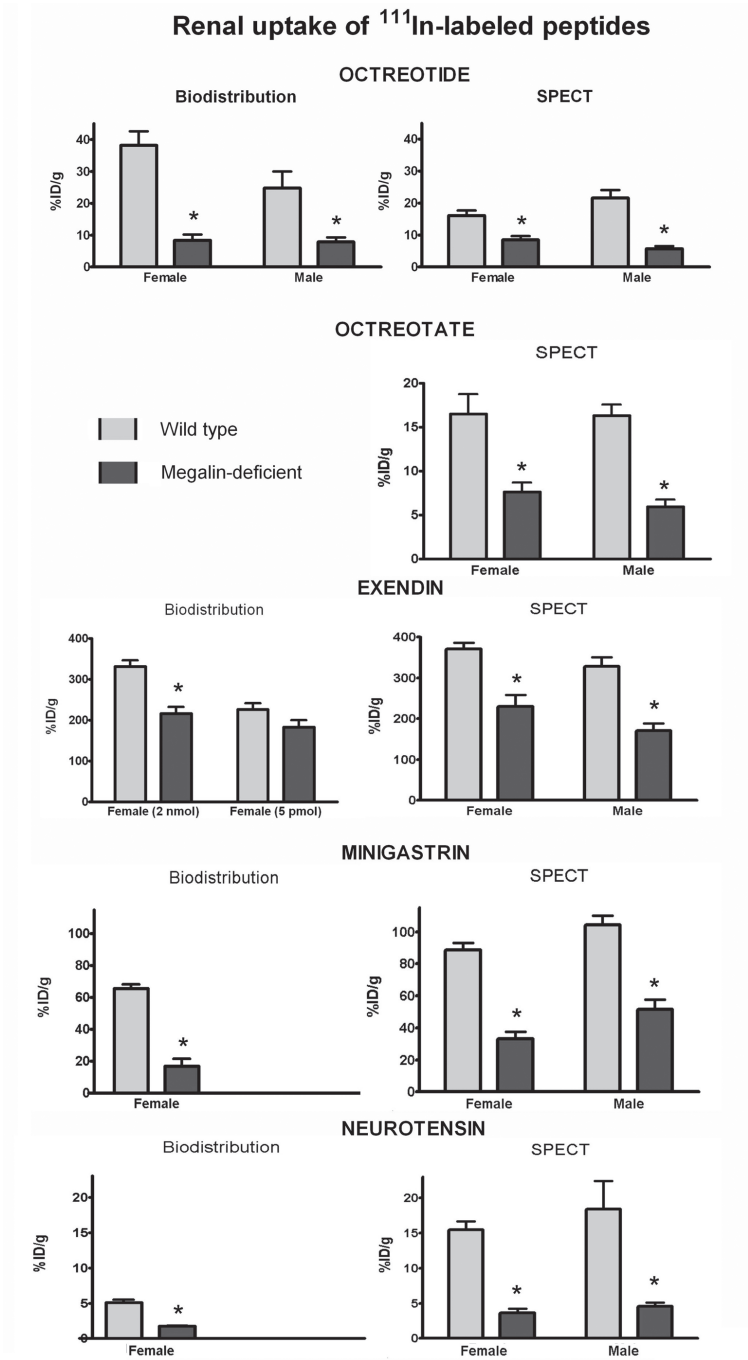

Figure 3: Renal uptake of ^{111}In-labelled peptides in wild-type and megalin-deficient mice, as measured by *ex vivo* biodistribution studies and SPECT 3 h p.i.. Results are presented as mean % ID/g, error bars indicate standard error of the mean. * indicates $p<0.05$

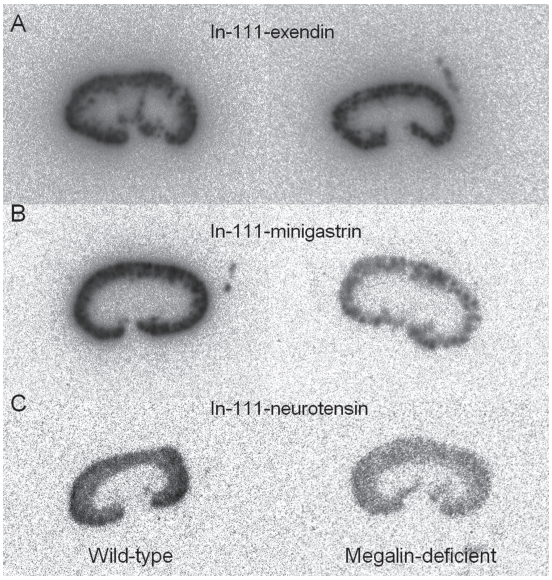

Figure 4: Autoradiography of kidneys of wild-type and megalin-deficient mice that received ^{111}In-exendin (A), ^{111}In-minigastrin (B) or ^{111}In-neurotensin (C).

difference in lung uptake was observed: both in wild-type and in megalin-deficient mice, the lung uptake was 11 %IA/g, much higher than in the animals that received 2 nmol of peptide.

Correlation between biodistribution and SPECT

The correlation between renal uptake values measured with biodistribution and SPECT was very good, as depicted in Figure 5. Spearman's rank correlation coefficient was 0.924 ($r^2 = 0.85$, $p<0.0005$).

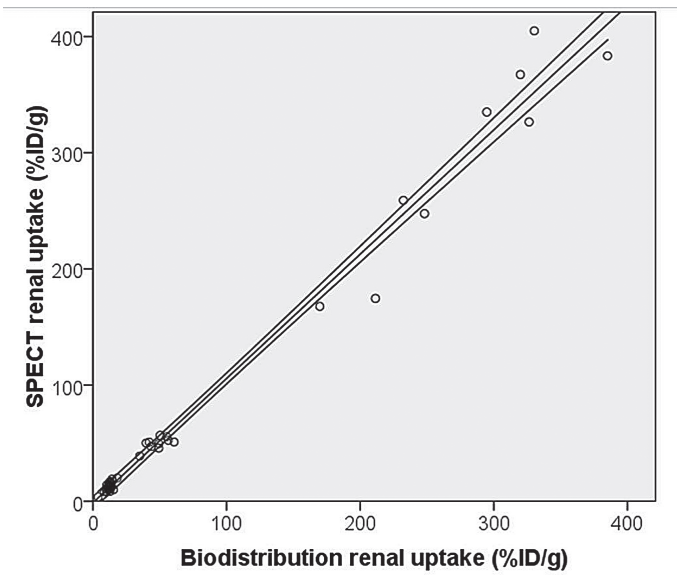

Figure 5: Correlation between renal uptake measured *ex vivo* and uptake measured with SPECT. Data from individual mice that were studied *ex vivo* after the final SPECT-scan (^{111}In-minigastrin and ^{111}In-exendin). Solid lines: linear fitted trend line with 95% confidence interval.

Discussion

In PRRT, the renal retention of radiolabelled peptides can lead to long-term nephrotoxicity, which limits the maximum radiation dose that can safely be administered. Recently, the multi-ligand receptor megalin was shown to be essential for the renal reabsorption of ^{111}In-octreotide [7]. In the present study we show that megalin is also involved in the proximal tubular reabsorption of ^{111}In-octreotate, ^{111}In-minigastrin ^{111}In-neurotensin and probably also of ^{111}In-exendin, and we confirm megalin's role in the reabsorption of ^{111}In-octreotide: the renal uptake of these peptides in megalin-deficient mice was reduced to 23% - 65% of the uptake in wild-type mice.

The remaining renal uptake of the radiolabelled peptides in the megalin-deficient mice may be due to residual megalin expression. The knock-out of megalin expression in the kidneys of these megalin$^{lox/lox}$;apoE^{Cre} mice occurs in a mosaic pattern, with a considerable percentage of tubular cells expressing normal levels of megalin, presumably caused by insufficient expression of Cre recombinase. Leheste et al. initially reported that approximately 10% of the proximal tubular cells of these mice express normal levels of megalin [12]. However, Motoyoshi reported residual megalin expression in 35%-50% of the proximal tubular cells in the same mouse strain [22]. Immunohistochemical staining of kidney sections in the present study confirmed incomplete reduction of megalin expression and considerable variation between individual mice. In addition to residual expression of megalin, part of the residual uptake may also be explained by the involvement of other uptake mechanisms in the reabsorption of these peptides, such as fluid phase endocytosis [23] or ligand-specific receptors. Proximal tubular expression of receptors for somatostatin, glucagon-like peptide-1 and cholecystokinin$_2$ has been described [24-26]. Another multiligand receptor, cubilin, is also involved in the proximal tubular reabsorption of several substances, including peptides and proteins [5, 27]. Cubilin lacks a transmembrane domain and is dependent on other transmembrane proteins such as megalin and amnionless for its internalisation [28, 29]. In megalin-deficient mice, the internalisation of cubilin may also be reduced. Therefore, involvement of cubilin in the reabsorption of the studied radiolabelled peptides cannot be excluded.

The data obtained in the current study with ^{111}In-exendin were paradoxical: significantly reduced renal uptake in megalin-deficient mice was measured when the animals received an imaging dose of 2 nmol, but no significant difference between the two groups was found at a biodistribution dose of 5 pmol ^{111}In-exendin. This paradox is probably caused by the residual megalin expression in the megalin-deficient mice, which may still be able to handle the 5 pmol dose, while it is saturated by the 2 nmol dose. Even though proximal tubular endocytosis is regarded as a low specificity, high capacity pathway, it can be blocked competitively by excesses of ligands [30, 31]. The difference between ^{111}In-exendin and the other peptides in this respect might be explained by a relatively high affinity of ^{111}In-exendin for megalin and/or associated receptors such as cubilin. This may also account for the very high baseline renal uptake of this peptide (225 – 371 %IA/g) and suggests that megalin is indeed involved in the renal reabsorption of ^{111}In-exendin. Since peptide doses used in PRRT are relatively high, the 2 nmol dose reflects the clinical situation better than the 5 pmol dose.

The renal uptake of the radiolabelled peptides at 3 h p.i. was similar in male and female mice. However, at 24 h p.i. the uptake of ^{111}In-octreotide and ^{111}In-octreotate was significantly lower in male mice, which has been described previously [13]. This suggests that the proximal tubular processing and retention of ^{111}In-labelled somatostatin analogues differ between male and female mice, leading to more rapid wash-out of radioactivity in males. However, the ratios of uptake between megalin-deficient and wild-type mice were comparable in both genders at both time points, which suggests that the role of megalin in the reabsorption of radiopeptides is independent of gender influences.

Reduced proximal tubular reabsorption of radiolabelled peptides is expected to cause increased excretion of the radiolabelled peptides in the urine without affecting plasma clearance, since glomerular filtration is not affected and reabsorbed radiolabelled peptides are not returned to the bloodstream. This is confirmed by our observation that the radioactivity concentrations of these radiolabelled peptides in the blood and in most organs other than the kidneys did not differ between megalin-deficient and wild-type mice. The one exception, lower uptake of ^{111}In-exendin in the lungs of megalin-deficient mice, was only observed at the high peptide dose of 2 nmol. At the lower dose of 5 pmol, no difference between megalin-deficient and wild-type animals was observed, and lung uptake was approximately 5-fold to 20-fold higher than in the 2 nmol groups. The lower uptake at higher peptide dose is most likely due to saturation of specific lung uptake, which was also described recently by Brom et al. [19]. The lower lung uptake in the megalin-deficient mice in this group may indicate involvement of megalin in the pulmonary uptake of this peptide. Megalin is expressed in the lungs [32], and this high-capacity receptor may contribute significantly to peptide uptake at higher concentrations, when specific glucagon-like peptide-1 receptor mediated uptake (high affinity but lower capacity) is saturated. Although the megalin$^{lox/lox}$;apoE^{Cre} mice are described to be kidney-specific megalin deficient mice [12], apoE is also expressed in the lungs [33], which may cause reduced expression of megalin in the lungs of these animals. Possible lower expression of megalin in the lungs is unlikely to have affected the renal uptake of the studied peptides, as their concentrations in blood and other organs did not differ significantly between the megalin-deficient and wild-type mice.

In this study the renal uptake measured *ex vivo* and on SPECT images correlated very well. This confirms the previously reported accuracy of the small animal SPECT system [34]. Small animal SPECT is an accurate alternative to biodistribution studies, enabling serial measurements *in vivo* and possibly reuse of animals in multiple experiments. However, SPECT remains much less sensitive than *ex vivo* organ measurement, which means that relatively high radiation doses have to be administered and only organs with a considerable uptake can reliably be quantified. This can lead to complications: in the present study, mice that received a relatively high kidney radiation dose from ^{111}In-exendin developed long-term kidney damage - which is described and analysed in detail elsewhere [21]. This necessitated repeating the experiments in a new set of mice. In addition, high activity doses require administration of a high peptide dose, as the maximum specific activity is limited. Peptide dose can influence a peptide's pharmacokinetics and biodistribution [19, 35]. As outlined above, the higher peptide dose needed for SPECT better reflects the PRRT dose.

Our results indicate that megalin plays an important role in the renal reabsorption of these diverse ^{111}In-labelled peptides. The variety of radiolabelled peptides and other ligands that are taken up via megalin suggests that it might also be involved in the renal reabsorption of other radiolabelled peptides. Insight into the role of megalin in the renal uptake of these radiolabelled peptides can help to develop new strategies for the reduction of renal peptide reabsorption, thus reducing the risk of nephrotoxicity after PRRT. For example, administration of an excess of other megalin ligands can reduce the uptake of radiolabelled peptides by competitive binding to megalin. Our group recently showed that co-injection of peptides derived from albumin, an important ligand of megalin, reduces the renal uptake of ^{111}In-labelled octreotide, exendin and minigastrin in rats [36]. Succinylated gelatin, a peptide-based plasma expander, has also been shown to interfere with proximal tubular reabsorption: a transient low molecular weight proteinuria was reported after administration of Gelofusine, with urinary loss of β_2-microglobulin, a ligand of megalin [5, 30, 37]. We previously found that co-administration of succinylated gelatin also reduced the renal uptake of ^{111}In-labelled octreotide, exendin and minigastrin in rats and of ^{111}In-octreotide in humans [38-40]. Other compounds that have been studied for the reduction of renal uptake of radiolabelled peptides are less universally applicable than succinylated gelatin and albumin. Lysine, a positively charged amino acid, is used clinically to reduce nephrotoxicity of somatostatin analogue PRRT [10]. However, Gotthardt et al. showed that lysine did not reduce the renal uptake of negatively charged minigastrin analogues, whereas negatively charged polyglutamic acids did reduce uptake of ^{111}In-minigastrin, but not of the positively charged ^{111}In-octreotide [9]. These observations suggest that different mechanisms are involved in the uptake of these radiolabelled peptides and seems to contradict our present findings that megalin is involved in the renal uptake of both peptides. However, megalin contains four large binding domains [6], and lysine and polyglutamic acid may bind selectively to distinct regions of these domains, thereby only interfering with the binding of specific radiolabelled peptides. The peptide mixtures in succinylated gelatin and albumin fragments are more likely to block several binding regions on the megalin receptor, thereby interfering with the binding and uptake of several different radiolabelled peptides.

In conclusion, the multiligand receptor megalin plays an important role in the renal reabsorption of ^{111}In-labelled octreotide, octreotate, minigastrin, exendin and neurotensin. This knowledge can be used to develop new methods to reduce the renal retention of these peptides, thus reducing the risk of nephrotoxicity and improving the possibilities for safe and effective PRRT.

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Reduction of renal uptake of radiolabelled octreotate and kidney protection

Chapter 3

Dose-response effect of Gelofusine on renal uptake and retention of radiolabelled octreotate in rats with CA20948 tumours

Marleen Melis[1], Magda Bijster[1], Monique de Visser[1], Mark W. Konijnenberg[2], Jan de Swart[1], Edgar J. Rolleman[1], Otto C. Boerman[3], Eric P. Krenning[1] and Marion de Jong[1]

[1] Erasmus MC, dept. of Nuclear Medicine, Rotterdam, The Netherlands
[2] Research & Development, Covidien, Petten, The Netherlands
[3] UMC St. Radboud, dept. of Nuclear Medicine, Nijmegen, The Netherlands

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Chapter 3.1

Abstract

Introduction: Peptide receptor radionuclide therapy using β⁻-emitting radiolabelled somatostatin analogues like DOTA,Tyr³-octreotate, shows beneficial results in patients suffering from somatostatin receptor overexpressing tumours. However, after high-dose therapy partial renal reabsorption of radiopeptides may lead to nephrotoxicity. Co-infusion of lysine/arginine lowers renal retention of these radiopeptides without affecting tumour uptake. Recently co-administration of Gelofusine has been described to have a comparable kidney-protecting effect in rats. In the present study optimal dosing of Gelofusine co-administration was studied in tumour-bearing rats.

Methods: Doses of 40, 80, 120 or 160 mg/kg Gelofusine were co-injected with 15 µg DOTA,Tyr³-octreotate, labelled with 3 MBq ^{111}In for biodistribution (24 h post injection, n = 4 per group) and with 60 MBq ^{111}In for micro-SPECT imaging experiments at 3 h, 24 h and 48 h post injection. An additional group of rats received 80 mg/kg Gelofusine plus 400 mg/kg lysine co-injection. Biodistribution studies were performed both in older (475 g) and younger (300 g) rats, the latter bearing CA20948 tumours.

Results: Co-injection of 40 mg/kg Gelofusine resulted in 40-50% reduction of renal uptake and retention of ^{111}In-DOTA,Tyr³-octreotate, whereas higher doses further increased the reduction to 50-60% in both groups of rats. Combining Gelofusine and lysine caused 70% reduction of renal uptake. The uptake of radiolabelled octreotate both in somatostatin receptor-expressing normal tissues and tumours was not affected by Gelofusine co-injection.

Conclusions: In rats co-injection of 80 mg/kg Gelofusine resulted in maximum reduction of renal retention of ^{111}In-DOTA,Tyr³-octreotate, which was further improved when combined with lysine. Tumour uptake of radiolabelled octreotate was not affected, resulting in an increased tumour to kidney ratio.

Introduction

The treatment of patients with somatostatin receptor-positive tumours with peptide receptor radionuclide therapy (PRRT) has shown convincing beneficial effects [1, 2]. The radiolabelled peptides are rapidly cleared via the glomeruli in the kidneys into the urine, but a low percentage is reabsorbed and retained in the cortical proximal tubules [3, 4]. After glomerular filtration a fraction of the administered peptides is internalized via endocytic receptors, in this process megalin has been demonstrated to play an essential role [5, 6]. Transfer of the radiopeptides to the lysosomes is followed by degradation of the peptide, after which amino acid chelate conjugates are trapped in the lysosomes of the tubular cells [7], delivering a high radiation dose to the renal cortex during PRRT. The maximum tolerated dose in PRRT in patients is not exactly known. However, from data from external beam radiation therapy a limit of 23 Gy on the kidney has been adapted as the upper limit of the dose that can be administered safely [8]. Because of the differences with external beam irradiation individual dosimetry has been introduced recently in PRRT. This is to correct for the characteristic features of the radionuclide and of the patient, like inhomogeneous intra-organ distribution, shorter penetration range, dose rates following exponential decay and patient-specific geometry. Calculation of the biological equivalent dose (BED), with a correction for CT-assessed kidney volume and dose fractionation, has been adapted resulting in more accurate prediction of kidney toxicity. Using an upper limit of a BED of 40-45 Gy has been described to be safe to prevent nephrotoxicity during PRRT [9-11]. Co-infusion of lysine and arginine (Lys/Arg) has become a standard procedure in our institution during PRRT with ^{177}Lu- or ^{90}Y-labelled somatostatin analogues, reducing the renal retention of the radiopeptides with approximately 35% [12-14]. These positively charged amino acids probably interfere in the megalin-mediated reabsorption process [4]. As a result higher radioactivity doses can be administered without risk of nephrotoxicity. In some cases, however, amino acid infusion may lead to nausea and vomiting, sometimes even to hyperkalaemia [13].

Recently, the reduction of renal retention of radiopeptides by co-infusion of the plasma expander Gelofusine was described [15, 16]. A transient low molecular weight proteinuria [17, 18] induced by Gelofusine led to a 40% reduction of renal reabsorption of radiolabelled octreotide, both in animals [15, 19, 20] and in humans [16]. The combination of both Lys and Gelofusine appeared to have an additive effect on the reduction of renal uptake of radiolabelled somatostatin analogues [19, 20]. This pointed to different mechanisms of interfering in the reabsorption process in the renal proximal tubules.

Most of these experiments have been performed with a fixed dose of Gelofusine. The aim of the current study was to investigate the dose-response effect of Gelofusine on renal retention of the ^{111}In-labelled somatostatin analogue DOTA,Tyr^3-octreotate. Biodistribution studies were performed in rats bearing CA20948 tumours expressing somatostatin receptors. Co-infusion of Lys has been described to have no effect on the tumour uptake of radiolabelled somatostatin analogues [21]; the effect of Gelofusine (with or without Lys) on receptor-mediated tumour uptake has not been described yet.

Materials and methods

Radionuclides, peptide, chemicals

$^{111}InCl_3$ was purchased from Covidien (Petten, The Netherlands). DOTA,Tyr^3-octreotate was obtained from BioSynthema (St Louis, MO, USA). Radiolabelling was performed according to previously published procedures [22]. The labelling efficiency exceeded 99%, as confirmed by thin-layer chromatography [22]. Specific activity of ^{111}In-DOTA,Tyr^3-octreotate was 3 MBq/15 µg peptide for biodistribution studies and 60 MBq/15 µg peptide for NanoSPECT imaging experiments. Gelofusine (40 g/l) was obtained from Braun Medical (Oss, The Netherlands) and L-lysine from Sigma (Zwijndrecht, The Netherlands). Shortly before use a 400 mg/ml L-lysine solution in saline was prepared.

Biodistribution experiments

Animal studies were conducted in agreement with the Animal Welfare Committee requirements of our institution using generally accepted guidelines. For all experiments male Lewis rats (Harlan, Horst, The Netherlands) were used (n=4 per group for biodistribution studies). A group of young, slim rats was used at 14 weeks of age (mean body weight of 300 g), and a group of old, heavier rats at 45 weeks of age (mean body weight of 475 g) at the time of biodistribution.

The young rats were subcutaneously inoculated with 500 µl of a CA20948 tumour cell suspension [23], prepared from 5 g crude tumour tissue in 100 ml saline, at both sides in the shoulder region. On day 24 after inoculation the biodistribution and imaging experiments were performed.

For biodistribution studies rats were anaesthetized with isoflurane/O2 and injected with Gelofusine (or saline in control animals) intravenously via the tail vein. The volume ranged from 300 µl to 2.000 µl, depending on the administered dose ranging from 40 mg/kg to 160 mg/kg. In the experiment with the young rats one extra group received both 80 mg/kg Gelofusine and 400 mg/kg L-lysine. The injection of Gelofusine was immediately followed by the administration of ^{111}In-DOTA,Tyr^3-octreotate. A peptide dose of 15 µg was used because this amount is required to administer the optimal activity dose of 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate in CA20948 tumour-bearing rats [21]. For the biodistribution studies 3 MBq ^{111}In-DOTA,Tyr^3-octreotate/15 µg in 250 µl was injected via the dorsal vein of the penis, whereas for imaging of the young tumour-bearing rats, one extra rat per group received 60 MBq ^{111}In-DOTA,Tyr^3-octreotate/15 µg in 250 µl.

After euthanasia at 24 h post injection (p.i.), organs and tumours were dissected and blood samples were taken. Organs and tumours were weighed and radioactivity was measured in a gamma counter (Wallac, 1480 Wizard 3", Perkin Elmer, Turku, Finland). Uptake of radioactivity was expressed as percentage of injected activity per gram tissue (%IA/g). Data were expressed as percentage of control. For autoradiography one kidney and the tumours of each animal were frozen embedded in OCT compound (Tissue Tek, Sakura, Zoeterwoude, The Netherlands) using isopentane cooled in liquid nitrogen.

MicroSPECT/CT imaging

One additional rat from each group of the CA20948 tumour-bearing rats was imaged with the four-headed multi-pinhole NanoSPECT/CT camera (Bioscan Inc., Washington DC, USA) [24]. Rats were anaesthetized with isoflurane/O_2. Nine pinhole apertures with a diameter of 2.5 mm were used on each head, with a field of view (FoV) of 24 mm. Settings of the ^{111}In energy peaks were 171 and 245 keV. Based on the CT topogram, a body range of 85 mm ranging from neck to bottom was scanned in 24 min with 60 s per projection. A 6 min CT at 45 kV_p was acquired. Animals were imaged at 3, 24 and 48 h p.i. to measure the retention of ^{111}In-DOTA,Tyr^3-octreotate and allow calculation of the kidney and tumour dose.

Using the InVivoScope software quantification of the amount of radioactivity in the volume of interest (VOI) of kidneys and tumours was performed. Each tumour nodule inside a CA20948 tumour was analysed separately. The amount of radioactivity in the VOI was expressed in MBq/ml tumour.

To achieve accurate quantification, the camera was calibrated by scanning a phantom, representing the attenuation of rats, filled with a known amount of ^{111}In activity.

Ex vivo autoradiography

Frozen kidneys and CA20948 tumours were cut into sections of 10 µm (Microm Cryo-Star HM 560 M, Walldorf, Germany). Autoradiographs of the sections were made by exposing them to SR phosphor imaging screens (Perkin Elmer, Groningen, The Netherlands) in X-ray cassettes. After 24-72 h screens were read by a Cyclone phosphor imager and analysed with OptiQuant 03.00 image processing system (Perkin Elmer, Groningen, The Netherlands). After exposure the sections were stained with haematoxylin and eosin (HE). Based on the results of this staining regions of interest (ROI) were determined. OptiQuant software was used to quantify the intensity of radioactivity and expressed in digital light units (DLU)/mm^2. Of each tumour five sections were analysed and at least five ROI per section.

Dosimetry

The dosimetry was based on the MIRD schema to calculate the dose by the product of the organ residence times and the S values for dose per cumulated activity. Radiation doses to the kidneys and CA20948 tumours were determined. The residence time of ^{111}In was determined by the kinetics of the radioactivity retention at three time points after administration. The kidney S values were calculated as described earlier [25], adapted to the actual mass of the kidneys, and the absorbed dose in mGy/MBq was calculated. This dose was calculated separately for the whole kidney and for the cortex, because the majority of the radioactivity was retained in the cortex containing glomeruli and proximal tubules. Only the self-absorption dose from activity uptake in the kidneys was calculated. Tumour S values also were dependent on the actual mass of each tumour. Olinda/EXM software was used for calculation of the absorbed dose in mGy/MBq [26].

Statistics

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student t test.

Results

In rat biodistribution studies the renal uptake of 15 mg ^{111}In-DOTA,Tyr^3-octreotate was examined, without or with co-injection of increasing doses of Gelofusine (ranging from 40 to 160 mg/kg). The retained amount of radioactivity without Gelofusine co-administration was 4.6 ± 0.3 or 4.1 ± 0.1 % IA/g in the young and older rats, respectively, at 24 h p.i.. A dose of 40 mg/kg Gelofusine significantly reduced the renal retention of ^{111}In-DOTA,Tyr^3-octreotate. (Figure 1A, B). This lowest dose of Gelofusine induced a reduction of renal uptake of 40% in young

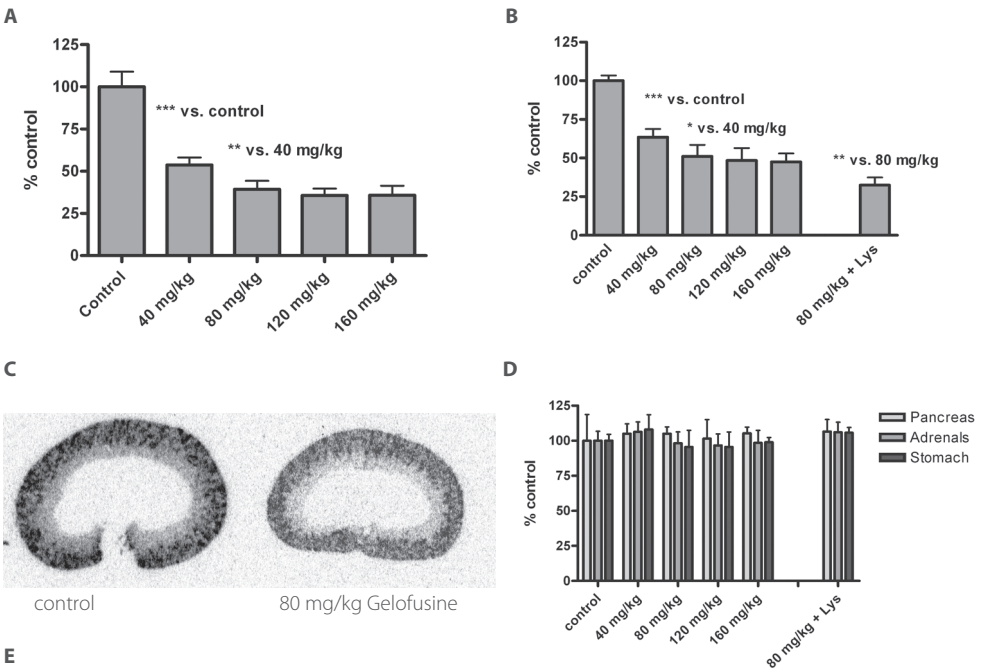

Figure 1: Biodistribution at 24 h p.i. of 15 µg ^{111}In-DOTA,Tyr^3-octreotate in rats, without or with co-injection of Gelofusine in increasing doses ranging from 40 to 160 mg/kg. In young rats Gelofusine 80 mg/kg in combination with Lys 400 mg/kg was tested as well. Uptake was calculated as % injected activity/gram (%IA/g). The %IA/g of the control group was set at 100%, values obtained in Gelofusine groups were expressed as percentage of control values; n = 4 per group.
A: Renal retention in 45-week-old rats with a mean body weight of 475 g
B: Renal retention in 14-week-old rats with a mean body weight of 300 g
C: Kidney sections after *ex vivo* autoradiography demonstrating localization of ^{111}In-DOTA,Tyr^3-octreotate 24 h p.i. in a representative kidney from the control and Gelofusine 80 mg/kg group of the 45-week-old rats
D: Uptake in pancreas, adrenals and stomach in 14-week-old rats
E: Uptake in CA20948 tumours in 14-week-old rats
* p <0.05; ** p <0.001; *** p <0.005

and of 50% in older rats. When a dose of 80 mg/kg was given a further reduction of uptake in the kidneys was found of 50% and 60%, respectively, compared to controls. With the 120 and 160 mg/kg Gelofusine doses no further reduction of the renal retention was measured; therefore the 80 mg/kg dose seemed sufficient to induce the maximum achievable reduction of renal retention of ^{111}In-DOTA,Tyr^3-octreotate. The maximum total volume of Gelofusine and radiopeptide that was administered to the heavy rats was more than 2000 µl; no side effects were observed.

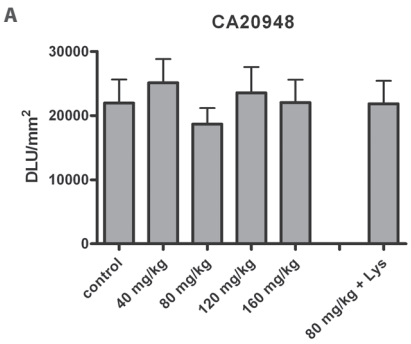

Figure 2: *Ex vivo* autoradiography of CA20948 after biodistribution study with ^{111}In-DOTA,Tyr^3-octreotate, without or with co-injection of increasing doses of Gelofusine.
A: Quantification of intensity of retained radioactivity in frozen sections of CA20948 tumours after *ex vivo* autoradiography of which the biodistribution results are shown in Figure 1E. Amount of radioactivity is expressed in DLU/mm^2. Only regions containing tumour cells were analysed, as demonstrated in the HE stained section. No significant differences were found.
B: Examples of CA20948 tumour sections after *ex vivo* autoradiography.

Ex vivo autoradiography of kidney sections demonstrated that the localization of the radioactivity was not affected by addition of Gelofusine (Figure 1C); radioactivity mainly localized in the cortex and to a lesser extent in the outer medulla. When the 80 mg/kg Gelofusine dose was combined with Lys co-administration a significant additive effect on reduction of renal uptake of radiolabelled octreotate was measured; 68% reduction compared to control values was achieved (Figure 1B). In the biodistribution study in the young rats it was found that the administration of large amounts of Gelofusine, with or without Lys, did not affect the uptake of ^{111}In-DOTA,Tyr^3-octreotate in somatostatin receptor-expressing organs (Figure 1D) and subcutaneously grown somatostatin receptor-expressing CA20948 pancreatic tumours (Figure 1E). Quantification of retained radioactivity in CA20948 tumour sections in *ex vivo* autoradiograms confirmed that Gelofusine co-administration had no significant effect on tumour uptake of radiolabelled octreotate (Figure 2A, B).

Tumour to kidney activity uptake ratios at 24 h p.i. of the control, the Gelofusine 80 mg/kg and the combination of Gelofusine and Lys situation were 0.18 ± 0.08, 0.30 ± 0.12, 0.38 ± 0.23 respectively. This was also nicely visualized in the NanoSPECT/CT images of CA20948 tumour-bearing rats (Figure 3A) [27]. Quantification of the tumour uptake of ^{111}In-DOTA,Tyr^3-octreotate 24 h p.i. in the images revealed again that increasing doses of Gelofusine did not affect tumour uptake of radiolabelled octreotate expressed in MBq/ml tumour (Figure 3B).

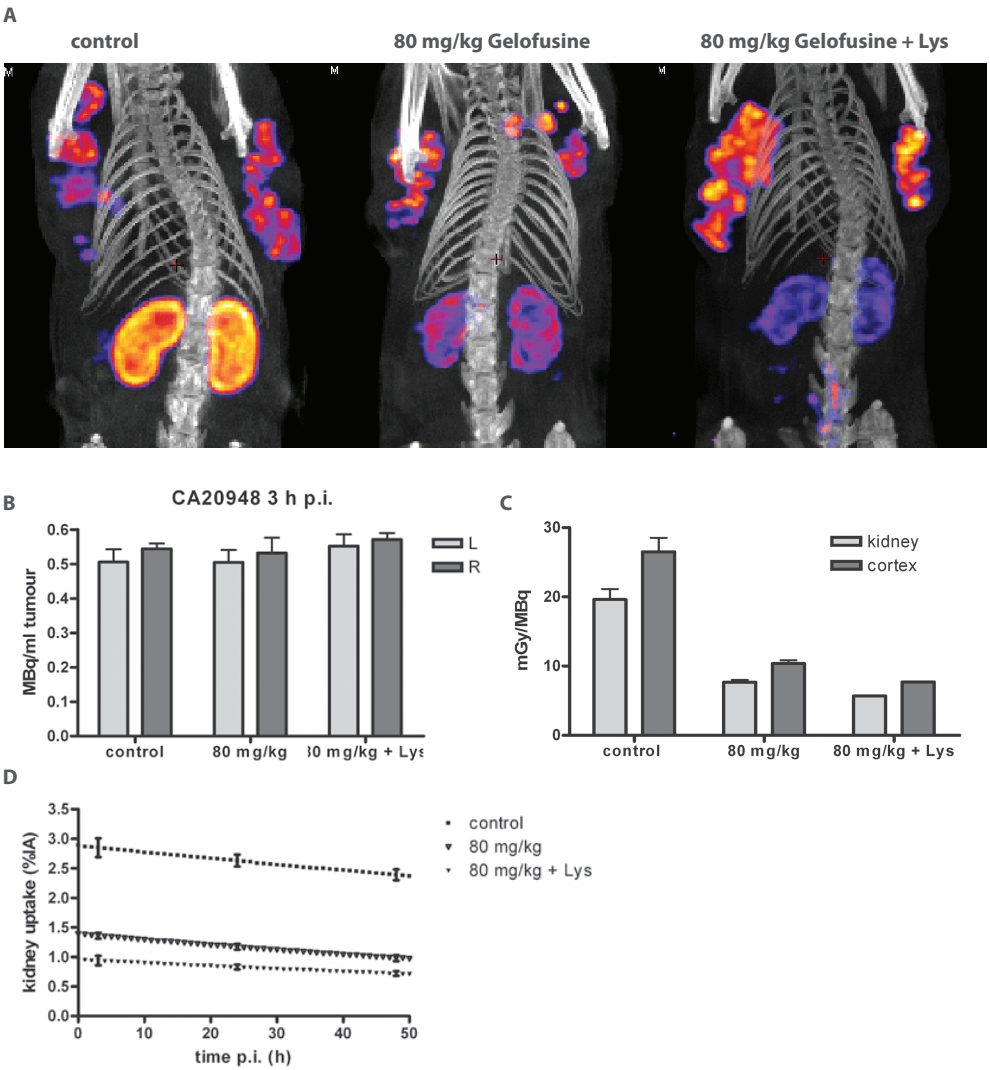

Figure 3: NanoSPECT/CT imaging, quantification and dosimetry.
A: NanoSPECT/CT images of CA20948 tumour-bearing rats, 3 h p.i. of 15 µg ^{111}In-DOTA,Tyr^3-octreotate, labelled with 60 MBq of ^{111}In, without or with co-injection of Gelofusine 80 mg/kg (and 400 mg/kg Lys). Earlier published in [27].
B: Quantification of retained radioactivity in CA20948 tumours of imaged rats using InVivoScope software. Amount of radioactivity was expressed in MBq/ml tumour. Each tumour nodule inside a CA20948 tumour was analysed separately. No significant differences were found.
C: Residence time of ^{111}In in kidneys, as determined in NanoSPECT/CT images at 3, 24 and 48 h p.i. of 15 µg ^{111}In-DOTA,Tyr^3-octreotate. Wash-out of ^{111}In was plotted for three rats: control, with Gelofusine 80 mg/kg alone and combined with 400 mg/kg Lys.
D: Dose calculation of ^{111}In-DOTA,Tyr^3-octreotate for whole kidney or renal cortex only, expressed in mGy/MBq ^{111}In. Renal radiation dose in a control rat is compared with rats receiving Gelofusine 80 mg/kg alone or combined with 400 mg/kg Lys as co-administration.

Images of the rats acquired at 3, 24 and 48 h p.i. were analysed to determine the kidney and tumour dosimetry. The kinetics of the washout of ^{111}In-DOTA,Tyr^3-octreotate from the kidneys and CA20948 tumours were similar in the control and the Gelofusine (+ Lys) groups; kidney data are shown in Figure 3C. After determination of the correct S values based on the actual masses of both kidneys and tumours, the absorbed doses were calculated. The dose on the renal cortex in the rats that received Gelofusine 80 mg/kg appeared to be 40% of the dose in the rats without renal protection, whereas the dose in the rats that received the combination of Gelofusine with Lys was only 30% of the dose in the control group as shown in Figure 3D. The total absorbed radiation dose caused by ^{111}In to the renal cortex in the control group was 1.7 ± 0.1 Gy, whereas the doses to the CA20948 tumours varied between 0.3 and 0.75 Gy and was independent of administration of renal protection agents.

Discussion

Reduction of renal retention of radiolabelled somatostatin analogues is an important issue in PRRT, since after treatment with therapeutic doses of ^{90}Y-labelled octreotide or octreotate nephrotoxicity has been described [28, 29], especially when renal doses exceeding 23 Gy were applied [14, 30]. The partial reabsorption of somatostatin analogues can be reduced by co-infusion with the cationic amino acids lysine and arginine during the administration of therapeutic doses of ^{90}Y- or ^{177}Lu-labelled somatostatin analogues. This is a commonly used method nowadays, resulting in about 35% reduction of renal uptake of radioactivity [2, 13]. Approximately 30% of the patients suffer from nausea and 15% from vomiting as well during the 4 h infusion of 2.5% lysine and 2.5% arginine in 1 l of saline, in spite of administration of the antiemitic granisetron [31]. Hyperkalaemia has not been demonstrated with these amounts of amino acids, but was only found in patients receiving a total dose of 75 g lysine [13]. Therefore, increasing the Lys dose is no option to improve the reduction of renal uptake. Research to further reduce renal retention of such high-energy radiolabelled peptides is warranted to increase the therapeutic window of PRRT.

The net charge of radiolabelled peptides is an important factor influencing renal retention [32]. The Lys residue in octreotide and octreotate seems responsible for the relatively high uptake in kidney. In a preclinical study comparing different neurotensine analogues it was shown that Lys co-administration also reduced renal retention of other radiolabelled peptides, but this only occurred when a Lys residue formed part of the peptide [33]. This points to a competitive mechanism which has been revealed to be, at least partly, a megalin-mediated process. This negatively charged multi-ligand scavenger receptor which is expressed on renal proximal tubules plays a key role in the endocytosis of radiolabelled somatostatin analogues [4, 6].

Gelofusine is a succinylated gelatine consisting of polypeptides with an average molecular weight of 30 kDa. It is used as a plasma expander in critically ill patients. ten Dam et al. described that after Gelofusine infusion an increased amount of α_1- and β_2-microglobulin was excreted in the urine, pointing to a disturbed protein reabsorption process [17]. Based on this

observation Gelofusine was co-administered with radiolabelled somatostatin analogues and offered a reduction of renal uptake of radiolabelled octreotide to an extent comparable to that of cationic amino acids, as recently described in animals and in healthy volunteers [15, 16]. The exact mechanism of the reducing effect of Gelofusine is still unknown. When the renal uptake of several non-somatostatin peptide analogues was studied, it appeared that their renal retention could be reduced as well, whereas in the cases of gastrin and exendin analogues Lys co-injection had no effect [19, 34]. Gotthardt et al. suggested that Lys and poly-glutamic acid only interfere with one of the four clusters of anionic amino acid repeats of megalin responsible for binding of a variety of ligands, while Gelofusine administration acts on all four clusters enabling reduced retention of all radiolabelled peptides tested thus far [19, 35].

To further reduce uptake and retention of radioactivity in the kidneys the combination of both agents was tested, demonstrating an additive effect. This combination of agents is promising, because more cycles of therapeutic doses could be safely administered in clinical PRRT using this combination without exceeding the mentioned 23 Gy absorbed kidney dose or 45 Gy BED limit. Until now in most experiments in rats and mice a fixed dose of Gelofusine was used [15, 19]. In the study of Rolleman et al. [20], a limited dose-response effect was suggested in the range between 50 and 80 mg/kg. In the current study this observation was confirmed. 40 mg/kg Gelofusine caused already a reduction of renal retention of ^{111}In-DOTA,Tyr^3-octreotate, which was further increased with a 80 mg/kg dose. These doses were independent of the body weight of the animals, since comparable results were obtained in both groups without significant difference. The 80 mg/kg dose appeared to be the dose being sufficient to induce maximum reduction of renal retention, since higher doses did not improve the reduction of kidney uptake of radiolabelled octreotate. Apparently maximal induction of proteinuria and/or blocking of receptors responsible for endocytosis already is achieved at this 80 mg/kg Gelofusine dose.

Comparing literature data with the currently described results, it is clear that the percentage of reduction of renal uptake by Gelofusine co-administration in a 80 mg/kg dose is comparable for all somatostatin analogues tested (^{111}In-DTPA,Tyr^3-octreotide, ^{177}Lu-DOTA,Tyr^3-octreotate, ^{111}In-DOTA,Tyr^3-octreotate) and is independent of the administered amount of peptide (0.5 mg, 15 mg), while the % IA/g kidney of control animals ranged from 1 to 4% IA/g [15, 19, 20].

The potential effect of Gelofusine on tumour uptake of ^{111}In-DOTA,Tyr^3-octreotate was investigated as well. The biodistribution experiment showed no interference of Gelofusine with radioactivity uptake in CA20948 tumour, although the tumour uptake varied largely due to the irregularly shapes and structures of the tumours, whereas the mean volume of the tumours was similar in all groups, although with a high variability. When radioactivity in separate tumour nodules in the frozen sections was quantified using *ex vivo* autoradiography, variability of tumour uptake was smaller and mean DLU/mm^2 was not reduced when Gelofusine was co-administered. Furthermore, the recently developed method to quantify the amount of radioactivity which is retained in imaged organs and tumours using microSPECT/CT, also confirmed that tumour uptake of ^{111}In-DOTA,Tyr^3-octreotate was not influenced by Gelofusine with or without Lys [20, 24].

Quantification of the retained renal radioactivity by imaging the same rat at several time points after injection of the radiolabelled octreotate allowed estimation of the radiation dose to the kidneys and tumours. The clearance rate of ^{111}In-DOTA,Tyr^3-octreotate from the kidneys and the tumours was similar in the control and in the renal protection groups. This confirms that Gelofusine and Lys had a transient effect on the renal reabsorption mechanism and only interfered in the initial retention of radiopeptides in the proximal tubules, but had no effect on the wash out of the radiometals. The induced reduction in radiation dose to the whole kidney or to cortex alone was comparable to the reduction found in the biodistribution study: up to 70%, which is very favourable for kidney protection when therapeutic radionuclides like ^{177}Lu or ^{90}Y will be applied.

Taken together our results indicating significant renal protection but unaffected uptake in somatostatin receptor-expressing tumours and organs, warrant a randomized cross-over trial to test the combination of amino acid infusions with or without Gelofusine in patients receiving PRRT with ^{177}Lu-DOTA,Tyr^3-octreotate in our centre.

It is hard to translate our reported optimal dose of 80 mg/kg Gelofusine for renal uptake reduction of ^{111}In-DOTA,Tyr^3-octreotate in rats, which was administered as a bolus injection, to the clinical situation where Gelofusine will be co-infused during several hours. The Gelofusine dose that was administered in the healthy volunteers was a bolus of 40 mg/kg per 10 min followed by a 3 h infusion of 0.8 mg/kg per min (144 mg/kg), resulting in a total dose of almost 200 mg/kg over a 3-h period of time [16]. In Bad Berka 450 ml of 4% Gelofusine solution was infused in 4 h combined with Lys/Arg, during PRRT, which means a dose of 225 mg/kg per 4 h for a patient with a body weight of 80 kg [36].

A drawback of the use of Gelofusine might be the risk of an allergic reaction in less than 1% of the patients, probably because of its bovine origin [37, 38]. Therefore patients need to be strictly monitored. When a significantly reduced renal retention of radioactivity can be obtained with extra Gelofusine administration, an adapted regimen to protect kidneys from renal toxicity during PRRT may be used in future. This will allow administration of more cycles of PRRT without risk of nephrotoxicity, while a higher tumour radiation dose will be achieved.

Due to crossfire from circulating radioactivity in the blood the bone marrow is the second organ at risk during PRRT. The clearance of radioactivity from the blood was not delayed by the administration of Gelofusine and Lys. Therefore, especially when the cumulative dose will be increased further, monitoring of blood cell counts is needed to detect haematological toxicity, like cytopaenia or myelodysplastic syndrome [2].

Conclusion

In rats a Gelofusine dose of 80 mg/kg body weight induced maximal reduction of 50-60% of renal retention after administration of a therapeutic peptide dose of 15 mg of ^{111}In-DOTA,Tyr^3-octreotate. Combination of this dose of Gelofusine with 400 mg/kg L-lysine resulted in a 70% reduced kidney uptake, while the somatostatin receptor-specific uptake in pancreas, stomach, adrenals and CA20948 pancreatic tumour was not affected. Application of combined Lys/Arg and Gelofusine infusions during PRRT in patients will enlarge the therapeutic window.

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Kidney protection by amifostine during peptide receptor radionuclide therapy (PRRT) in tumour-bearing rats; indications for two mechanisms of action

Marleen Melis, Flavio Forrer, Cristina Müller, Edgar J. Rolleman, Roelf Valkema, Eric P. Krenning and Marion de Jong

Erasmus MC, dept. of Nuclear Medicine, Rotterdam, The Netherlands

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Chapter 3.2

Abstract

Introduction: Megalin-mediated renal retention of radiolabelled somatostatin analogues may result in nephrotoxicity during clinical PRRT; co-infusion of cationic amino acids can reduce the renal uptake though. The radical scavenger amifostine is being used as cytoprotector in external beam and chemotherapy, whereas protection from long-term nephrotoxicity in rats during PRRT with ^{177}Lu-DOTA,Tyr^3-octreotate has also been demonstrated. Here we aimed to further investigate the renal protection mechanism of amifostine and potential interference with anti-tumour effects during PRRT.

Methods: *In vitro* uptake studies were performed with ^{111}In-DOTA,Tyr^3-octreotate using BN-16 and CA20948 cells, expressing megalin/cubilin- and somatostatin receptors (sst) respectively, in combination with amifostine or WR-1065, the active metabolite of amifostine. In CA20948 tumour-bearing rats biodistribution and therapy studies were performed using 3 MBq ^{111}In-DOTA,Tyr^3-octreotate (0.5 or 15 µg peptide) or 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (15 µg) respectively with or without amifostine co-administration, either s.c. or i.v..

Results: Surprisingly, addition of amifostine and WR-1065 reduced uptake of ^{111}In-DOTA,Tyr^3-octreotate in BN-16 cells to 66% and 55% of control, respectively, but not in CA20948 cells. In agreement with this, amifostine decreased renal uptake of 15 µg radiolabelled octreotate in biodistribution studies to 62% of control level after s.c. and to 39% after i.v. co-administration, but tumour uptake was not affected. This was consistent with complete tumour remission after PRRT in the presence of amifostine.

Conclusions: Our results support evidence that amifostine may provide renal protection during PRRT, both by reduction of the absorbed kidney radiation dose and by the earlier reported radical scavenging, whereas anti-tumour effects were not attenuated.

Introduction

Patients with metastasized somatostatin receptor over-expressing tumours can significantly benefit from peptide receptor radionuclide therapy (PRRT) using ^{177}Lu- or ^{90}Y-labelled somatostatin analogues, e.g. Tyr^3-octreotate [1, 2]. However, megalin receptor-mediated retention of radiopeptides in the renal proximal tubules may lead to a high renal radiation dose and therefore to long-term renal damage [3, 4]. Renoprotection by co-infusion of the cationic amino acids lysine and arginine (Lys/Arg) is commonly applied during administration of PRRT, because a reduced renal uptake of 60% of control levels can be achieved [5, 6] which may be further improved when combined with co-infusion of the gelatin-based plasma expander Gelofusine [7, 8]. Thus, inhibition of the initial renal uptake of radiopeptides is a successful way to reduce the risk of late renal dysfunction. On the other hand kidney protection can also be achieved by mitigation of radiation-induced tissue damage by radioprotective drugs such as amifostine [9, 10].

The radical scavenger amifostine, or Ethyol or WR-2721, was developed by the Walter Reed Army Institute of Research to protect people from radiation effects during a nuclear war. It is approved by the FDA to be used during radiotherapy [11, 12] and chemotherapy [13-15] to protect healthy tissues such as kidneys, salivary glands and progenitor cells in bone marrow [16]. The mechanisms of action of the antioxidant amifostine have been reported to include scavenging of oxygen radicals reducing development of inter-strand cross-links, hydrogen atom donation and induction of intracellular hypoxia by auto oxidation [17, 18]. The pro-drug amifostine is converted into the active compound WR-1065 after dephosphorylation by the enzyme alkaline phosphatase (AP) which is present on plasma membranes of e.g. arteriolar endothelium of various normal tissues and proximal tubular epithelium in the kidney [19]. The molecular structures of amifostine and WR-1065 are shown in Figure 1.

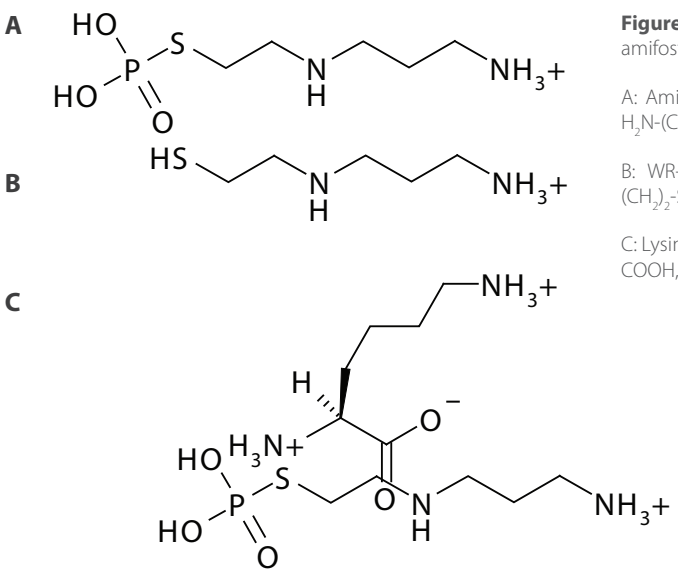

Figure 1: Molecular structures of amifostine, WR-1065 and lysine.

A: Amifostine = WR-2721= $C_5H_{15}N_2O_3PS$ = H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-S-PO_3H_2, MW = 214.2

B: WR-1065= $C_5H_{14}N_2S$ = H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-SH, MW= 134.3

C: Lysine= $C_6H_{14}N_2O_2$ = H_2N-$(CH_2)_4$-CH(NH_2)-COOH, MW= 142.2

In rats administration of amifostine protected kidneys from radiation-induced fibrosis and tubular atrophy beyond 150 days after external beam irradiation [20], confirming the antioxidant effects of amifostine [21]. We previously reported that co-administration of amifostine also protected kidneys from long-term toxicity after irradiation by ^{177}Lu-DOTA,Tyr^3-octreotate during PRRT in non-tumour-bearing rats [9]. Potential protection of the tumour against the therapeutic effects of PRRT by amifostine could not be examined in that study though. Sound vascularisation and high capillary AP activity in normal tissues compared to neovascularisation, less AP expression and a relatively low pH value in tumour tissues, support the tissue selectivity and preferential conversion of amifostine and uptake of WR-1065 in normal cells [22-24]. Hence attenuation of tumour therapy by amifostine is not expected, although preclinical reports are ambiguous on this issue [22, 25, 26].

Because the combination of amifostine and somatostatin analogue-based PRRT was not studied before in tumour-bearing animals, in the current study we aimed to gain more insight into the renal protection mechanism of amifostine during PRRT and into potential tumour protection, i.e. interference of amifostine with anti-tumour effects. Uptake of ^{111}In-DOTA,Tyr^3-octreotate was studied *in vitro* using megalin/cubilin receptor-expressing BN-16 cells and sst-expressing CA20948 tumour cells, with or without amifostine or WR-1065 and also with Lys as positive control inhibiting somatostatin analogue uptake in BN-16 cells. *In vivo* biodistribution studies using ^{111}In-DOTA,Tyr^3-octreotate were performed in CA20948 tumour-bearing rats, with or without co-administration of amifostine. Furthermore, tumour-bearing rats were treated with ^{177}Lu-DOTA,Tyr^3-octreotate, with or without amifostine, and were followed beyond 150 days post therapy (p.t.) to monitor anti-tumour effects.

Materials and methods

Radionuclides, peptide, chemicals

$^{177}LuCl_3$ was obtained from IDB (Baarle Nassau, The Netherlands) and $^{111}InCl_3$ was purchased from Covidien (Petten, The Netherlands). DOTA,Tyr^3-octreotate and octreotide were supplied by BioSynthema (St Louis, MO, USA). Radiolabeling was performed according to previously published procedures with a labelling efficiency exceeding 95%, as confirmed by thin-layer chromatography [27, 28].

The specific activity of ^{111}In-DOTA,Tyr^3-octreotate for the *in vitro* uptake experiments was 50-80 MBq/µg peptide. In biodistribution studies ^{111}In-DOTA,Tyr^3-octreotate was administered either as 3 MBq/15 µg or 3 MBq/ 0.5 µg peptide. A peptide amount of 15 µg was administered because this amount is required to label the therapeutic dose of 555 MBq ^{177}Lu to DOTA,Tyr^3-octreotate for PRRT in CA20948 tumour-bearing rats [29]. A peptide amount of 0.5 µg was applied as well because in earlier studies we showed that this resulted in the highest uptake in sst-expressing tissues and CA20948 tumours [30].

Human serum albumin (HSA) kits (Vasculocis) were purchased from IBA Molecular Benelux (Louvain-la-Neuve, Belgium) and ^{99m}Tc-labelled according to the provided procedure. Final ^{99m}Tc-HSA concentration was 10 MBq/2mg/ml.

Amifostine (Ethyol) was purchased from Pinnacle biologics (Almere, The Netherlands) in vials containing 500 mg, to be reconstituted in 9.7 ml saline. WR-1065 was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Institutes of Health (Bethesda, MD, USA). L-lysine and bovine serum albumin (BSA) were obtained from Sigma (Zwijndrecht, The Netherlands).

In vitro BN-16 and CA20948 cell assays

Rat yolk sac carcinoma epithelial (BN-16) cells, expressing megalin/cubilin receptors, were kindly provided by Prof. P.J. Verroust from the department of Anatomy, University of Aarhus, Denmark [31, 32]. BN-16 and CA20948 cells [33] were cultured at 37^{0}C in DMEM (Invitrogen, Breda, The Netherlands) supplemented with 10% foetal calf serum (FCS). Cells were seeded into 12-well plates and cultured until confluence. Before addition of 10^{-9}M radiopeptide (plus amifostine, WR-1065 or Lys) cells were rinsed twice with 2 ml warm PBS (pH 7.4) and incubated for 1h at 37^{0}C with 1 ml serum-free DMEM and rinsed twice again with 2 ml warm PBS (pH 7.4). Amifostine, WR-1065 or Lys were prepared as 2.10^{-2}M stock solution in Ringer's solution (B. Braun Medical, Oss, The Netherlands) with 10mM HEPES (pH 7.4). Dilutions of 2.10^{-3}M and 2.10^{-4}M were prepared. Per well 500 µl of the three concentrations of amifostine, WR-1065 or Lys were added in triplicate or 500 µl Ringer's solution was added as negative control [34]. To each well 500 µl of 2.10^{-9}M ^{111}In-DOTA,Tyr^3-octreotate was added and the well-plates were incubated for 1h at 37°C. Cells were rinsed twice with Ringer's solution after which 1 ml of 1 M NaOH was added to lyse the cells. The lysates were counted in a gamma counter (Perkin Elmer, Wallac, 1480 Wizard 3", Turku, Finland). This protocol was also followed to measure the uptake of 3.10^{-3}M ^{99m}Tc-HSA, to check BN-16 megalin receptor expression, because albumin is one of the main megalin ligands. Octreotide (10^{-5}M) or BSA (10 mg/well) were added as positive controls for specific inhibition of ^{111}In-DOTA,Tyr^3-octreotate or ^{99m}Tc-HSA uptake, respectively.

Counts were expressed as percentage of added radioactivity (%A) per mg protein of cell lysate, the latter determined using a commercially available colorimetric assay (BioRad, Veenendaal, The Netherlands). Effects of co-incubation with the different agents were expressed as percentage of ^{111}In-DOTA,Tyr^3-octreotate uptake in control wells.

Animal experiments

Animal studies were conducted in accordance with the guidelines of the Animal Welfare Committee. Male Lewis rats with a mean body weight (BW) of ~250 gram were purchased from Harlan (Horst, The Netherlands).

1. Biodistribution

CA20948 tumour cells were cultured in DMEM supplemented with 10% FCS and ~10^6 cells in 500 µl Hank's balanced salt solution (Invitrogen, Breda, The Netherlands) were inoculated s.c. in both flanks of the rats. Two weeks after inoculation the biodistribution experiments were

performed, n=4 rats per group. Rats were anaesthetized with isoflurane/O_2 and injected with 1 ml of amifostine solution (50 mg amifostine, or ~200 mg/kg) or saline (control animals) via the tail vein. The injection of amifostine was followed either immediately or after 30 min by the administration of ^{111}In-DOTA,Tyr^3-octreotate via the penis vein. One group received amifostine s.c. immediately before radiolabelled octreotate. This biodistribution assay was performed using either 0.5 µg or 15 µg ^{111}In-DOTA,Tyr^3-octreotate peptide.

After euthanasia at 24 h post injection (p.i.) tumours, kidneys and other organs were dissected and blood samples were taken. Tissues were weighed and radioactivity was measured in the gamma counter. Uptake of radioactivity was expressed as percentage of injected activity per gram tissue (%IA/g). Data obtained in amifostine-treated rats were expressed as percentage of data obtained in control rats.

2. Peptide receptor radionuclide therapy (PRRT)

One to two weeks after inoculation of CA20948 cells into one flank, when the tumour reached a size of 4-10 cm^2, rats received 555 MBq/15 µg ^{177}Lu-DOTA,Tyr^3-octreotate or saline as control, injected via the penis vein (250 µl). Thirty min before administration of ^{177}Lu-DOTA,Tyr^3-octreotate, two groups of rats were injected with 1 ml amifostine (50 mg, or 200 mg/kg), either s.c. (AMI sc) or i.v. followed by s.c. doses of 10 mg for 5 days (AMI iv-sc). Rats in the ^{177}Lu-DOTA,Tyr^3-octreotate only group received saline instead of amifostine. BW and tumour size were determined twice a week up to 150 days post therapy (p.t.). The 2 largest perpendicular tumour diameters were measured using a calliper and the tumour volume was calculated by multiplication and expressed in cm^2. Loss of more than 10% of original BW and tumour size beyond 15 cm^2 were indications to euthanize the animal.

Statistics

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Student t test. Concerning the differences in BW (Figure 4A) analysis was performed using ANOVA. Six time points (0, 2, 10, 15, 20 and 22 weeks p.t.) and the four treatments were included as independent variables and absolute BW as dependent variable.

Results

BN-16 cells are known to express megalin/cubilin receptors [31], whereas sst are not present on these cells. On the other hand, CA20948 cells express sst on their cell membranes [30]. Since albumin is one of the main ligands of megalin receptors their expression was checked using ^{99m}Tc-HSA, resulting in an uptake level in BN-16 cells of more than 20 %A/mg cellular protein after 1 h of incubation at 37^{0}C, which could be nearly completely blocked by an excess of 10 mg BSA (data not shown). The uptake level of ^{111}In-DOTA,Tyr^3-octreotate in BN-16 cells was 0.7 %A/mg protein and 13.5 ± 0.8 %A/mg protein in CA20948 cells; these levels were set at 100% for the two cell lines. Addition of excess BSA or octreotide inhibited uptake of ^{111}In-DOTA,Tyr^3-octreotate in BN-16 cells; BSA to 49.2 ± 6.4 % and octreotide to 73.6 ± 5.6 % of control. In CA20948 cells octreotide completely blocked the uptake of radiolabelled oc-

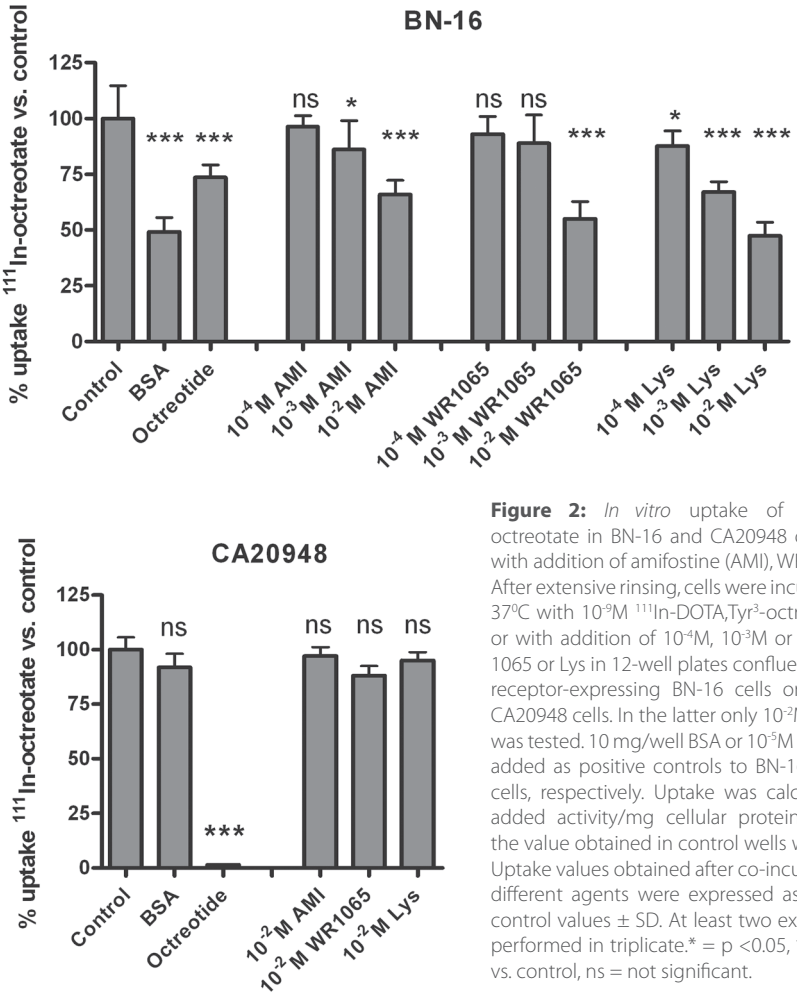

Figure 2: *In vitro* uptake of ^{111}In-DOTA,Tyr^3-octreotate in BN-16 and CA20948 cells without or with addition of amifostine (AMI), WR-1065 or Lys. After extensive rinsing, cells were incubated for 1 h at 37^{0}C with 10^{-9}M ^{111}In-DOTA,Tyr^3-octreotate, without or with addition of 10^{-4}M, 10^{-3}M or 10^{-2}M AMI, WR-1065 or Lys in 12-well plates confluent with megalin receptor-expressing BN-16 cells or sst-expressing CA20948 cells. In the latter only 10^{-2}M of each agent was tested. 10 mg/well BSA or 10^{-5}M octreotide were added as positive controls to BN-16 and CA20948 cells, respectively. Uptake was calculated as % of added activity/mg cellular protein (%A/mg) and the value obtained in control wells was set at 100%. Uptake values obtained after co-incubation with the different agents were expressed as percentage of control values ± SD. At least two experiments were performed in triplicate.* = p <0.05, *** = p <0.0001 vs. control, ns = not significant.

treotate whereas no competitive effect of BSA was found, confirming that these cells only express somatostatin- and no megalin/cubilin-receptors (Figure 2). In BN-16 cells amifostine or WR-1065 co-incubation showed significant inhibition of ^{111}In-DOTA,Tyr^3-octreotate uptake in a dose dependent way, WR-1065 to 55.0 ± 7.8 % and amifostine to 65.9 ± 6.3% of control at 10^{-2} M. Co-incubation with 10^{-2}M Lys reduced uptake of ^{111}In-labelled octreotate to 47.4 ± 6.1% of control, comparable with WR-1065 and BSA. In CA20948 cells no significant competitive effects of these three agents on ^{111}In-DOTA,Tyr^3-octreotate uptake were found (Figure 2).

In vivo biodistribution studies in tumour-bearing rats were performed with 0.5 or 15 µg of ^{111}In-DOTA,Tyr^3-octreotate, to simulate the effect of amifostine co-administration with both diagnostic and PRRT peptide amounts. The uptake of ^{111}In-DOTA,Tyr^3-octreotate 24 h p.i. in kidneys and especially in CA20948 tumours and sst-expressing tissues such as pancreas, stomach and adrenals, was dependent on the peptide amount (Table 1). Radioactivity levels in the kidneys

%IA/g ± SD	0.5 µg	15 µg
Kidney	6.85 ± 0.45	8.54 ± 1.46
CA20948	7.62 ± 1.48	1.65 ± 0.33
Pancreas	8.27 ± 0.87	0.99 ± 0.06
Adrenals	9.00 ± 1.91	1.15 ± 0.09
Stomach	1.29 ± 0.20	0.14 ± 0.01

Table 1: Biodistribution data 24 h p.i. of 0.5 or 15 µg ^{111}In-DOTA,Tyr^3-octreotate in rats without co-administration of amifostine.
Uptake in kidneys, sst-expressing CA20948 tumours and organs (pancreas, adrenals and stomach) was calculated as % injected activity/gram (%IA/g) ± standard deviation (SD). n = 4 rats per group. Difference in renal uptake between the two groups was not significantly different (p=0.07), whereas the uptake in sst-expressing tissues was highly significantly different (p<0.0001).

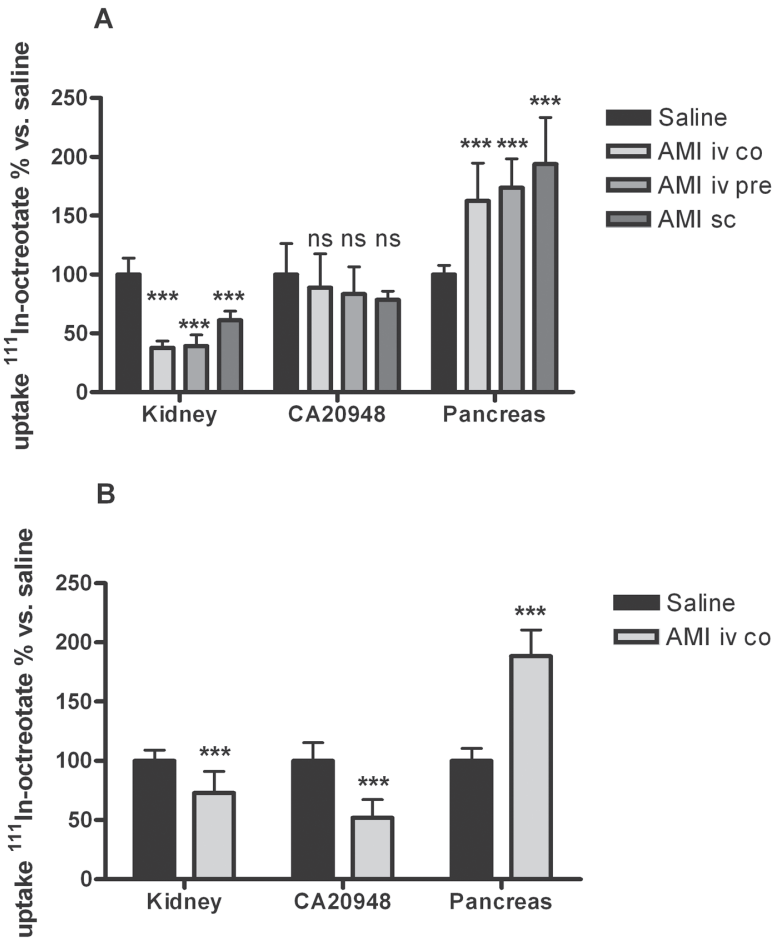

Figure 3: Biodistribution data 24 h p.i. of 15 µg (A) or 0.5 µg (B) ^{111}In-DOTA,Tyr^3-octreotate (3 MBq) in CA20948 tumour-bearing rats without or with co-administration of 50 mg amifostine (AMI) via different routes of injection.
AMI iv co = intravenously (i.v.) co-injection of AMI with ^{111}In-DOTA,Tyr^3-octreotate, AMI iv pre = i.v. injection of AMI 30 min before ^{111}In-DOTA,Tyr^3-octreotate, AMI sc = subcutaneous (s.c.) injection of AMI immediately before ^{111}In-DOTA,Tyr^3-octreotate. Uptake was calculated as % IA/g and the value of the saline group was set at 100%. Uptake values obtained in AMI groups were expressed as percentage of control values ± SD. n = 4-6 rats per group. *** = p <0.0001 vs. saline group. Differences between renal uptake using 15 µg ^{111}In-DOTA,Tyr^3-octreotate in AMI sc group vs. AMI iv co and AMI iv pre were significantly different as well (p <0.0001 and p=0.0002 respectively), ns = not significant.

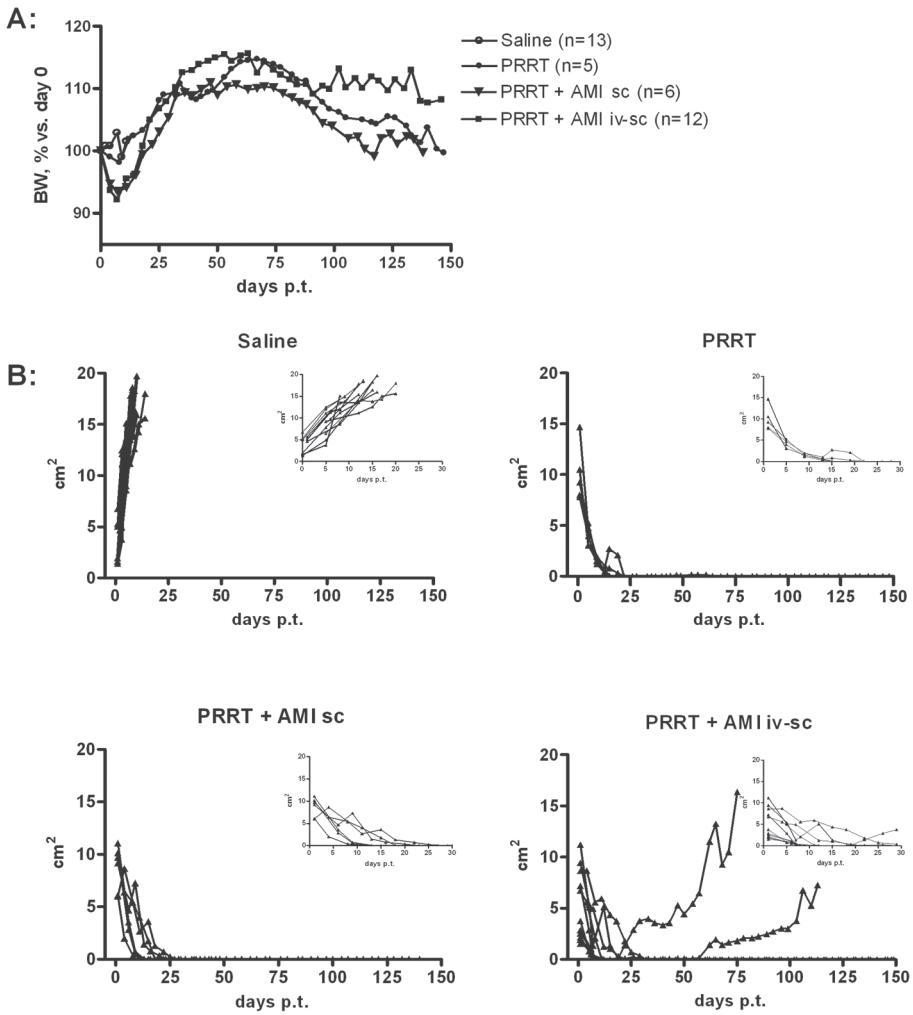

Figure 4: PRRT using 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (15 µg) in CA20948 tumour-bearing rats without or with co-administration of amifostine (AMI) via different routes of injection.
Saline = group without PRRT and AMI (n=13), PRRT = group i.v. ^{177}Lu-DOTA-Tyr^3-octreotate without AMI (n=5), AMI sc = group s.c. injection of 50 mg AMI immediately before ^{177}Lu-DOTA-Tyr^3-octreotate (n=6), AMI iv-sc = group i.v. injection of 50 mg AMI 30 min before ^{177}Lu-DOTA,Tyr^3-octreotate, followed by 5 daily s.c. doses of 10 mg AMI (n=12).
A: Body weight (BW) over time p.t., monitored twice a week up to day 150 p.t., expressed as percentage of initial BW. At 22 weeks p.t. the BW of the PRRT AMI iv-sc group was significantly higher than the BW of the PRRT AMI sc group (ANOVA, $p<0.05$, Scheffé's correction for multiple comparisons).
B: Tumour sizes over time p.t., measured twice a week up to day 150 p.t. Rats in the saline group had to be euthanized when tumour size > 15 cm^2. Tumour response in PRRT and PRRT AMI sc group was 100%, in PRRT AMI iv-sc group 83%.

of both amounts of ^{111}In-DOTA,Tyr^3-octreotate were not significantly different. Sst-mediated uptake levels in tumours and organs, however, were significantly less when the high 15 µg peptide dose was administered compared to tissues from rats that were injected with the 0.5 µg octreotate amount [30].

Three ways of co-administration of amifostine were tested in biodistribution experiments with the 15 µg peptide amount (Figure 3). The s.c. route led to 39% reduction of the renal retention, but i.v. administered amifostine even caused a 61-62% reduction of renal radioactivity. On the other hand, a reduction of renal retention of 27% was found when amifostine was co-injected i.v. with 0.5 µg ^{111}In-DOTA,Tyr^3-octreotate. Concerning sst-mediated uptake, amifostine co-administration with the 15 µg amount of radiolabelled octreotate caused no significant effect on CA20948 tumour uptake, but a nearly twofold increased level of radioactivity in sst-expressing tissues (pancreas, adrenals and stomach) was detected. This phenomenon of increased uptake levels in sst-expressing organs was also encountered when the low, 0.5 µg peptide amount was administered, but in this case a significant 48% reduction of the tumour uptake was encountered.

Monitoring body weight (BW) after PRRT is being used as a marker of the state of health of treated rats. Non-tumour bearing, non-treated control rats gain BW steadily over time [9]. In the current experiment the non-treated tumour-bearing animals had to be euthanized because of too large tumours at day 20 after start of the experiment, so they were lost for follow-up (Figure 4). Administration of amifostine via both routes induced a transient BW loss in tumour-bearing rats during the first week after PRRT with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (Figure 4A). From day 25 on, BW was similar to that of the rats that received PRRT without amifostine. All PRRT-treated rats afterwards gained weight until day 35 post therapy (p.t.) and stabilized until day 90 after which loss of BW was observed. Rats from the iv-sc AMI group lost less BW than the other two groups. Significant BW differences at the six tested time points between PRRT+AMI iv-sc vs PRRT+AMI sc and PRRT only treatments were found ($p<0.001$).

In this study, the very effective therapeutic effects of 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate on CA20948 tumours were clearly illustrated again. At day 25 p.t. all tumours were not palpable anymore (100% complete response). However, in 2 out of the 12 rats in the AMI iv-sc PRRT group tumours escaped at 22 and 60 days p.t., resulting in a 83% final tumour eradication vs. the 100% score in the other PRRT groups.

Discussion

Amifostine (Ethyol, WR-2721) is currently being applied as a cytoprotective agent for normal tissues against harmful effects of both ionizing irradiation or chemotherapeutic agents. Clinical applications include prevention of external beam irradiation-induced xerostomia in patients suffering from head and neck carcinoma and reduction of renal toxicity in patients treated with cisplatin because of advanced ovarian cancer [12, 16].

In several studies the pharmacokinetics of amifostine has been reported in the literature. Amifostine, either administered intraperitoneally (i.p), i.v. or s.c, is very rapidly cleared from the blood; within 6-30 min. p.i. only 6-10% of the parent drug is left, while 5-10 min p.i. the maximum tissue concentrations of the active metabolite WR-1065 are reached in liver and kidneys [25, 35-37]. A comparative study in cynomolgus monkeys using both i.v. and s.c. routes of amifostine administration demonstrated that the WR-1065 peak level after s.c administration was reached later, declined more slowly and remained at a higher level than after i.v. administration [37]. In the initial clinical studies in which amifostine was co-administered, the i.v. route was recommended. Because amifostine should be infused within half an hour before irradiation logistical issues complicated incorporation into clinical practice. Furthermore, common adverse effects were associated with this i.v. route of administration. Transient hypotension occurred in ~50% of cases and interruption of amifostine infusion was indicated when a fall of 20% in systolic blood pressure was observed [15, 18]. Besides hypotension, dose dependent side effects such as nausea, vomiting and local skin reactions occurred [18]. In a clinical study s.c. administered amifostine appeared to be well tolerated by 85% of patients, showing less adverse events such as hypotension [38], whereas the radioprotection efficacy was similar to studies applying i.v. amifostine administration [11]. Moreover the s.c. route is simpler and saves time in clinical practice.

Radioprotective effects of amifostine were described in several preclinical studies. In rats amifostine offered long-term kidney protection when administered i.p. in a 200 mg/kg BW dose 30 min before 6 Gy external kidney irradiation [20]. The same amifostine dose, administered either i.v. or s.c., was described to protect rats efficiently from mucositis after a 15 Gy dose of external beam irradiation, whereas lower doses accomplished less beneficial effects. No significant differences in cytoprotective effects were found using either route of administration, however a prolonged beneficial effect after s.c. administration was found [39]. Earlier we described renal protective effects of amifostine after a 35-70 Gy absorbed kidney radiation dose induced by 278 or 555 MBq ^{177}Lu-DOTA,Tyr^{3}-octreotate PRRT [9]. In this study a dose of i.v. 200 mg/kg BW was administered as well, followed by 7 daily s.c. doses of 25 mg/kg. These daily maintenance doses were thought to be essential to sustain a protective WR-1065 level during the first week post therapy (p.t.), because of the short half-life of WR-1065. Scavenging of oxygen radicals delivered after ^{177}Lu-radiation in the kidneys by WR-1065 seemed to have protected the renal function because of maintained BW and ^{99m}Tc-DMSA uptake, less urinary protein excretion and relatively low serum creatinine and urea levels after PRRT plus amifostine compared to the group that received PRRT without amifostine. Therefore, we chose this administration route of amifostine again for one of the groups in the current PRRT study and compared it with a group that received a single s.c. amifostine dose of 200 mg/kg shortly before PRRT. The BW of the AMI iv-sc group of rats stabilized beyond 90 days p.t. vs. a decline in BW in the PRRT only and AMI sc groups (Figure 4A) confirming a higher level of well-being [9]. This supported the hypothesis that continuous presence of WR-1065 during the first days after administration of PRRT is essential for long-term kidney protection.

The potential risk of undesired tumour protection by amifostine is an issue being discussed since co-treatment was started. In some early preclinical radiotherapy studies using high radiation doses and high amounts of amifostine, undesired tumour protection seemed to occur

[25]. Recent reviews and meta-analyses concluded that anti-tumour efficacy of chemo- and radiotherapy was not compromised by amifostine though [12, 18, 26, 40, 41]. In the current rat study tumour protection by amifostine from anti-tumour effects was also not found. All tumours showed a complete response after injection of 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (15 µg), although over time in the AMI iv-sc group two out of 12 rats encountered a relapse of tumour growth. This unaltered beneficial therapeutic effect is in agreement with the unaffected *in vitro* uptake of radiolabelled octreotate in CA20928 cells in the presence of amifostine and WR-1065 and also with the *in vivo* results of the CA20948 biodistribution study using 15 µg ^{111}In-DOTA,Tyr^3-octreotate, in which at 24 h p.i. similar uptake levels were reached in rats with or without amifostine co-administration.

The fact that renal radioactivity was reduced after amifostine co-administration in the ^{111}In-DOTA,Tyr^3-octreotate biodistribution experiments was unexpected. AP content is abundant in kidney and liver, as well as in other types of normal tissues, in epithelium, connective tissue and vasculature [35]. So the conversion from amifostine into WR-1065 will take place locally in the kidneys, and WR-1065 will remain present because only a limited amount appeared to be excreted into the urine [36]. Therefore this positively charged amifostine metabolite may act as a competitor in the megalin-mediated renal reabsorption of radiolabelled octreotate. This hypothesis is based on the fact that the positively charged amino acids lysine (and arginine) interfere with the reabsorption process of radiolabelled somatostatin analogues, which has been demonstrated by *in vitro* assays using the megalin receptor-expressing BN-16 cells [5, 34, 42]. In this *in vitro* assay addition of WR-1065 and, to a lesser extent, the parent drug amifostine also reduced the *in vitro* uptake of ^{111}In-DOTA,Tyr^3-octreotate to a similar level as was reached with BSA and Lys (Figure 2). This is a clear indication that these positively charged agents interfered in the megalin-mediated uptake of the radiolabelled peptide and might therefore represent a mechanism responsible for reduction of renal retention of ^{111}In-DOTA,Tyr^3-octreotate *in vivo*.

Administration of amifostine 30 min prior to PRRT was adapted from radioprotection protocols to enable metabolization of WR-1065 before generation of harmful oxygen species by irradiation. Since the WR-1065 blood level already peaks at 5-10 min after amifostine administration, this 30 min time interval is not needed to obtain less renal radioactivity after injection of the radiopeptides, in line with the fact that no significant differences in renal uptake were detected between pre- or co-injection of amifostine (Figure 3A). However, the WR-1065 peak level after s.c. amifostine administration occurs later and is lower, explaining the decrease of reduction of renal radioactivity in this group (Figure 3A). So, although the s.c. route of administration of amifostine seemed to be more favourable in studies concerning cytoprotection after ionizing radiation because of less side-effects [38, 39] in the present study concerning kidney protection by amifostine during PRRT, i.v. co-administration proved to be more effective in reducing the initial renal retention of radiolabelled octreotate than the s.c. route.

The percentage of reduction of renal radioactivity by i.v. amifostine co-injection was less if 0.5 µg ^{111}In-DOTA,Tyr^3-octreotate was injected compared to 15 µg, i.e. 27 vs 61% reduction, respectively (Figure 3B). Since we did not register blood pressure in the rats, we only can speculate that a transient drop in systolic blood pressure induced by the bolus injection of

amifostine reduced the renal clearance rate of the radiopeptides. This was most obvious with the high amount of peptide [15]. Due to a longer circulation time of a higher concentration of ^{111}In-DOTA,Tyr^3-octreotate the uptake in the sst-expressing organs could have reached the higher levels as compared to tissues from rats without amifostine co-administration. This is in contrast however with the decreased uptake in CA20948 tumours when 0.5 µg ^{111}In-DOTA,Tyr^3-octreotate was used. In rats the pharmacokinetics of the binding of radiolabelled somatostatin analogues to sst-expressing normal tissues or to tumours appeared to be different [30, 43]. Rat CA20948 tumour uptake of ^{111}In-DOTA,Tyr^3-octreotide was optimal using a peptide mass of 0.5 µg, while pancreas showed an increasing uptake until 5 µg per rat. We postulate that hypotension may have hampered the blood flow into the tumour, vascularised by newly formed blood vessels, and affected the optimal, high tumour uptake realized with 0.5 µg radiolabelled octreotate. Amifostine will not be used during diagnostic application of somatostatin analogue based scintigraphy when low peptide amounts are administered; therefore this reduced tumour uptake will not be an issue in clinical practice.

The earlier described protective effects on late kidney function and histology when amifostine was given together with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate were attributed to the radical scavenger properties of the amifostine derivative WR-1065 because of interference in the cascade of events leading to the development of fibrosis in the kidney [9]. In the current study we discovered that also the initial renal uptake and retention of ^{111}In-DOTA,Tyr^3-octreotate was reduced when amifostine was co-administered. Therefore two mechanisms may have contributed to the kidney protective effect of amifostine: a combination of a lower absorbed kidney radiation dose because of reduced renal uptake and an attenuation of the process leading to long-term renal fibrosis elicited by free oxygen species. To discriminate between the effects mediated by these two mechanisms, in future experiments long-term kidney protection will be monitored after administration of amifostine prior to PRRT and compared to the protection after amifostine administration post PRRT.

Conclusions:

Co-administration of the cytoprotector amifostine with radiolabelled octreotate reduced the initial renal uptake and retention of this peptide. There is strong evidence that this effect is based on partial inhibition of the megalin-mediated renal reabsorption of radiopeptides by amifostine and/or WR-1065. Therefore amifostine may provide kidney protection when applied during PRRT both by reduction of the absorbed kidney radiation dose and by radical scavenging reducing late development of radiation induced renal fibrosis as reported earlier. Furthermore, we were able to demonstrate that amifostine did not affect tumour response after ^{177}Lu-DOTA,Tyr^3-octreotate PRRT in rats. Thus, the application of amifostine during clinical PRRT might be a promising strategy to reduce the risk of radiation induced nephrotoxicity.

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Micro-SPECT to monitor renal function after renal retention of radiopeptides

Chapter 4

From outside to inside? Dose dependent renal tubular damage after high-dose peptide receptor radionuclide therapy in rats measured with *in vivo* ^{99m}Tc-DMSA-SPECT and molecular imaging

F. Forrer, E. Rolleman, M. Bijster, M. Melis, B. Bernard, E.P. Krenning, and M. de Jong

Department of Nuclear Medicine, Erasmus MC Rotterdam, The Netherlands

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Chapter 4.1

Abstract

Introduction: In peptide receptor radionuclide therapy (PRRT), the dose-limiting organ is most often the kidney. However, the precise mechanism and the exact localization of kidney damage during PRRT have not been fully elucidated. We studied renal damage in rats, after therapy with different amounts of ^{177}Lu-DOTA,Tyr^3-octreotate and investigated ^{99m}Tc-DMSA as a tool to quantify renal damage after PRRT.

Methods: Twenty-nine rats were divided over three groups and injected with either 0, 278 or 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate, leading to about 0, 46 and 92 Gy to the renal cortex. More than 100 days after therapy, kidney damage was investigated using ^{99m}Tc-DMSA (Dimercaptosuccinic acid) SPECT (single photon emission computed tomography), autoradiography, histology, and blood analyses.

Results: In vivo SPECT with ^{99m}Tc-DMSA resulted in high resolution (<1.6mm) images. The ^{99m}Tc-DMSA uptake in the rat kidneys was related inversely with the earlier injected activity of ^{177}Lu-DOTA,Tyr^3-octreotate and correlated inversely with serum creatinine values. Renal *ex vivo* autoradiograms showed a dose dependent distribution pattern of ^{99m}Tc-DMSA. ^{99m}Tc-DMSA SPECT could distinguish between the rats that were injected with 278 or 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate, whereas histological damage grading of the kidneys was nearly identical for these two groups. Histological analyses indicated that lower amounts of injected radioactivity caused damage mainly in the proximal tubules, while as well the distal tubules were damaged after high dose radioactivity.

Conclusion: Renal damage in rats after PRRT appeared to start in a dose dependent way in the proximal tubules and continued to the more distal tubules with increasing amounts of injected activity. *In vivo* SPECT measurement of ^{99m}Tc-DMSA uptake was highly accurate to grade renal tubular damage after PRRT.

Introduction

Peptide receptor radionuclide therapy (PRRT) with radiolabelled somatostatin analogs has become an important tool in the management of neuroendocrine tumors. Convincing results were found for both objective tumor response and quality of life [1-4]. During PRRT using somatostatin analogs labelled with ß-emitters like ^{90}Y and ^{177}Lu, usually the kidney is the dose-limiting organ [5, 6].

Although the major part of the radiopharmaceutical is excreted into the urine, the partial reabsorption in the tubular cells leads to a considerable radiation dose to the radiosensitive kidneys [7, 8]. It was shown recently that the localization of the radiopeptide in the kidney is not homogeneous, but predominately in the cortex where it forms a striped pattern, with most of the radioactivity centered in the inner cortical zone [9]. In the only study that included biopsies of human kidneys after PRRT, mainly thrombotic microangiopathy was found despite minor tubular atrophy and interstitial fibrosis [10]. In addition it is known that PRRT can lead to radiation nephritis [11, 12]. The precise mechanism of renal damage however is not fully elucidated; especially the localization of the most pronounced damage has not been identified yet.

The potential coherence of the inhomogeneous distribution of radioactivity in the kidney and the localization of damage is highly relevant since a number of radionuclides potentially suitable for therapy are available. The β$^-$-emitters ^{90}Y, ^{177}Lu and ^{131}I are widely used for therapies with a number of vectors [13, 14]. Several therapy studies were performed with the Auger emitter ^{111}In and several new radionuclides, including α-emitters, are under investigation [15, 16]. The different physical characteristics of these radionuclides result in a different tissue penetration range of the therapeutic particles and will therefore lead to a different distribution pattern of absorbed radiation dose in the kidney [17].

The aim of this study was to investigate rat kidneys more than 100 days after injection of different amounts of ^{177}Lu-DOTA,Tyr^3-octreotate. The highest activity injected was intended to induce severe kidney damage. The kidneys were investigated with a number of methods: *ex vivo* autoradiography, histological analysis with different staining methods and measurement of serum creatinine to get a complete overview of function and morphology. As it is known that radiopeptides are absorbed partially in the tubular epithelial cells, *in vivo* SPECT (single photon emission computed tomography) with ^{99m}Tc-DMSA (dimercaptosuccinic acid), as a marker for renal tubular damage [18, 19], were acquired with a dedicated animal SPECT camera (NanoSPECT), that allows absolute *in vivo* quantification of renal ^{99m}Tc-DMSA-uptake [20]. The ^{99m}Tc-DMSA scintigrams were performed to evaluate the value of this tracer in the follow up of renal function after PRRT in rats in order to develop a sensitive method to follow renal function over time.

Materials and Methods

Radiopharmaceuticals

^{177}Lu-DOTA,Tyr^3-octreotate was synthesized and labelled as described previously [21]. The ^{99m}Tc-DMSA (Dimercaptosuccinic acid) kit was purchased from GE Healthcare (Buckinghamshire, United Kingdom) and labelled according to the indicated procedure.

Animal studies

Animal experiments were performed in compliance with the regulations of the institution and with generally accepted guidelines governing such work. Twenty nine young, male Lewis-rats (Harlan, Horst, The Netherlands) with a body weight of 250 - 300 grams were divided over 3 groups. The control group consisted of 9 rats. Ten rats were i.v. injected with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate and 10 rats with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. In 20 rats (5 controls, 7 of the 278 MBq group and 8 of the 555 MBq group) SPECT scans with ^{99m}Tc-DMSA were acquired. Since renal damage is late toxicity, the scans were acquired between 109 - 146 days after the injection of ^{177}Lu-DOTA,Tyr^3-octreotate. The ^{99m}Tc-DMSA uptake in the kidneys was quantified. Thereafter, an autoradiogram of the ^{99m}Tc-DMSA uptake was performed in 6 rats (two from each group). Kidneys from all animals were analysed histologically.

Animal SPECT (NanoSPECT) and software

SPECT imaging was performed with a four-headed multiplexing multi-pinhole NanoSPECT (Bioscan Inc., Washington D.C., USA). Each head is outfitted with an application-specific tungsten collimator with 9 pinholes. For this study we imaged with rat apertures that are comprised of a total of 36, 2 mm diameter pinholes imaging a cylindrical field of view that is 60 mm in diameter by 24 mm in length. These rat apertures provided a reconstructed resolution below 1.6 mm at 140 keV with an average sensitivity of 1100 cps/MBq across the field of view (FOV). The images are acquired in a step-and-shoot helical scan-mode which allows to image a defined range from 24 to 270 mm according to the region to be imaged. The energy-peak for the camera was set at 140 keV. The window width was ± 10%. The rats were scanned 4-6 hours after the injection of 50 MBq ^{99m}Tc-DMSA. An acquisition time of 30 seconds per projection was chosen resulting in total acquisition times ranging from 6-9 minutes per animal. The data were reconstructed iteratively with the HiSPECT© software (Bioscan), a dedicated ordered subsets-expectation maximisation (OSEM) software package for multiplexing multi-pinhole reconstruction. The NanoSPECT was calibrated with a phantom, approximately of the size of the animals, filled with a known activity of ^{99m}Tc such that voxel values in the reconstruction provide a proper estimate of the activity level without further calculation.

A volume of interest (VOI) was drawn manually around both kidneys; the 3D activity distribution within the VOI was then summed to determine the uptake. Because of the favourable biodistribution of ^{99m}Tc-DMSA, limited to the kidneys, the VOI could be drawn generously to prevent partial-volume effects at the edges. All measured activities were corrected for decay and expressed as % injected activity [%IA]. The injected activity was determined by measuring the syringe in a dose-calibrator before and after injection of the animal. The difference was defined as the injected activity. The quantification of the VOI was performed with the INTERVIEW XP© software (Mediso Ltd., Budapest, Hungary). After imaging the rats were sacrificed.

Autoradiography

In six animals (2 from each group) after euthanasia one kidney was removed, quickly frozen on liquid nitrogen cooled isopentane and processed further for autoradiography. The tissue was embedded in TissueTek (Sakura, Zoeterwoude, The Netherlands) and processed for cryosectioning as described previously [22]. Briefly, tissue sections (10 µm) were mounted on glass slides. The sections were exposed to SR phosphor imaging screens (Packard Instruments Co., Canberra CT, USA) for 1 d in radiographic cassettes. The screens were analysed using a Cyclone phosphor imager and a computer-assisted OptiQuant 03.00 image processing system (Packard).

Histology

Immediately after removing from the animal, kidneys were fixed in 10% neutral buffered formalin, trimmed and processed by standard techniques for embedding in paraffin. Four-micron sections were cut and stained with haematoxylin-eosin (HE) or periodic acid-Schiff reagent (PAS). The microscopic renal damage score (RDS) was graded blinded to the treatment protocol ranging from 0 (no damage) to 4 (severe damage). The criteria for these grades are listed in Table 1.

The PAS stained sections were used for better differentiation between proximal and distal tubules.

Table 1: Criteria for the histological kidney damage score.

Grade	Overview	Glomeruli	Tubules
1	More or less normal aspect High cell count glomeruli	Apoptotic cells in the endothelium Inflammatory infiltrate	Apoptotic cells Rough protein staining Little dilated Normal BM No protein cylinders
2	Dilation of tubules Damaged tubule cells	Like grade 1	More apoptotic cells More pronounced dilation BM thickened Little protein cylinders in tubules Regenerating cells (mitotic activity)
3	Stronger dilated tubules Cell rich infiltrate Regenerating tubules PAS: thickened BM PAS: protein cylinders	Vascular lumina smaller, few erythrocytes Sometimes shrinkage	Flat epithelium, partly total loss of epithelium Strong dilation Inflammatory infiltrate Regeneration present Protein cylinders More pronounced BM thickening
4	Heavily dilated tubules Heavily thickened BM Protein cylinders	Like grade 3 More optical empty space due to shrinkage glomeruli	Like grade 3, but more protein cylinders Periferal fibrosis

Abbreviation: BM = basal membrane

Blood analyses

Blood chemistry and haematological parameters were determined by standard hospital analysis procedures.

Statistics

To correlate the results, Pearson's correlation coefficient was calculated. The Student t test was used to test significance of differences. A p-value <0.05 was considered significant.

Results

The administration of ^{177}Lu-DOTA,Tyr^3-octreotate to the rats was straightforward. No acute discomfort was observed in the rats treated. After inclusion, the body weight of the rats from all groups dropped slightly by not more than 5%. After this initial decline, the body weight of the control rats increased continuously as expected. In contrast, the body weight of the rats treated with 278 MBq initially increased slightly and then remained stable, while the body weight of the rats treated with 555 MBq initially increased and dropped beyond approximately 70 days after PRRT. (Figure 1A-C).

By SPECT with ^{99m}Tc-DMSA in all rats both kidneys could be visualized although kidneys in the group injected with 555 MBq where visible only faintly. The spatial resolution of the images was high with a spatial resolution below 1.6 mm. Differentiation between functional parenchyma characterized by tracer accumulation and the cold regions indicative of the renal

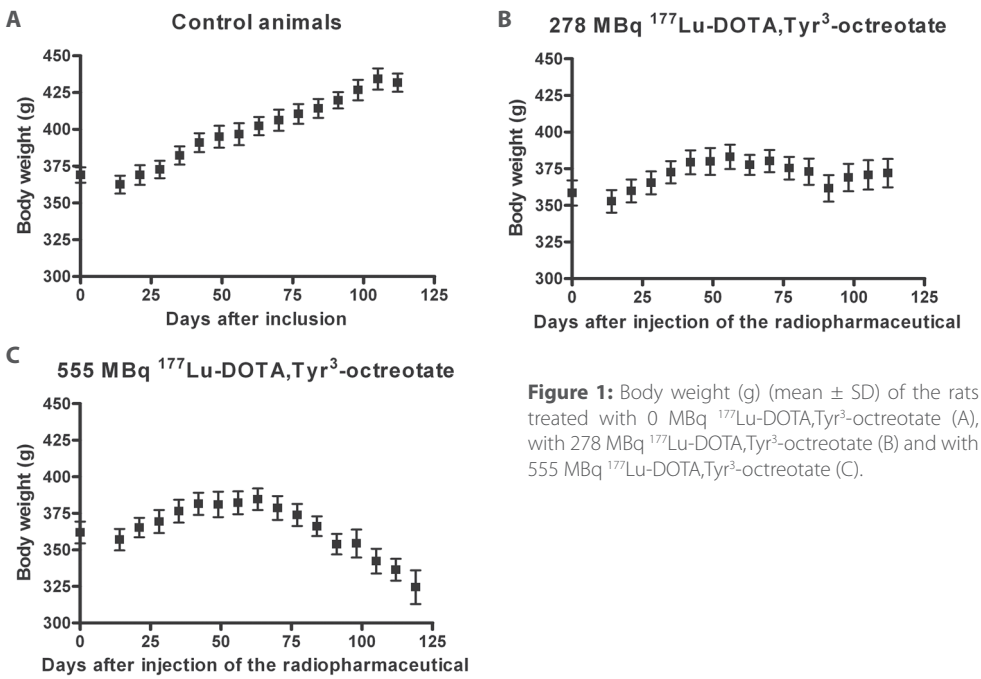

Figure 1: Body weight (g) (mean ± SD) of the rats treated with 0 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (A), with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (B) and with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate (C).

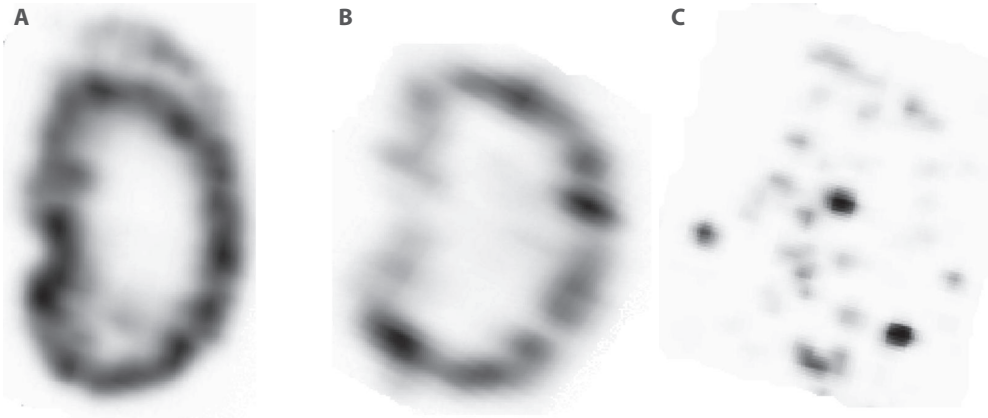

Figure 2: Coronal SPECT slices of rat kidneys acquired 4 – 6 h after the injection of 50 MBq ^{99m}Tc-DMSA. The slices correspond to the kidneys in Figure 3 and 4. (A) is from a control animal, (B) from an animal 115 days after therapy with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate and (C) from an animal 109 days after therapy with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate.

pelvis was easily possible (Figure 2A-C). The renal uptake of ^{99m}Tc-DMSA was significantly different between the three groups (all $p < 0.01$). The mean values ± SD were 23.2 ± 1.2 %IA for the control group, 9.9 ± 6.3 %IA for the group injected with 278 MBq and 1.4 ± 0.5 %IA for the group injected with 555 MBq.

Figure 3A-C show examples of the autoradiograms with ^{99m}Tc-DMSA from rat kidneys after treatment with 0, 278 and 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. Figure 3A shows a normal distribution of ^{99m}Tc-DMSA in a control rat with a high accumulation in the renal cortex. In

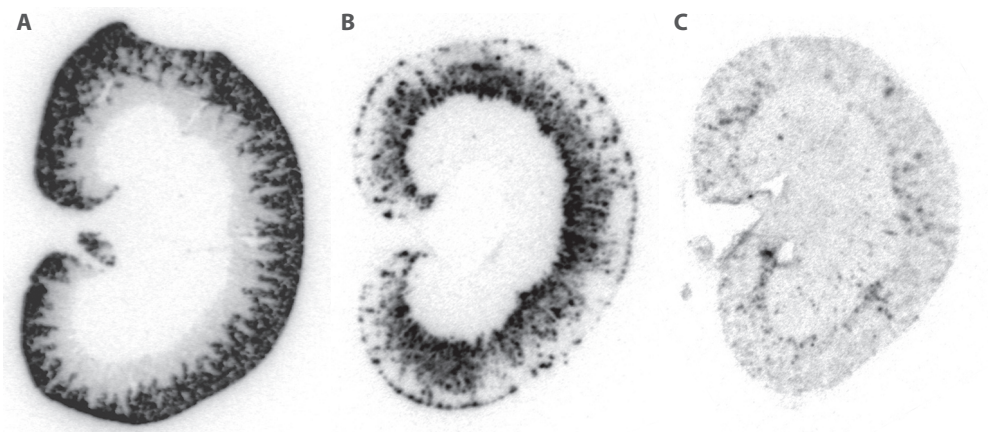

Figure 3: Renal autoradiograms after an *in vivo* injection of 50 MBq ^{99m}Tc-DMSA. The rats were sacrificed 6 h after injection. Figure 3A shows the autoradiogram of a control animal with a normal radioactivity distribution. Figure 3B shows the autoradiogram of an animal 115 days after therapy with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. Figure 3C shows the autoradiogram of an animal 109 days after therapy with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. The images correspond to the kidneys in Figure 2 and 4.

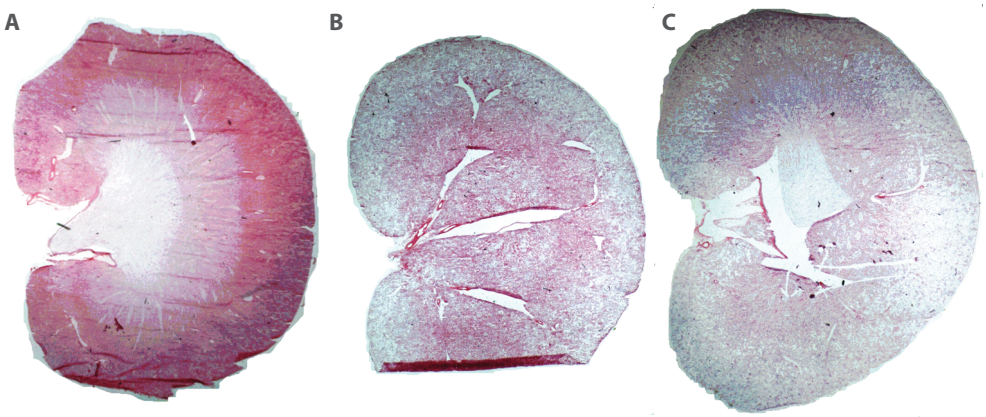

Figure 4: (A-C) Low power overviews (3x) of coronal histological slices of rat kidneys, stained with HE. The slices correspond to Figures 2 and 3.

contrast, in figure 3C showing an autoradiogram of a rat treated with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate, hardly any ^{99m}Tc-DMSA uptake can be seen, indicating severely damaged tubular function. Figure 3B shows an autoradiogram of a rat treated with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. Here, intermediate renal ^{99m}Tc-DMSA uptake was found. The distribution pattern of the radioactivity was obviously different compared to that of the control rat. We found a "shift" of the radioactivity from the cortex to the outer medulla. The corresponding SPECT scans are displayed in Figure 3A-C. A very good match between SPECT and autoradiograms was found, underlining the high accuracy of the SPECT images.

In the third row (Figure 5A-C) the corresponding histological HE stained overview images of adjacent sections of those providing the autoradiograms are shown. A dose dependent loss of eosinophile cytoplasm can be seen already in the low power (3x) overview.

The detailed histology showed, as expected, no significant abnormalities in the control rats. One kidney was scored with a damage score of 2, all other kidneys did not show any damage and were scored 0. An example of a histological, PAS stained slice of a control rat is shown in Figure 5A.

In the rats treated with 555 MBq, detailed histology revealed intense changes in the proximal and distal tubules ^{177}Lu-DOTA,Tyr^3-octreotate. A mixed picture with inhomogeneous nuclei, apoptotic and necrotic cells was found (Figure 5C). In all kidneys of this group we found extensive protein leakage into the tubules and collecting tubes. Furthermore interstitial nephritis with inflammation cells was found. The glomeruli however showed none or only very mild changes. Based on the criteria given in Table 1 all kidneys of the rats treated with 555 MBq were histologically scored as grade 4 damage.

The rats treated with 278 MBq showed severe histological damage as well. Especially the proximal tubules were heavily damaged with atrophy, dilatation, apoptotic nuclei and necrosis. However, in contrast to the kidneys of the rats that were treated with 555 MBq there was a

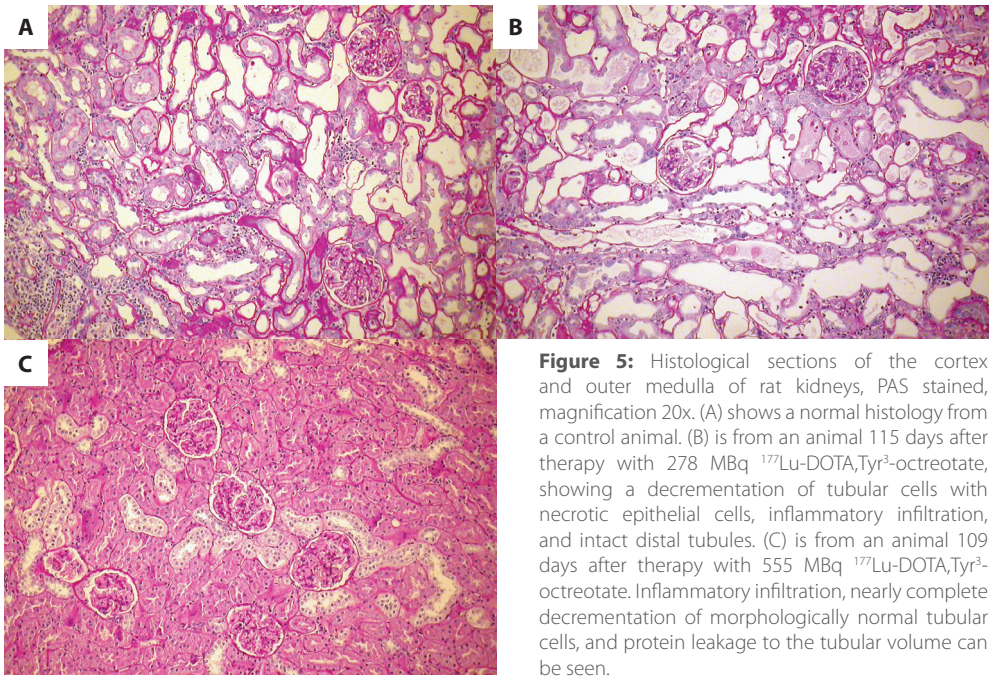

Figure 5: Histological sections of the cortex and outer medulla of rat kidneys, PAS stained, magnification 20x. (A) shows a normal histology from a control animal. (B) is from an animal 115 days after therapy with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate, showing a decrementation of tubular cells with necrotic epithelial cells, inflammatory infiltration, and intact distal tubules. (C) is from an animal 109 days after therapy with 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate. Inflammatory infiltration, nearly complete decrementation of morphologically normal tubular cells, and protein leakage to the tubular volume can be seen.

notable number of tubules that did not show histological damage. The PAS staining revealed that these were mainly distal tubules, located in the outer medulla. In addition to the tubular damage, signs of interstitial nephritis with inflammation cells were found as well (Figure 5B). The tubular damage was accentuated in the cortex which reflects the uptake of ^{99m}Tc-DMSA very well. The histological scoring resulted in one grade 2 score, one grade 3 and 8 grade 4 scores. Thus, regarding only the histological score, no significant difference to the group treated with 555 MBq was found (p=0.18).

At the day of sacrifice a blood sample was drawn by cardiac puncture to measure the serum creatinine values. The results (mean ± SD) were 36.5 ± 17.5 µmol/l in the control group, 129.7 ± 79.9 µmol/l in the group injected with 278 MBq, and 425.3 ± 219.2 µmol/l in the group injected with 555 MBq. All differences between all groups were significant ($p<0.05$) (Figure 6). Furthermore a significant ($p<0.01$) correlation was found between the creatinine values and and the %IA determined by SPECT.

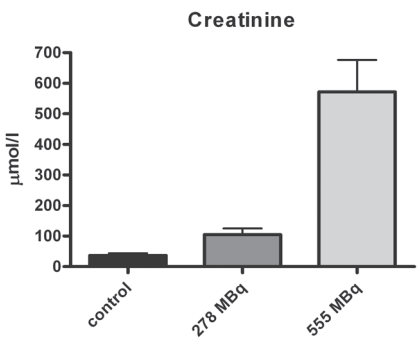

Figure 6: Serum creatinine values (µmol/l, mean ± SD) of the three groups at the day of sacrifice.

Discussion

High dose PRRT could cause severe renal damage in rats as well as in humans because of the radiation absorbed dose to the kidney during therapy [5, 8, 10, 12]. Recently a number of new drugs were introduced with potential to reduce renal toxicity during PRRT, but the effectiveness of these drugs during PRRT remains to be proven in patients [23-26]. Worthwhile to be highlighted are the studies with amifostine because this is the first drug investigated for PRRT that does not aim at reducing the renal uptake but which acts as a radical scavenger to reduce systemically the toxic effects of the radiation. Because amifostine is acting by a completely different mechanism, a combination with drugs that reduce the renal uptake appears most promising [23].

To improve kidney protection during PRRT it is important to understand the mechanism of renal damage. One step towards a better understanding could be close monitoring of kidney function after PRRT *in vivo* and over time. The newly available dedicated small animal gamma cameras offer the possibility to investigate physiological processes in the same animal over time. However a tracer, easily available for daily routine, needs to be defined. The relation that was found between ^{99m}Tc-DMSA uptake and serum creatinine values as well as the relation between ^{99m}Tc-DMSA uptake and the injected activity of ^{177}Lu-DOTA,Tyr^3-octreotate indicate that ^{99m}Tc-DMSA is an accurate marker for renal function after PRRT in these animals.

The only work containing histological data from human kidneys after PRRT reports, despite minor fibrosis and tubular atrophy, mainly thrombotic microangiopathy (involving glomeruli, arterioles, and small arteries). These pathological changes were comparable to the changes found after external beam radiation when the kidney is within the field of radiation. The data for this study were generated investigating kidney biopsies of patients after treatment with DOTA,Tyr^3-octreotide labelled with ^{90}Y, a high energy β^--emitter [10].

Recently published data showed that the highest concentration of radiolabelled peptides in rat kidneys and in human kidneys as well was found in the proximal tubular cells [9, 22]. The multiligand scavenger receptor megalin appeared to play a crucial role for the reabsorption of radiopeptides into the tubular cells [27]. Using the high energy β^--emitter ^{90}Y, emitting β particles with a maximum energy of 2.27 MeV, will result in a fairly homogenous energy distribution over the whole kidney including a high radiation absorbed dose to the glomeruli [28]. For this study ^{177}Lu was used, emitting β particles with a maximum energy of 0.50 MeV which results in a significant lower tissue penetration range of these particles and a different energy distribution over the kidney. Taking into account the space between tubules and glomeruli as well as microdosimetric aspects, a lower radiation absorbed dose to the glomeruli when using ^{177}Lu compared to ^{90}Y can be expected. It was calculated recently that other radiolanthanides with even lower energy β^--particles could improve the energy distribution further [16]. Recently several articles were published using α emitters for internal radiation therapy [28-30]. After the administration of ^{213}Bi labelled DOTA,Tyr^3-octreotide to Lewis-rats, no histological changes were observed in kidney glomeruli and tubules. As a consequence of the treatment with 22.2 MBq of ^{213}Bi-DOTA,Tyr^3-octreotide merely mild interstitial nephritis was observed. It is very likely that the physical characteristics of the radionuclide used might have a strong

influence on kidney damage and might be one of the reasons why no histological changes in the glomeruli were found in this study using ^{177}Lu.

The estimated radiation absorbed dose to the kidney is high after single dose administration of 278 or 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate in rats. Dosimetric calculations showed that injecting these activities into rats resulted in doses to the cortex of approximately 46 and 92 Gy respectively and approximately 35 and 70 Gy respectively to the whole kidney [17]. For the treatment of patients a maximum tolerated dose to the kidneys of 23 Gy is generally accepted although this value is derived from external beam radiation, dealing with different properties especially concerning the dose rate and energy distribution within the kidney [6]. Taking into account the potential dose reduction by the co-infusion of amino acids, 278 MBq of ^{177}Lu-DOTA,Tyr^3-octreotate would result in approximately 23 Gy.

The results of this rat study strongly suggest that late renal damage after high dose ^{177}Lu-DOTA,Tyr^3-octreotate therapy is mainly tubular. This is supported by the results of the ^{99m}Tc-DMSA studies, being a marker for the renal tubular function. The dose dependent reduction of ^{99m}Tc-DMSA uptake in the rat tubules suggests that ^{177}Lu-DOTA,Tyr^3-octreotate provokes a dose dependent tubular damage.

In conclusion ^{99m}Tc-DMSA appears to be a good marker to quantify the extent of damage after PRRT. Using ^{99m}Tc-DMSA in combination with a dedicated small animal gamma camera will allow the following of renal function after PRRT in animals over time.

Localization and extent of damage respectively after PRRT was found to be dose dependent. While in rats treated with 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate kidney damage was found to be mainly in the proximal tubule, higher injected radioactivities resulted in a decline of morphologically undamaged tubules.

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Dynamic and static micro-SPECT in rats to monitor renal function after ^{177}Lu-labelled Tyr^3-octreotate radionuclide therapy

Marleen Melis[1], Jan de Swart[1], Monique de Visser[1], Saskia C. Berndsen[1], Stuart Koelewijn[1], Roelf Valkema[1], Otto C. Boerman[2], Eric P. Krenning[1], Marion de Jong[1]

[1] Erasmus MC, dept. of Nuclear Medicine, Rotterdam, the Netherlands
[2] Radboud University Medical Centre, dept. of Nuclear Medicine, Nijmegen, the Netherlands

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Chapter 4.2

Abstract

Introduction: High kidney radiation doses during clinical peptide receptor radionuclide therapy (PRRT) with β^--particle emitting radiolabelled somatostatin analogues will lead to renal failure several months after treatment, urging co-infusion of cationic amino acids lysine (Lys) and arginine to reduce the renal radiation dose. In rat PRRT studies renal protection by co-administration of Lys was confirmed, based on histological examination of kidney specimens indicating nephrotoxicity. In the current study we investigated dedicated small animal SPECT/CT renal imaging in rats to monitor renal function *in vivo* during follow-up of PRRT, with and without Lys.

Methods: Three groups of rats were imaged using the multi-pinhole NanoSPECT/CT camera: (1) controls and (2) rats >90 days post therapy (p.t.) with 460 MBq/15 µg ^{177}Lu-DOTA, Tyr^3-octreotate without or with (3) 400 mg/kg Lys co-injection as kidney protection (n=6 per group). At 90 and 140 days p.t. static kidney scintigraphy was acquired 2 h post injection of 25 MBq ^{99m}Tc-DMSA. In addition, dynamic dual isotope renography was performed using 50 MBq ^{111}In-DTPA and 50 MBq ^{99m}Tc-MAG3 at d100-120 p.t.

Results: ^{111}In-DTPA and ^{99m}Tc-MAG3 studies revealed a time-activity pattern comparable to those in patients with a peak at 2-6 min followed by decline of renal radioactivity. Reduced ^{111}In-DTPA, ^{99m}Tc-MAG3 and ^{99m}Tc-DMSA uptake indicated renal damage in the PRRT group (2), whereas the PRRT + Lys group (3) only showed a decrease of ^{99m}Tc-MAG3 peak activity. These results indicating nephrotoxicity in group (2) and renal protection in group (3) correlated with levels of urinary protein and serum creatinine and urea, and were confirmed by renal histology.

Conclusion: Quantitative dynamic dual isotope imaging using both ^{111}In-DTPA and ^{99m}Tc-MAG3 and static ^{99m}Tc-DMSA imaging in rats is feasible using small animal SPECT, enabling longitudinal monitoring of renal function. Especially ^{99m}Tc-MAG3 renography appears to be a more sensitive marker of tubular function after PRRT than serum chemistry or ^{99m}Tc-DMSA scintigraphy.

Introduction

Recent technical improvements of the resolution and sensitivity of dedicated small animal SPECT cameras opened new possibilities in preclinical research. Multi-pinhole collimated cameras achieve submillimeter spatial resolution and accurate quantification of the amount of radioactivity in tissues is feasible [1, 2]. Therefore the non-invasive technique of *in vivo* functional imaging can be used to follow-up longitudinal induction and progression of disease [3].

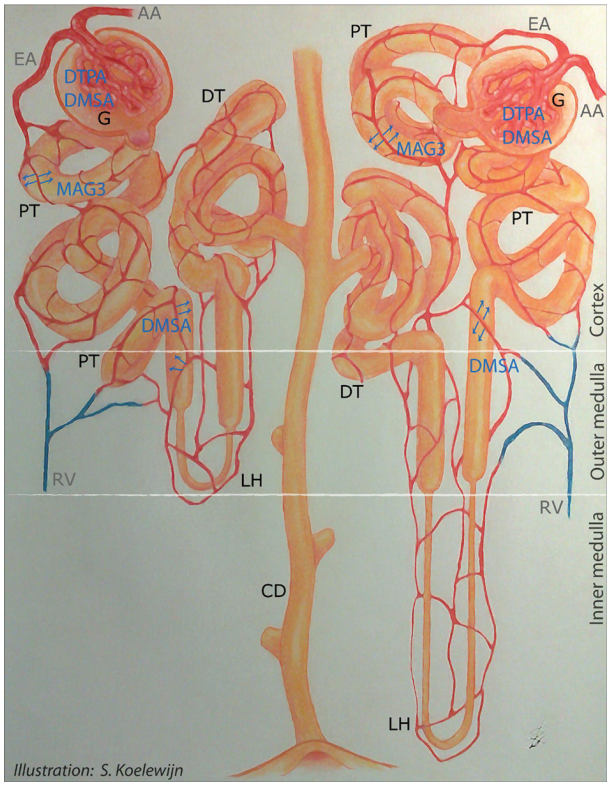

Figure 1: Blood enters the functional units of the kidneys, the nephrons, via an afferent arteriole (AA) of the renal artery. In the capillary network in the glomerulus (G), waste and useful molecules are filtered from the blood and transported into the proximal tubules (PT). Waste molecules are excreted in the primary urine via the loop of Henle (LH) and the distal tubules (DT) into the collecting duct (CD) leading to the renal pelvis and the bladder. In the first convoluted part of the PT megalin(/cubilin)-receptors are expressed playing a significant role in reabsorption of useful molecules. Extraction from blood in the efferent arteriole (EA) [27] directly into the PT is another route of excretion of molecules into the urine, which is the most prominent way of ^{99m}Tc-MAG3 clearance. ^{111}In-DTPA is only cleared via glomerular filtration and directly excreted into the urine, whereas ^{99m}Tc-DMSA is partly filtrated in the glomeruli followed by partial reabsorption in the PT. ^{99m}Tc-DMSA is also partially peritubularly extracted from the blood, primarily in the last straight part of the PT which may extent into the outer medulla of the kidney. RV = renal vein.

Peptide receptor radionuclide therapy (PRRT) in patients suffering from somatostatin receptor-overexpressing neuroendocrine tumours using radiolabelled somatostatin analogues like octreotate, shows beneficial results. Significant tumour response, survival benefit and improvement of quality of life is obtained [4]. However, partial reabsorption of the radiopeptides in the kidneys may lead to long-term nephrotoxic effects, especially when ^{90}Y-labelled analogues are administered [5, 6]. Recent developments to further improve tumour response rates include salvage therapy and combined therapy with both ^{90}Y- and ^{177}Lu-labelled radiopeptides, supporting the need for kidney protection [7-9]. Nowadays co-infusion of lysine/arginine during PRRT is a protective measure in the clinic resulting in a 35-40% reduction of the kidney radiation dose [10-12] also found in preclinical animal models [13]. Therapy with 555 or 278 MBq ^{177}Lu-DOTA,Tyr^3-octreotate resulted in complete remission (CR) [14] or 80% CR and 20% partial remission [15]

of somatostatin receptor expressing tumours in rats, respectively. PRRT with 555 MBq however, delivers a 60 Gy radiation dose to the kidneys inducing renal toxicity beyond 90 days after therapy [16, 17].

In patients renal function can be monitored by renography, in addition to measurements of serum creatinine and creatinine clearance as markers of glomerular filtration rate (GFR) [14, 18]. The uptake of ^{99m}Tc-radiolabelled dimercaptosuccinic acid (^{99m}Tc-DMSA) by the kidneys is directly related to tubular function. Although ^{99m}Tc-DMSA scintigraphy is clinically widely used to provide information on renal cortical morphology [19], the exact mechanism of renal handling is unknown. Peritubular extraction or absorption of plasma protein bound ^{99m}Tc-DMSA directly from the blood into the proximal tubular cells or glomerular filtration followed by tubular reabsorption are the two postulated mechanisms of ^{99m}Tc-DMSA uptake. Discussions on the relative contribution of each of these two pathways are ongoing [20-26]. Dynamic imaging of the clearance of radiolabelled diethylenetriaminepentaacetic acid (DTPA) monitors glomerular filtration, because this small molecule is only cleared via this route. ^{99m}Tc-labelled mercaptoacetyltriglycine (MAG3) is primarily rapidly extracted from the blood, besides a 10-15% fraction that is cleared via glomerular filtration. Extraction of ^{99m}Tc-MAG3 by basolateral uptake into cells lining proximal tubules is mediated by an active organic anion transport (OAT1) system, especially in the first convoluted part (S1 and S2) of the proximal tubules [27].

In the current study dynamic dual isotope imaging of ^{111}In-DTPA and ^{99m}Tc-MAG3 and static ^{99m}Tc-DMSA imaging (Figure 1) were performed to study glomerular filtration, tubular secretion and peritubular absorption in rats beyond 90 days post therapy (p.t.) with ^{177}Lu-DOTA,Tyr^3-octreotate PRRT, with or without kidney protection by lysine (Lys) co-administration and compared with untreated controls. For this purpose the multi-pinhole collimated small animal NanoSPECT/CT camera was used [24]. Data were correlated with urine and serum chemistry and kidney histology.

Materials and Methods

Animals

Animal studies were conducted in accordance with the guidelines of the Animal Welfare Committee of our medical centre. For all experiments male Lewis rats (Harlan, Horst, The Netherlands) were used (n=6-9 per group). Body weight (BW) was determined twice a week up to 140 days p.t. Urine samples were collected every three weeks p.t. in metabolic cages for 24 h.

Radionuclides, peptide, radiopharmaca and chemicals

$^{177}LuCl_3$ was obtained from IDB Holland (Baarle Nassau, the Netherlands) and DOTA,Tyr^3-octreotate was obtained from BioSynthema (St Louis, MO, USA). Radiolabelling was performed according to previously published procedures [29]. A PRRT dose of 460 MBq ^{177}Lu-DOTA,Tyr^3-octreotate/15 µg was used. A fresh 400 mg/ml L-lysine (Sigma, Zwijndrecht, The Netherlands) solution was prepared and co-injected with ^{177}Lu-DOTA,Tyr^3-octreotate in a 400 mg/kg BW dose [13].

$^{111}InCl_3$ as well as DMSA and MAG3 kits were purchased from Covidien (Petten, The Netherlands), the latter two being ^{99m}Tc-labelled according to the provided procedure. For ^{99m}Tc-DMSA the concentration was 100 MBq/ml for ^{99m}Tc-MAG3 250 MBq/ml. ^{111}In-DTPA was prepared by addition of 100 µl 4 mM DTPA to 400 MBq $^{111}InCl_3$ (pH 3.5-4) and adjusted with saline to a concentration of 250 MBq/ml.

Molecular imaging

Rats were imaged using a four-headed multi-pinhole SPECT/CT camera (NanoSPECT/CT, Bioscan Inc., Washington D.C., USA). Settings of the energy peaks (window width ± 10%) for ^{177}Lu were 55, 113 and 208 keV, for ^{111}In 171 and 245 keV and for ^{99m}Tc 140 keV. During ^{111}In and ^{99m}Tc dual isotope imaging no visible cross talk from the γ-rays emitted by ^{111}In into the 140 keV window was observed.

Nine pinhole apertures with a diameter of 2.5 mm were used on each camera head, with a transaxial field of view (FOV) of 60 mm. Based on the CT topogram, the axial scan length was 55 mm covering the renal region, which was used for all mentioned imaging procedures. Animals were imaged while anesthetized with isoflurane/O_2 and body temperature was maintained using a heated bed.

Four days p.t. rats were scanned to quantify the renal ^{177}Lu activity dose. ^{99m}Tc-DMSA scans were performed at day 90 and 140 p.t. Scans were acquired at 2 h post injection (p.i.) of 25 MBq ^{99m}Tc-DMSA, administered in 250 µl via the dorsal penis vein. ^{177}Lu-DOTA,Tyr^3-octreotate and ^{99m}Tc-DMSA images were acquired for 12 min using 24 projections of 60 s/projection per head.

Between 100-120 days p.t. dual-isotope dynamic scans were obtained. Before scans were started, rats were anesthetized and a 27G infusion set with a tubing length of 30 cm (Venisystems, Hospira, Sligo, Ireland) filled with saline supplemented with heparin (50 IE/ml), was inserted into a tail vein. Immediately after the start of the scan 200 µl (50 MBq) ^{99m}Tc-MAG3 followed by 200 µl (50 MBq) ^{111}In-DTPA was administered and flushed with 700 µl saline (dead volume

of catheter was 400 µl). 20 scans of 2 min each (16 projections per head, 9 s/projection) were acquired, resulting in a total acquisition time of 40 min. To study the influence of extra hydration on the efflux of ^{99m}Tc-MAG3 and ^{111}In-DTPA from the kidneys an extra group of control rats was imaged starting 45 min after intraperitoneal injection of 5 ml saline.

Quantification of the amount of radioactivity in a volume of interest (VOI) over the kidneys was performed using InVivoScope software (Bioscan). Detected counts in the VOI were converted into MBq using a correction factor obtained by scanning a water phantom with the same volume as a rat body to correct for attenuation, filled with a known amount of radioactivity. Radioactivity in VOI over the whole kidneys, or over a cortical part in ^{99m}Tc-MAG3 images, was quantified and expressed as percentage injected activity per kidney or cm^3 renal cortex in each of the 20 scans during renography to create a time-activity curve. Peak activity of ^{99m}Tc-MAG3 or ^{111}In-DTPA was defined as the mean activity 2-6 min p.i. and 2-4 min p.i. respectively.

Analytical procedures

Urinary protein content and serum urea and creatinine levels were measured as previously published [16]. At day 90 p.t blood was drawn from a tail vein before injection of ^{99m}Tc-DMSA and at euthanasia at day 140 p.t. blood was collected to store serum.

Ex vivo autoradiography and histology

As previously described the localization of ^{99m}Tc-DMSA in kidneys was visualized by *ex vivo* autoradiography [17] and the grade of renal damage was evaluated microscopically according to a scale from grade 0 (no damage), grade 1 (little tubular dilation), grade 2 (basal membrane thickening), grade 3 (shrinkage of glomeruli, flat tubule epithelium) to grade 4 (severe tubular and glomerular damage) as described earlier in detail [16].

Statistics

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Student t test.

Results

Quantification of ^{177}Lu in the kidneys four days p.t. with 460 MBq ^{177}Lu-DOTA,Tyr^3-octreotate showed that 3.8 ± 0.3 MBq was retained per kidney of the unprotected animals, whereas 2.0 ± 0.1 MBq ^{177}Lu localized in kidneys of Lys protected rats (Table 1); a significant reduction of renal retention of ~45%. Irradiation of the renal tubular cells by the β^--particles emitted by ^{177}Lu obviously led to renal problems starting from 50 days p.t in PRRT rats without Lys kidney protection. Instead of the normal BW increase over time, stabilization of the BW along with urinary protein loss was observed when compared to controls. Further increase of protein loss in urine and rise of urea and creatinine serum levels determined at day 90 p.t. confirmed development of renal damage (Table 1). Histological grading of renal damage at day 140 p.t. correlated with these observations. Both massive tubular and glomerular damage was observed in kidneys of

unprotected rats, whereas after Lys co-injection only minor abnormalities in tubular morphology were observed (Table 1).

	days p.t.	Control (p Control vs PRRT)	PRRT (p PRRT vs PRRT + Lys)	PRRT + Lys (p Control vs PRRT + Lys)
^{177}Lu uptake (MBq/kidney)	4	-	3.8 ± 0.3 (***)	2.0 ± 0.1
Body weight, % vs. day 0	90	134 ± 3 (***)	111 ± 6 (**)	127 ± 5 (*)
Body weight, % vs. day 0	140	143 ± 4 (***)	113 ± 11(**)	138 ± 6 ns
Protein in urine (mg/24h)	100	11 ± 2 (***)	62 ± 9 (***)	22 ± 8 (***)
Urea in serum (mmol/l)	90	5.8 ± 0.2 (***)	10.5 ± 2.2 (*)	7.2 ± 1.0 (**)
Urea in serum (mmol/l)	140	5.3 ± 0.5 (***)	32.6 ± 9.4 (***)	7.3 ± 0.6 (***)
Creatinine in serum (mmol/l)	90	21 ± 2 (***)	60 ± 5 (***)	37 ± 3 (***)
Creatinine in serum (mmol/l)	140	24 ± 1 (***)	108 ± 20 (***)	35 ± 4 (***)
Grading histological renal damage	140	0.4 ± 0.6 (***)	4 ± 0 (***)	1.3 ± 0.5 (*)
^{99m}Tc-DMSA (%IA/kidney)	90	13.7 ± 1.4 (***)	6.4 ± 2.4 (***)	12.5 ± 1.2 ns
^{99m}Tc-DMSA (%IA/kidney)	140	18.1 ± 1.9 (***)	5.6 ± 3.2 (***)	14.3 ± 1.1 (**)
^{99m}Tc-MAG3 (%IA/kidney, 2-6 min)	100-120	13.1 ± 2.6 (***)	4.3 ± 1.5 (***)	8.5 ± 1.6 (***)
^{99m}Tc-MAG3 (%IA/cm^3 cortex, 2-6 min)	100-120	6.9 ± 1.9 (***)	2.6 ± 1.3 (*)	3.8 ± 1.0 (***)
^{111}In-DTPA (%IA/kidney, 2-4 min)	100-120	4.7 ± 1.0 (**)	2.1 ± 1.4 ns	4.4 ± 0.6 ns

Table 1: Overview of results in control, PRRT and PRRT + Lys rats. Quantification of retained renal radioactivity: total MBq/kidney of ^{177}Lu, %IA/kidney for ^{99m}Tc-DMSA, peak activity expressed in %IA/kidney for ^{111}In-DTPA and ^{99m}Tc-MAG3 or peak activity expressed in %IA/cm^3 cortex for ^{99m}Tc-MAG3. Body weight of rats expressed as % of body weight at day 0. Urine and blood chemistry: protein loss in urine expressed in mg/24 h urine, serum urea and creatinine content expressed in mmol/l or µmol/l. Histological grading of renal damage expressed on a scale of 0-4. (*) = p<0.05, (**) = p<0.001, (***) = p<0.0001.

Tubular damage beyond day 90 p.t. was confirmed by ^{99m}Tc-DMSA static imaging (Table 1, Figure 2). Renal ^{99m}Tc-DMSA uptake in non-treated animals ranged from 14-19% IA per kidney, which was significantly decreased to 2-8% IA in PRRT treated rats ($p<0.0001$) and slightly decreased to 11-15 % IA in kidneys of rats co-injected with Lys ($p<0.001$). *Ex vivo* autoradiography confirmed these data.

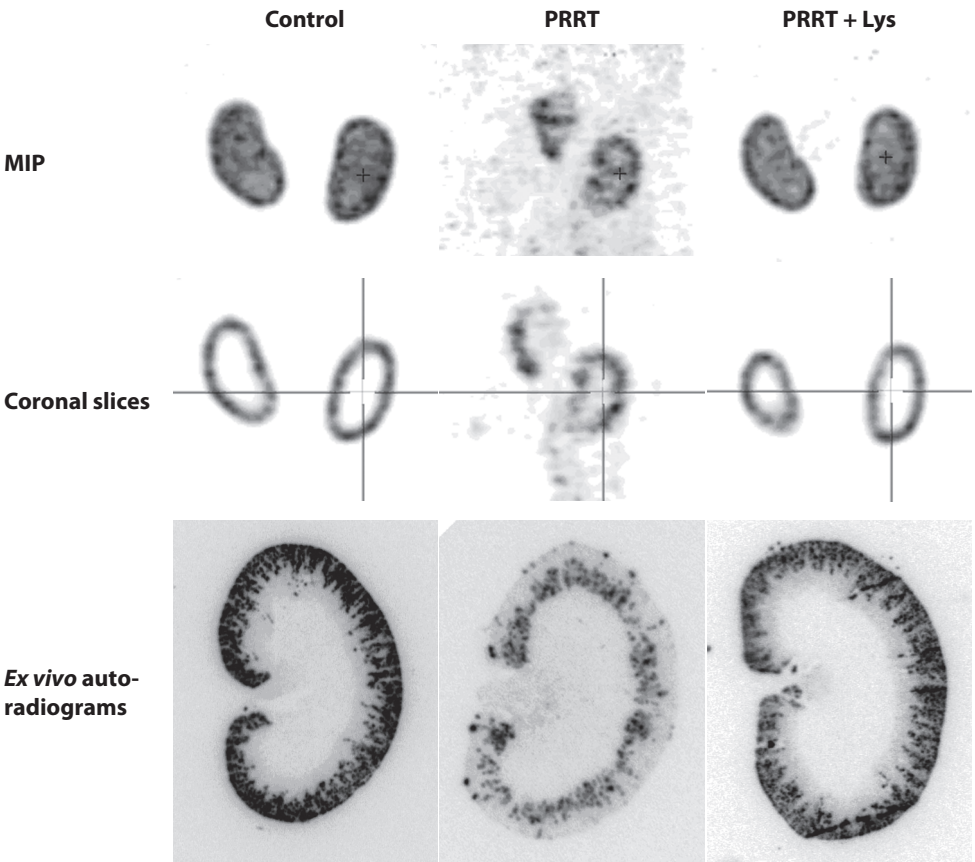

Figure 2: Typical examples of maximum intensity projections (MIP) (upper row) and coronal slices (middle row) of kidney images after ^{99m}Tc-DMSA scintigraphy performed at day 140 p.t. in non-treated Control rats (column left), PRRT-treated (column middle) or PRRT+Lys-treated rats (column right). *Ex vivo* autoradiograms of frozen kidney sections, prepared immediately after imaging, are shown in the lower row.

Dual isotope dynamic studies with ^{111}In-DTPA and ^{99m}Tc-MAG3 revealed a similar pattern in the time-activity curves as in humans with a peak of renal uptake for ^{111}In-DTPA at 2-4 min and of ^{99m}Tc-MAG3 at 2-6 min after administration, followed by a decline of renal radioactivity until most radioactivity was excreted at 20 min p.i. and stabilized until 40 min p.i. (Table 1, Figure 3). Quantification of total renal ^{99m}Tc-MAG3 radioactivity showed a mean peak activity in non-treated rats of 13.1% IA, while in PRRT rats only 4.3% IA was detected. Lys co-administration protected ^{99m}Tc-MAG3 extraction capacity of tubular cells after PRRT, because a significantly

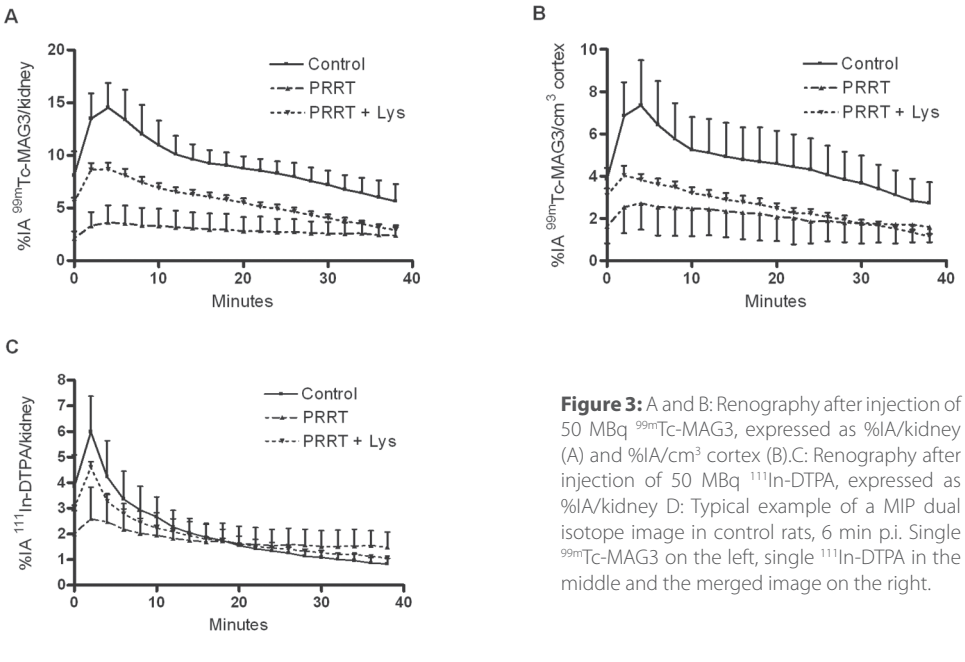

Figure 3: A and B: Renography after injection of 50 MBq ^{99m}Tc-MAG3, expressed as %IA/kidney (A) and %IA/cm^3 cortex (B).C: Renography after injection of 50 MBq ^{111}In-DTPA, expressed as %IA/kidney D: Typical example of a MIP dual isotope image in control rats, 6 min p.i. Single ^{99m}Tc-MAG3 on the left, single ^{111}In-DTPA in the middle and the merged image on the right.

higher uptake ($p<0.0001$) of 8.5% IA renal radioactivity was quantified in the PRRT+Lys group. Mean peak activity quantified only in cortical regions of the kidneys of these groups were 6.9, 2.6 and 3.8% IA/cm^3 cortex, respectively. ^{111}In-DTPA imaging revealed a small, but not-significant difference in peak activity between the control and PRRT+Lys groups (4.7 vs. 4.4% IA respectively), however. ^{111}In-DTPA uptake in the PRRT only group, was significantly less though (2.1% IA, $p=0.0003$), indicating that glomerular filtration was impaired after PRRT without kidney protection.

In clinical renography studies patients are hydrated, i.e. drinking 500 ml water 1 h before imaging, but are imaged with an empty bladder to stimulate efflux of radioactivity from the renal pelvis. Therefore, an extra group of control rats was imaged after extra hydration before the scan. The mean peak activities of ^{99m}Tc-MAG3 and ^{111}In-DTPA remained unaltered, while the excretion of ^{111}In-DTPA was unchanged and the efflux of ^{99m}Tc-MAG3 was moderately enhanced during the last phase between 20 and 40 min p.i. (data not shown).

Discussion

In this small animal SPECT study in rats non-invasive renal imaging using ^{99m}Tc-DMSA, ^{99m}Tc-MAG3 and ^{111}In-DTPA gave insight into both tubular and glomerular functions after high absorbed kidney radiation doses to monitor renal function as follow-up after PRRT. Simultaneous renography using dual isotope dynamic imaging with ^{99m}Tc-MAG3 and ^{111}In-DTPA was advantageous to restrict animal discomfort. Accurate quantification of renal radioactivity was obtained in short scans when 50 MBq of both ^{99m}Tc-MAG3 and ^{111}In-DTPA were administered leading to activity levels of 0.6-6 MBq per kidney for ^{99m}Tc, and 0.4-2.5 MBq for ^{111}In. Compared to clinical renography using frames of 15 s during a 20 min scan, the 2 min frames in the rats were relatively long, but had to be chosen because of the rotation time of the camera heads between projections. Recently Bioscan developed high sensitivity apertures with enlarged pinhole diameter offering improved time resolution. In the Linoview study [26] continuous dynamic SPECT imaging was performed using 15 s frames for 30 min p.i. of ^{99m}Tc-MAG3 and the newly developed U-SPECT dedicated small animal SPECT camera (Milabs, Utrecht, The Netherlands) also enables acquisition of shorter frames than used in this study [30].

Renographic ^{99m}Tc-MAG3 studies demonstrated more efficient elimination of the radioactivity from the kidneys in conscious mice than in anesthetized ones, indicating that the metabolic state of the animals is a confounding factor during dynamic renal imaging [26]. Although the clearance rate of ^{99m}Tc-MAG3 and ^{111}In-DTPA in our current study in anesthetized rats might not reflect the true physiological state, the three groups of rats to be compared were imaged under equal, controlled conditions. Furthermore, the efflux of the radiopharmaceuticals from the kidneys to the bladder could be hampered by a filled bladder. To stimulate emptying of the bladder by extra hydration of the rats 45 min before imaging, the efflux of ^{99m}Tc-MAG3 during the last phase of clearance was only slightly improved, while the peak uptake of ^{99m}Tc-MAG3 and ^{111}In-DTPA remained unchanged. Since this was the readout in this study, hydration of the rats was not considered essential.

Previous experiments demonstrated that approximately 3% of the radiopeptides is reabsorbed per kidney [16, 17]; a process being mediated by the multi-ligand scavenger receptor megalin, which is expressed in the first part of the cortical proximal tubules [31, 32]. Competition for the megalin receptor by Lys and radiolabelled octreotate, containing one Lys residue, might explain the reduction of renal retention found after co-administration with cationic amino acids [10, 13, 32]. In the current study PRRT was performed using 460 MBq of ^{177}Lu-DOTA,Tyr^3-octreotate, but long-term nephrotoxic effects were similar to those after 555 MBq PRRT, although the reduction of ^{99m}Tc-DMSA uptake was less severe [17]. The aberrant localization pattern in PRRT kidney ^{99m}Tc-DMSA autoradiograms (Figure 2) demonstrated that ^{99m}Tc-DMSA only was retained in the non-megalin receptor expressing S3 part of the proximal tubules, located in the outer medulla, in contrast to the localization in control kidneys. Since ^{111}In-DTPA renography demonstrated a very poor glomerular filtration in PRRT rats, ^{99m}Tc-DMSA will not or hardly be filtered and therefore not be reabsorbed in the proximal tubules. Therefore, peritubular extraction was the only route of ^{99m}Tc-DMSA retention in this part of the proximal tubules, as illustrated by the radioactivity localized in the outer medulla. The reduced level of retained ^{177}Lu in kidneys of PRRT+Lys rats protected from affected glomerular filtration and megalin-medi-

ated damage in the first convoluted part of the cortical proximal tubules, as demonstrated by the unimpaired ^{99m}Tc-DMSA localization pattern. A role of megalin in the tubular reabsorption of ^{99m}Tc-DMSA after glomerular filtration is confirmed by our observation that only 35-55% of ^{99m}Tc-DMSA renal uptake was found when we imaged kidney-specific megalin-deficient mice [28] compared to wild-type mice (data not shown). Also in Clcn5 knockout mice, a model of Dent's disease showing defective megalin and cubilin-mediated endocytosis, heavily reduced ^{99m}Tc-DMSA uptake was observed, which matched with results described in studies in patients suffering from Dent's disease or Fanconi syndrome [25, 26, 33]. Therefore the conclusion from Müller-Suur et al. that only peritubular extraction is responsible for renal ^{99m}Tc-DMSA uptake has been contradicted by these observations [22]

^{99m}Tc-MAG3 imaging showed no peak of tubular extraction in PRRT rats, both after total renal and cortex-only ^{99m}Tc-quantification; the latter method avoiding confounding radioactivity already excreted into the renal pelvis (Figure 3B). The clearance of ^{99m}Tc-MAG3 is not megalin- or cubilin receptor dependent as described in Clcn5 knockout mice [26], which we confirmed in megalin-deficient mice (data not shown), but is OAT1-mediated. This transport system is expressed in cells at the basolateral side of the convoluted proximal tubules and appeared to be inactive beyond 100 days after PRRT. Although Lys co-administration during PRRT significantly protects kidney function, yet ^{99m}Tc-MAG3 tubular extraction was clearly impaired when compared to control rats. It has been proposed earlier that ^{99m}Tc-DMSA was the optimal tracer to monitor and predict renal function [3, 34]. However, the current data suggest that ^{99m}Tc-MAG3 renography is a more sensitive method to quantify loss of function of cortical proximal tubules than ^{99m}Tc-DMSA scintigraphy after PRRT. Unaffected ^{99m}Tc-MAG3 renography is one of the inclusion criteria before ^{90}Y- or ^{177}Lu-based PRRT and is also used during follow-up in one centre [12, 35] next to GFR markers used in other medical centres [8, 14]. Although further clinical validation is warranted, introduction of routine ^{99m}Tc-MAG3 renography might be considered in more PRRT centres, especially in patients with compromised renal function. Also, patients receiving extra cycles of PRRT [7, 8], are appropriate candidates for ^{99m}Tc-MAG3 follow-up when the accepted safe limit of 23-27 Gy absorbed kidney radiation dose adapted from external beam radiation or the 37-45 Gy limit of the biologically equivalent dose (BED) on the kidney, taking into account the kidney mass and the non-uniform localization of renal radioactivity [36, 37] is being exceeded. The application of renal imaging using ^{99m}Tc-DMSA or ^{99m}Tc-MAG3 can be extended to follow-up renal proximal tubule function in other diseases when the kidney is the organ at risk [26, 38].

Conclusion

We demonstrated that quantitative renal imaging in rats is feasible using the NanoSPECT/CT small animal camera, enabling monitoring of renal function over time in a non-invasive way. Both static ^{99m}Tc-DMSA scintigraphy and dynamic dual isotope ^{111}In-DTPA and ^{99m}Tc-MAG3 renography demonstrated that tubular and glomerular functions were affected beyond 90 days after PRRT with 460 MBq ^{177}Lu-DOTA-Tyr^3-octreotate. Lys co-administration preserved kidney function as monitored by ^{111}In-DTPA and ^{99m}Tc-DMSA. These results were consistent with the

findings of kidney histology. Moreover, ^{99m}Tc-MAG3 renography seems to be a more sensitive marker of tubular function after PRRT than ^{99m}Tc-DMSA imaging, serum urea urinary protein content or histology. Therefore ^{99m}Tc-MAG3 scintigraphy might gain a more prominent role in clinical PRRT.

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Nephrotoxicity in mice after repeated imaging using ^{111}In-labelled peptides

Marleen Melis[1], Erik Vegt[2], Mark W. Konijnenberg[3], Monique de Visser[1], Magda Bijster[1], Marcel Vermeij[4], Eric P. Krenning[1], Otto C. Boerman[3] and Marion de Jong[1]

[1] Erasmus MC, dept. of Nuclear Medicine, Rotterdam, the Netherlands
[2] Radboud University Medical Centre, dept. of Nuclear Medicine, Nijmegen, the Netherlands
[3] Covidien, Research & Development, Petten, the Netherlands
[4] Erasmus MC, dept. of Pathology, Rotterdam, the Netherlands

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Chapter 4.3

Abstract

Introduction: We determined the renal radiation dose of a series of ^{111}In-labelled peptides using animal SPECT. Because the animals' health deteriorated, renal toxicity was assessed.

Methods: Wild-type and megalin-deficient mice were imaged repeatedly at 3- to 6-week intervals to quantify renal retention after injection of 40-50 MBq ^{111}In-DTPA-labelled peptides (octreotide, exendin, octreotate, neurotensin, and minigastrin analogues), and the absorbed kidney radiation doses were estimated. Body weight, renal function parameters, and renal histology were determined 16-20 weeks after the first scan and compared with those in naive animals.

Results: Because of high renal retention, ^{111}In-DTPA-exendin-4 scans resulted in a 70 Gy kidney radiation dose in wild-type mice. Megalin-deficient kidneys received 20-40 Gy. The other peptides resulted in much lower renal doses. Kidney function monitoring indicated renal damage in imaged animals.

Conclusions: Micro-SPECT enables longitudinal studies in one animal. However, long-term nephrotoxic effects may be induced after high renal radiation doses, even with ^{111}In-labelled radiotracers.

Introduction

Malignant cells often express a high density of membrane receptors for regulatory peptides such as somatostatin, gastrin and exendin [1]. Radiolabelled peptide analogues specifically targeting these receptors are used as either diagnostic or therapeutic tools in tumour imaging and radionuclide therapy. Peptide receptor radionuclide therapy (PRRT) using ^{90}Y- or ^{177}Lu-labelled somatostatin analogues (Tyr^3-octreotide or Tyr^3-octreotate) have led to significant therapeutic results in terms of tumour response, survival, and quality of life [2]. ^{90}Y and ^{177}Lu both are β^--particle-emitting radionuclides, whereas ^{111}In emits mainly γ-rays and additionally Auger and conversion electrons (CEs). The mean range in tissue is 3900 mm (β^-) and 200 mm (β^-) for ^{90}Y and ^{177}Lu, respectively, and 0.02-10 mm (Auger) and 200-500 mm (CE) for ^{111}In. When ^{111}In-labelled analogues were applied during PRRT, less therapeutic benefit was generated [3, 4], probably because of the short range of the high linear energy transfer of Auger electrons, without cross-fire effect. In line with these observations, ^{111}In-octreotate was more efficacious for the treatment of smaller, than larger, tumours in CA20948 tumour-bearing rats [5].

A potential side effect of PRRT with somatostatin analogues is late renal damage due to partial renal retention of radioactivity in the proximal tubules. Nephrotoxic effects in patients have been described after administration of ^{90}Y-PRRT [6], but rarely after ^{177}Lu-PRRT [7], and not after ^{111}In-PRRT [3].

The scavenging receptor megalin, facilitating the renal reabsorption of many proteins and peptides in the proximal tubules, has proven to be essential for renal reabsorption of radiolabelled octreotide in mice. The uptake of ^{111}In-octreotide was significantly less in kidney-specific megalin-deficient mice than in wild-type (WT) mice in biodistribution studies [8], similar to the reducing effect of the co-administration of lysine in WT animals [9]. In this study, we examined the role of megalin in the renal retention of ^{111}In-labelled somatostatin analogues and other peptides by imaging WT and megalin-deficient mice using a dedicated animal micro-SPECT/CT camera. In consecutive experiments, five different ^{111}In-labelled peptide analogues were administered to the same animals at intervals of at least three weeks [10]. Relatively high-activity doses of ^{111}In (40-50 MBq per mouse) were injected, permitting the accurate quantification of renal radioactivity. The renal uptake of all tested peptides in megalin-deficient mice was only 23%-62% of the uptake in WT mice [10].

The purpose of the current study was to assess the effects of high-dose SPECT imaging using ^{111}In-labelled peptide analogues with regard to risk of nephrotoxicity. The total absorbed radiation dose to the kidneys of WT and megalin-deficient mice was calculated. These results were correlated with renal histology and kidney function parameters, which were determined approximately 20 weeks after the start of the experiment.

Materials and Methods

Animals

Animal studies were conducted in accordance with the guidelines of the Animal Welfare Committee of both medical centres. Kidney-specific megalin-deficient mice (Megalin$^{lox/lox}$;apoE^{Cre}), generated as described previously [11], were kindly provided by Thomas E. Willnow (Berlin, Germany). Animals were bred locally, and animals expressing the apoE^{Cre} gene were identified by polymerase chain reaction analysis. Megalin-deficient animals were compared with WT C57Bl/6 mice (Harlan); five animals of each strain and sex were imaged. Urine, serum, and kidneys from naive animals were tested for reference values.

Radionuclides and peptides

$^{111}InCl_3$ was purchased from Covidien (Petten, the Netherlands). Diethylenetriamine-pentaacetic acid (DTPA) peptides, as listed in Table 1, were radiolabelled as described previously [12, 13]. Specific activity was 20 MBq/nmol of peptide, with a radiochemical yield exceeding 95%, as confirmed by thin-layer chromatography [12].

Table 1: Structure of investigated peptide analogues, their molecular weight, and manufacturers

Peptide	Structure	MW	Company
Octreotide	DTPA-octreotide	1000	Covidien, Petten, The Netherlands
Octreotate	DTPA,Tyr^3-octreotate	1000	Mallinckrodt, St Louis, MO, USA
Exendin	DTPA-Lys^{40}-exendin-4	4200	Peptide Specialty Laboratories GmbH Heidelberg, Germany
Neurotensin	DTPA-G(Pip)^6,G(Pam)^8,tBuG^{12}-neurotensin$^{6-13}$	815	BioSynthema, St Louis Mo, USA
Minigastrin 0	DTPA-D-Glu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH$_2$)	1832	kindly provided by Dr. M. Behe, Marburg, Germany

Molecular imaging

Mice were imaged using a four-headed multi-pinhole SPECT/CT camera (NanoSPECT/CT, Bioscan Inc., Washington D.C., USA). Scans were obtained at 3 and 24 h after the injection of 40-50 MBq ^{111}In-DTPA-peptide (2 nmol) while the animals were anesthetized with isoflurane/O_2 and body temperature was maintained using a heated bed. The imaging procedure was repeated in these animals after at least three weeks of decay of ^{111}In. The order and intervals of the experiments are listed in Table 2. After the final scan, the animals were euthanized; relevant tissues were dissected and weighed, and their activity was determined in a γ-counter.

Nine-pinhole apertures with a diameter of 1.4 mm were used on each head of the camera, the field of view was 16 mm. On the basis of the CT topogram, a body range of 30 mm covering the renal region was scanned in 24 min, with 120 s per projection. Settings of the ^{111}In energy peaks were 171 and 245 keV. The amount of radioactivity in a volume of interest of the kidneys was quantified using InVivoScope software (Bioscan, Inc.). Detected counts were converted to

MBq using a correction factor obtained by scanning a phantom, filled with a known amount of ^{111}In activity, with the same volume as the mouse body to correct for attenuation.

Dosimetry

The mathematic dosimetry model for mice as developed by Hindorf et al. [14] was used. The residence time of ^{111}In was assumed to follow a single exponential clearance pattern. Furthermore, only the kidney self-radiation dose was assumed. The renal uptake of ^{111}In-labelled octreotide in male mice and ^{111}In-labelled neurotensin in both sexes was too low to quantify at 24 h after injection; therefore, the clearance rate was assumed to be similar to that of ^{111}In-labelled octreotide in female mice.

Analytical procedures

Urinary protein was measured using a commercially available colorimetric assay purchased from BioRad (Veenendaal, The Netherlands). Urine was collected for 24 h in metabolic cages, 12 weeks (male mice) or 16 weeks (female mice) after ^{111}In-DTPA-exendin-4 imaging. At euthanasia, four or three weeks later, respectively, serum was collected to determine urea and creatinine levels using standard clinical chemistry procedures.

Histology

One of the dissected kidneys was fixed in 10% buffered formalin and embedded in paraffin. Sections (4 µm) were cut, stained with haematoxylin-eosin or periodic acid-Schiff reagent, and evaluated microscopically to score renal damage according to a scale from grade 0 (no damage) to grade 4 (severe damage) as described previously [15].

Results

The absolute amount of retained radioactivity in WT female controls 3 h after injection ranged from 5% (neurotensin) to 10-15% (somatostatin analogues), 65% (minigastrin), and 225% (exendin) injected activity per gram of kidney. All peptides tested showed less renal uptake in megalin-deficient mice than in WT controls (reduction ranging from ~40% [exendin] to ~55% [minigastrin], ~60% [somatostatin], and ~75% [neurotensin]) [10]. The uptake values combined with the S value [14], related to kidney mass as determined after euthanasia, were used to calculate the absorbed kidney dose after each scan. As an example, this calculation is shown in Table 3 for ^{111}In-DTPA-exendin-4, resulting in absorbed kidney radiation doses ranging from 20-40 Gy in megalin-deficient to more than 70 Gy in WT mice (Table 2).

Surveillance of the animals revealed that female WT mice showed some subdued but still responsive behaviour approximately 16 weeks after this dose. Body weight loss was observed as well (data not shown). The relationship with increased urinary protein levels was not significant, but a trend of elevated levels in imaged animals was found (Figure 1). Some weeks later, serum urea and creatinine levels were found to be elevated in scanned animals - creatinine both in WT and megalin-deficient mice, urea only in WT animals (Figure 1). Grading of

Table 2: Absorbed kidney radiation doses (mean ± SD) after subsequent injection of ^{111}In-labelled peptides

absorbed kidney dose (Gy)		Female mice			Male mice	
	Time (wk)	WT	Megalin-deficient	Time (wk)	WT	Megalin-deficient
octreotide	0 wk	2.1 ± 0.5	1.0 ± 0.2	0 wk	1.9 ± 0.5*	0.4 ± 0.2 *
exendin	4 wk	73 ± 9	38 ± 11	4 wk	72 ± 6	21 ± 4
octreotate	13 wk	3.3 ± 1.0	1.1 ± 0.3	9 wk	2.9 ± 0.6	0.5 ± 0.1
neurotensin	20 wk	0.8 ± 0.2 *	0.16 ± 0.04 *	13 wk	0.7 ± 0.2 *	0.16 ± 0.04 *
minigastrin	23 wk	28 ± 4	5 ± 3	20 wk	30 ± 2	7 ± 1

Clearance assumed to be equivalent to octreotide in females.
Data are mean ± SD.

histological renal damage revealed that the most pronounced effects were found in scanned female WT mice, showing thickening and necrosis of tubular basal lamina and glomerulosclerosis. In megalin-deficient mice, similar histological damage was detected but less frequently (Figure 2).

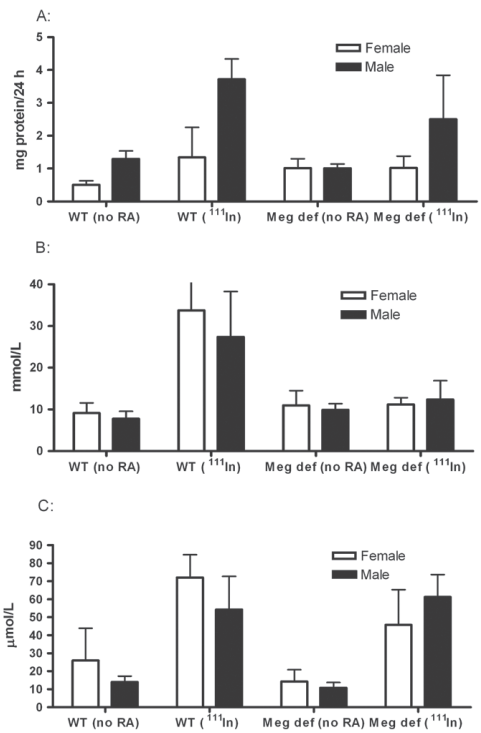

Figure 1: Urine and serum chemistry of both sexes of WT and kidney-specific megalin-deficient mice, either non-scanned (no RA) or scanned (^{111}In).
A: Urinary protein (mg/24 h) measured 12-16 weeks after ^{111}In-DTPA-exendin-4 imaging.
B and C: Urea in serum (mmol/l (B) and creatinine in serum (µmol/l) (C), both determined at 16-19 weeks after ^{111}In-DTPA-exendin-4 imaging.
n = 2-8 per group. Values are presented as mean ± SD. Meg def = megalin-deficient

Table 3: Calculation of absorbed kidney dose after ^{111}In-DTPA-exendin-4

		3 h p.i.		24 h p.i.				
	Injected activity (MBq)	**total MBq**	**% Injected activity**	**total MBq**	**% Injected activity**	**kidney mass (mg)**	**mGy/MBq**	**absorbed dose (Gy)**
WT F	42.4 ± 3.0	27.2 ± 2.6	64.0 ± 2.2	15.5 ± 1.7	44.4 ± 2.9	140 ± 13	1.66 ± 0.15	73 ± 9
Meg def F	42.6 ± 4.7	16.1 ± 3.9	37.8 ± 8.1	8.5 ± 2.4	24.6 ± 6.2	156 ± 17	0.86 ± 0.23	38 ± 11
WT M	39.2 ± 3.5	33.2 ± 3.3	69.3 ± 1.4	16.3 ± 1.4	42.7 ± 3.5	189 ± 11	1.46 ± 0.10	72 ± 6
Meg def M	35.5 ± 3.1	17.5 ± 2.9	42.0 ± 8.2	7.0 ± 1.3	20.7 ± 4.8	225 ± 14	0.50 ± 0.11	21 ± 4

Data are mean ± SD.

Discussion

Recent improvements in resolution and sensitivity of small-animal SPECT cameras opened new possibilities in preclinical research, including serial imaging of the same animal, leading to reduction of animal numbers [16]. In this study in mice, we quantified renal uptake of several ^{111}In-labelled peptides, enabling dosimetric calculations. Although the sensitivity of multi-pinhole micro-SPECT cameras is relatively high, we aimed for 1 MBq in the targeted organ to allow accurate quantification. In this comparative study, we administered 40-50 MBq of each ^{111}In-labelled peptide to achieve reliable quantification of renal retention, even in megalin-deficient mice with an expected renal uptake lower than that in WT mice. However, renal damage developed at approximately 16 weeks after an unexpectedly high (38-70 Gy) kidney radiation dose. On the basis of these results, we had to repeat imaging of some peptides in naive animals to exclude the possibility that results were affected by impaired renal function.

Patients treated with ^{111}In-DTPA-octreotide PRRT did not encounter long-term nephrotoxic effects after a cumulative kidney dose of 45 Gy [3], whereas a safe kidney dose limit of 23 Gy was adapted from external-beam radiation [17]. These patients acquired the total ^{111}In-dose, fractionated in a minimum of eight cycles with at least two week intervals, whereas the mice in the current study received the 40- to 70-Gy kidney dose after a single administration. Kidney protection by dose fractionation also was observed after ^{177}Lu-octreotate PRRT in rats [15].

Dose calculations were based on published S values for ^{111}In in mouse kidneys [14]; however, other authors described different, but comparable, S values [18, 19]. All are voxel-based models using either geometrical shapes representing organs [14] or more realistic shapes [18, 19], assuming homogenous distribution of the radioactivity. This may lead to underestimation of the effective dose, because renal radioactivity is primarily localized in the cortex, resulting in higher radiation doses to the tubules (and glomeruli) [20]. Another reason for dose underestimation might be the assumption of exponential clearance based on only two time points. Acquiring images at

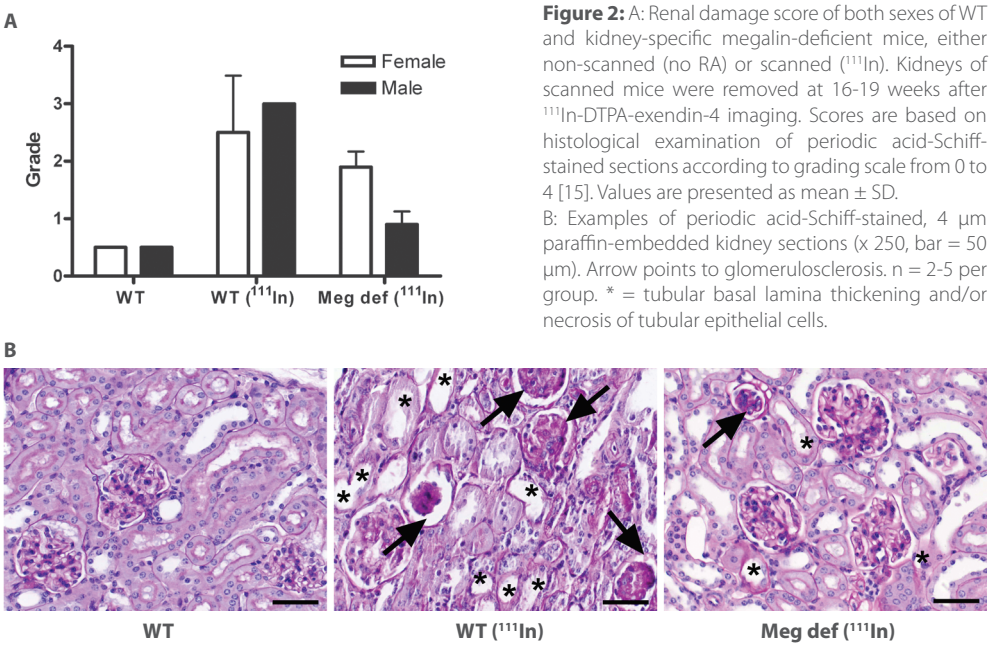

Figure 2: A: Renal damage score of both sexes of WT and kidney-specific megalin-deficient mice, either non-scanned (no RA) or scanned (^{111}In). Kidneys of scanned mice were removed at 16-19 weeks after ^{111}In-DTPA-exendin-4 imaging. Scores are based on histological examination of periodic acid-Schiff-stained sections according to grading scale from 0 to 4 [15]. Values are presented as mean ± SD.
B: Examples of periodic acid-Schiff-stained, 4 µm paraffin-embedded kidney sections (x 250, bar = 50 µm). Arrow points to glomerulosclerosis. n = 2-5 per group. * = tubular basal lamina thickening and/or necrosis of tubular epithelial cells.

more, especially later, time points will provide better insight into the clearance and residence time of radiolabelled peptides and lead to more accurate dosimetry. In addition, biodistribution studies should be performed when relatively low renal uptake has to be quantified [12]. In humans, the total kidney dose delivered by ^{111}In is partially due to γ-irradiation; this dose is less important in mice because of much smaller kidneys. Taken together, these issues hamper comparison between mice and humans concerning effects of absorbed kidney doses.

Body weight loss was the first indication of nephrotoxicity in these mice. Renal dysfunction was confirmed by elevated urinary protein content and by elevated urea and creatinine serum levels, both to a moderate extent. Microscopic evaluation of kidney sections revealed histological damage in both tubuli and glomeruli. So, after megalin-mediated reabsorption of ^{111}In-labelled peptides into proximal tubular cells, the glomeruli were also affected. The maximal range of Auger electrons (0.02-10 mm), and especially the longer range of CEs (200-500 mm), is sufficient to reach adjacent glomeruli. In the future – in addition to the chemical and histological studies that have been performed [16] - we will perform renographic studies using ^{99m}Tc-DMSA (dimercaptosuccinic acid), ^{99m}Tc-MAG3 (mercapto acetyl tri glycine), and ^{111}In-DTPA to gain insight into both tubular and glomerular function after high absorbed kidney doses.

In retrospect ^{111}In-DTPA-exendin-4 imaging should have been performed as the last peptide in the series, and the injected activity dose could have been less - 10 MBq instead of 40-50 MBq.

Conclusion

An absorbed kidney dose of more than 40 Gy due to renal accumulation of ^{111}In-labelled peptides resulted in long-term nephrotoxicity in mice. The small-range, low-energy Auger electrons and CEs emitted by ^{111}In caused both tubular and glomerular damage. Micro-SPECT offers the opportunity to perform follow-up studies in one animal by consecutive scanning. However, the risk of side effects due to high radiation doses must be considered.

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Summary, additional studies and future directions

Samenvatting, aanvullende studies en richtlijnen voor de toekomst

Chapter 5

Summary

Specific tumour targeting using radiolabelled peptide analogues is an attractive modality to visualize primary tumours and tumour metastases. In addition it can be applied to treat tumour patients. Tumours originating from neuroendocrine cells often overexpress hormone receptors; e.g somatostatin-, gastrin- or glucacon-like peptide-1 (GLP-1)- receptors [1]. These receptors can be targeted using radiolabelled somatostatin, minigastrin/CCK or exendin peptide derivatives, respectively.

In 1994 the somatostatin analogue ^{111}In-DTPA-octreotide (Octreoscan®) was approved by the FDA for clinical γ-camera imaging, i.e. peptide receptor scintigraphy (PRS) for diagnostic purposes to monitor the disease in patients with NET. Octreoscan® is characterized by high affinity for somatostatin receptor (sst) subtypes 2, 3 and 5 [2], whereas most neuroendocrine tumours overexpress sst2 [3]. Since ^{111}In is emitting therapeutic Auger- and conversion electrons next to γ-radiation, ^{111}In-DTPA-octreotide was tested for peptide receptor radionuclide therapy (PRRT), but objective tumour responses were rare [4].

Tyr^3-octreotate, with a very high affinity to sst2, was developed [5, 6] and DOTA-chelation enabled stable radiolabelling with the therapeutic β$^-$-emitters ^{90}Y and ^{177}Lu. Nowadays PRRT with ^{90}Y-DOTA,Tyr^3-octreotide or ^{177}Lu-DOTA,Tyr^3-octreotate has been implemented in several centres [7-9], with favourable therapeutic results compared to historical controls [10-12]. According to SWOG criteria ^{177}Lu-DOTA,Tyr^3-octreotate PRRT resulted in ~55% of the patients in an objective response, while in ~30% stable disease was found and in ~15% of the patients progression of disease was still encountered [7].

Besides specific binding of radiolabelled somatostatin peptide analogues to tumour cells, uptake is also found in the spleen, liver and kidneys. The first publications on clinical ^{90}Y-DOTA,Tyr^3-octreotide PRRT reported that late nephrotoxicity occurred in about 10% of patients leading to end stage renal disease [13, 14]. Since these alarming reports the cumulative absorbed kidney doses during PRRT were limited to 23-27 Gy, based on experiences with EBRT [15], or to 37-45 Gy applying the principle of the biological equivalent dose (BED), correcting a.o. for the effect of dose fractionation, regional distribution of the radioactivity and kidney size [16-18]. Furthermore, co-infusion of cationic amino acids during the administration of PRRT reduced the renal retention of radiolabelled somatostatin analogues with approximately 40% offering kidney protection [13, 19, 20]. Recent reviews evaluating the incidence of long-term renal toxicity after PRRT describe very few cases of renal damage when ^{177}Lu-DOTA,Tyr^3-octreotate with amino acids was applied [11, 16]. However, in 30% of patients receiving ^{90}Y-DOTA,Tyr^3-octreotide PRRT low grade renal toxicity was observed with rising creatinine levels and loss of creatinine clearance [16, 21] illustrating the need of kidney protection during PRRT.

In *Chapter 1.1* the aim of the current research to identify the mechanism of renal uptake of radiolabelled peptides in order to design ways to protect the kidneys from harmful side effects of PRRT was introduced.

A broad overview of the current status of kidney protection during PRRT using radiolabelled somatostatin analogues is been given in *Chapter 1.2*, which focussed mainly on the basic amino acids lysine and arginine and their interference with the renal reabsorption of radiolabelled somatostatin analogues. This method is successfully used in the clinical setting during PRRT nowadays. Recently, also Gelofusine and albumin fragments were defined as agents achieving similar reductions of renal retention of different radiopeptides upon co-administration.

For research into prevention of renal damage elicited by irradiation during PRRT the uptake mechanism responsible for renal uptake and retention of radiolabelled peptide analogues was investigated The conclusion that megalin plays an important role in the renal uptake of radiopeptides appeared to be helpful to develop new strategies for kidney protection as is described in *Chapter 2*.

The level of renal uptake of radiolabelled somatostatin, bombesin, neurotensin, minigastrin and CCK analogues covered a range from very low (CCK) to very high (minigastrin) uptake in the kidneys of rats and mice, as is reported In *Chapter 2.1*. The rank order of CCK < bombesin = neurotensin < octreotide << minigastrin was similar in both species, with a faster washout from mouse kidneys. Moreover, we found no gender difference in the renal uptake in rats, whereas in mice up to four times higher levels of retained renal radioactivity was found for octreotide and bombesin analogues in females vs. males. In clinical studies no gender differences in renal uptake of radiopeptides are described [17], therefore we concluded that the rat is a favourable model over the mouse to study the renal uptake of newly developed radiopeptides.

The localization of the radioactivity in the kidney as determined by *ex vivo* autoradiography was similar for all tested radiopeptides: most radioactivity was localized in the renal cortex, less in the outer medulla and no radioactivity was detected in the renal pelvis. This pattern correlated with the results of immunohistochemical staining of kidney sections using antibodies recognizing megalin, which is predominantly expressed in the first convoluted parts of the proximal tubules (PCT), located in the renal cortex, whereas less expression in the proximal straight tubules (PST) located in the outer medulla is found.

The suggestion that the net negatively charged multi-ligand scavenging receptor megalin plays a role in the renal uptake of radiopeptides was confirmed in the studies described in *Chapter 2.2*. First, using micro-autoradiography, it was demonstrated that the localization of radioactivity was restricted to the proximal tubules in the renal cortex. Radioactivity was not observed in the glomeruli or distal tubules. Next, three agents; lysine, sodium maleate and cisplatin, were co-administered in rats with radiolabelled somatostatin analogues because of their potential interference with the active endocytic processes mediated by megalin in the proximal tubules in the reabsorption of many small proteins and peptides. All tested agents resulted in renal uptake reduction of radioactivity to 60%, 15% and 70%, respectively. An excess of the positively charged amino acid lysine co-administered with radiolabelled octreotide, containing one lysine residue, probably interfered with the reabsorption of this compound resulting in decreased renal radioactivity. The agent sodium maleate induces reduction of the

energy metabolism of cells and inhibition of the membrane reconfiguration during endocytosis. This might explain the huge reduction of radioactivity levels in the kidneys after pre- or co-treatment with maleate. The chemotherapeutic drug cisplatin often induces nephrotoxic side effects, but only in PST, explaining the relatively low reduction of renal uptake of ^{111}In-DTPA-octreotide. Because of serious side effects after both maleate and cisplatin, these agents are not suited for clinical implementation in kidney protection regimes during PRRT.

Kidney-specific megalin-deficient mice offered the opportunity to investigate the renal uptake and retention of ^{111}In-DTPA-octreotide in these animals versus wild-type mice with normal renal megalin expression. The first results are described in *Chapter 2.3*. Molecular imaging at 4 and 24 h after injection of ^{111}In-DTPA-octreotide was performed using a dedicated small animal SPECT camera. After the last scan the amount of radioactivity per kidney was quantified. In both sexes a striking 70%-85% reduction of uptake in the megalin-deficient kidneys was found, which was confirmed by *ex vivo* autoradiography of frozen sections of the kidneys.

In the next study using these megalin-deficient animals, described in *Chapter 2.4*, the renal uptake of ^{111}In-labelled neurotensin, exendin, and minigastrin analogues was determined and compared to that of octreotide and octreotate. Quantification of the uptake and retention in the kidneys of megalin-deficient and wild-type mice was based on micro-SPECT. Consecutive imaging experiments in the same mice were performed with 3-9 week intervals. Separately biodistribution studies were performed. Less uptake of all tested radiopeptides was observed in the kidneys of the megalin-deficient mice compared to wild-type controls. Thus, the renal reabsorption of radiopeptides from various peptide families, with different sizes and with variable net charges appeared to be at least partly megalin-mediated.

Based on the conclusion that megalin plays an important role in renal reabsorption after administration of radiopeptides, Gelofusine was tested for reduction of renal uptake of radiopeptides. This plasma expander consists of negatively charged succinylated gelatin, with a mean molecular weight of 30 kDa. Gelofusine is used in the clinic to treat hypovolemia in states of shock in critical care patients. A transient loss of proteins and peptides is detected in the patients' urine after infusion, pointing to a temporal drop in efficiency of renal reabsorption [22]. Indeed, in a study in healthy volunteers, Gelofusine co-infusion with Octreoscan® offered a similar reduction of the renal retention as co-infusion with cationic amino acids [23]. In *Chapter 3.1* we determined the optimal Gelofusine dose to be co-administered in rats to obtain the highest reduction of renal uptake of ^{111}In-DOTA,Tyr^3-octreotate. A dose of 80 mg/kg body weight was sufficient to accomplish 50%-60% reduction of renal retention, this was even improved to 70% reduction when Gelofusine was combined with lysine. Furthermore we demonstrated that the tumour uptake of ^{111}In-DOTA,Tyr^3-octreotate was not affected by co-administration of Gelofusine and lysine, offering a threefold increase of the tumour to kidney ratio versus control.

Another agent, the radical scavenger or anti-oxidant amifostine is applied as cytoprotector during clinical radiotherapy to attenuate side effects in healthy tissues induced by reactive oxygen radicals resulting from irradiation. Previous investigations in rats proved that co-administration of amifostine during ^{177}Lu-DOTA,Tyr^3-octreotate PRRT reduced long-term neph-

rotoxic effects [24]. In the study as described in *Chapter 3.2* we aimed to elaborate on the mechanism underlying the kidney protection by amifostine and to rule out possible tumour protection. The effects of co-administration of amifostine with radiolabelled octreotate was tested in tumour-bearing rats, both in biodistribution studies 24 h after injection of ^{111}In-DOTA,Tyr^3-octreotate and in a PRRT study using ^{177}Lu-DOTA,Tyr^3-octreotate with a follow-up of >120 days post therapy (p.t.). No interference of amifostine with tumour uptake or therapeutic effects was demonstrated. However, the initial renal uptake was decreased to 40% of control level when amifostine was co-injected. This unexpected phenomenon was confirmed in an *in vitro* assay using the megalin-receptor expressing BN-16 cell line. Co-incubation of amifostine or its active metabolite WR-1065 reduced binding of ^{111}In-DOTA,Tyr^3-octreotate to these cells to 66% and 55% of control level, respectively. These data suggest that amifostine/WR1065 reduced initial renal radioactivity levels in a megalin-mediated way, comparable to the reduction obtained with lysine co-administration. Therefore, besides scavenging of harmful oxygen species interfering in the cascade of events leading to renal fibrosis, a lower level of retained β^--emitting ^{177}Lu in the kidneys could have led to kidney protection during PRRT when amifostine is co-administered [24].

The development of nephropathology after irradiation is a long-term process taking several months in rats. Monitoring of kidney function over time can be performed by measurements of serum creatinine, urea or urinary protein levels. Another non-invasive way to detect the onset of renal failure is imaging of the uptake or clearance of radiotracers specific for visualization of kidney function using a γ-camera. ^{99m}Tc-DMSA, a tracer retained in the cortical tubules, has been tested to monitor the kidney function in rats during the follow-up of PRRT using a dedicated small animal SPECT/CT camera. The results of these studies are described in *Chapter 4.1*. The dose dependent level of renal damage >100 days after 278 or 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotate PRRT inversely correlated with ^{99m}Tc-DMSA uptake per kidney based on static imaging. The higher the absorbed renal radioactivity dose, the lower the ^{99m}Tc-DMSA uptake when measured 100-150 days after PRRT. Moreover the pattern of retained renal ^{99m}Tc-DMSA demonstrated specific loss of function of cortical tubules; only in the PCT after the low PRRT dose and in the PST as well after the high PRRT dose. In *Chapter 4.2* monitoring of rat kidney function after 460 MBq ^{177}Lu-DOTA,Tyr^3-octreotate PRRT with or without lysine co-injection is described, using the tracers ^{111}In-DTPA, ^{99m}Tc-MAG3, and ^{99m}Tc-DMSA. ^{111}In-DTPA and ^{99m}Tc-MAG3 were used to image the dynamic processes of glomerular filtration (^{111}In-DTPA) and tubular secretion (^{99m}Tc-MAG3). In control rats ^{111}In-DTPA and ^{99m}Tc-MAG3 showed time-activity curves with a peak a few minutes after injection, followed by rapid secretion into the tubules and excretion into the urine. When renal damage was developed >90 days after PRRT, hardly any peak activity of both agents could be detected. In rats that received PRRT plus lysine co-administration to protect the kidneys, ^{111}In-DTPA clearance and ^{99m}Tc-DMSA uptake remained unaffected. In contrast, ^{99m}Tc-MAG3 clearance still showed an aberrant pattern. We concluded that ^{99m}Tc-MAG3 is a more sensitive marker of early renal damage induced after PRRT than ^{99m}Tc-DMSA uptake or serum chemistry measuring creatinine and urea levels.

In *Chapter 4.3* we describe that nephrotoxic effects were detected in mice after consecutive imaging experiments using ^{111}In-labelled radiopeptides. This was unexpected, because in early clinical ^{111}In-DTPA-octreotide PRRT studies no renal damage was encountered, even

after a renal absorbed dose of 45 Gy, thus clearly exceeding the accepted safety limit of 23 Gy. However, it appeared that the very high renal uptake and retention of ^{111}In-exendin (225% IA/g kidney) delivered a 70 and 40 Gy dose in kidneys of wild-type and megalin-deficient mice, respectively, leading to renal failure in both mouse strains >90 days after the ^{111}In-exendin injection. Thus, a high renal radiation dose by γ-radiation and Auger- and conversion electrons emitted by ^{111}In, may also cause long-term nephrotoxicity. Small animal SPECT enables monitoring of disease or treatment in one animal over time, but the risk of an accumulated radiation dose to the kidneys exceeding the safety limit has to be considered, as shown in this chapter.

Additional studies

Given the fact that megalin plays a pivotal role in the renal reabsorption of various radiolabelled peptides, potential inhibition of this process by co-administration of albumin derivatives, was a logical step for further investigation because albumin is one of the main ligands of megalin. Intact albumin (68 kDa) is only marginally filtered in the glomeruli, therefore enzymatically cleaved fractions of albumin (FRALB) were co-administered with ^{111}In-DTPA-octreotide in rats, resulting in less renal uptake than in control rats [25]. This confirmed the hypothesis that co-administration of albumin fractions decreases the level of megalin-mediated reabsorption of somatostatin analogues. In a follow-up study well-defined synthetic peptides derived from albumin, with varying charges also were tested as specific inhibitors of renal uptake. Co-administration of a 36 amino acid peptide (3.8 kDa) derived from the C-terminal end of albumin (ALBPEP), offered a broad reduction of renal uptake of various radiopeptides, ranging from 26% (exendin) and 33% (octreotide) to 88% (gastrin) inhibition [26].

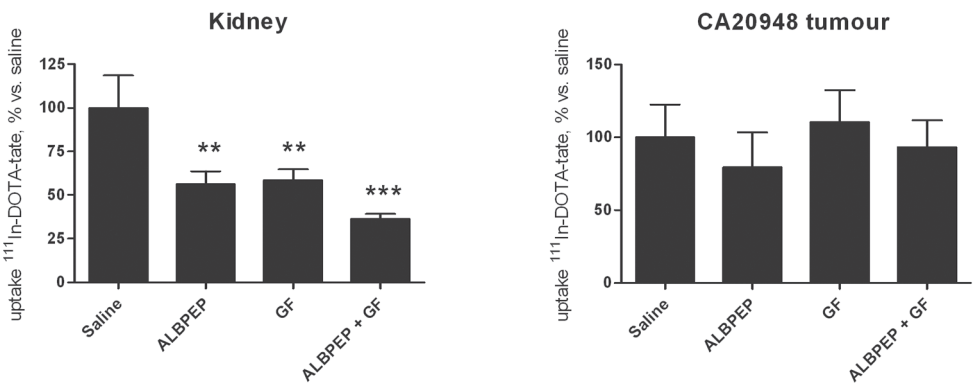


Figure 1: Biodistribution data 24 h after injection of 15 µg ^{111}In-DOTA,Tyr^3-octreotate (^{111}In-DOTA-tate) in rats with or without co-administration of albumin derived peptide (ALBPEP (4 mg/rat)) and/or Gelofusine (GF (80 mg/kg)). Uptake in kidneys and sst-expressing CA20948 tumours was calculated as % injected activity/gram (%IA/g) ± SD and the value of the saline group was set at 100%. n = 4 rats per group. Difference in renal uptake vs. saline group of single ALBPEP and GF groups (** = $p<0.01$) and the ALBPEP+GF (*** = $p<0.001$) group were significant, whereas the difference between ALBPEP+GF and single ALBPEP or GF also was significantly different (** vs. ALBPEP, *** vs GF). No significant differences were found for CA20948 tumour uptake.

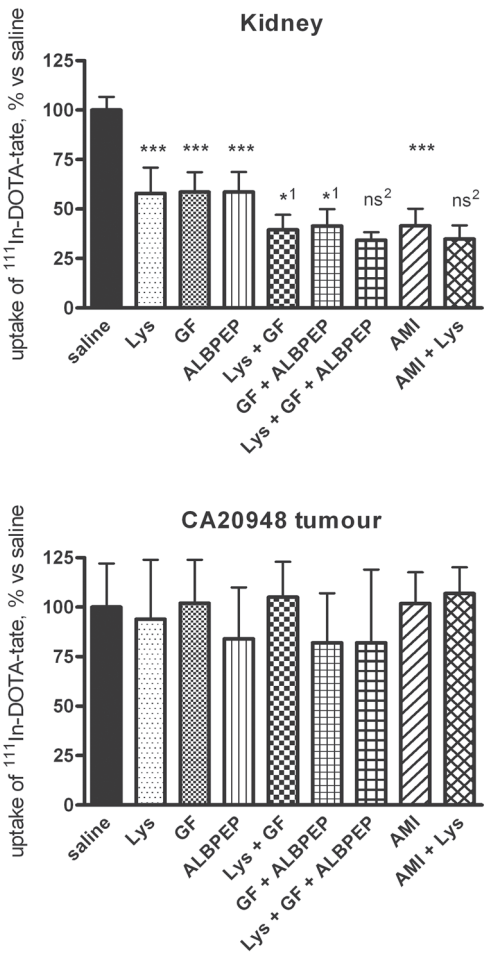


Figure 2: Biodistribution data 24 h after injection of 15 µg ^{111}In-DOTA,Tyr^3-octreotate (^{111}In-DOTA-tate) in rats with or without co-administration of Lys (400 mg/kg), GF (80 mg/kg), ALBPEP (4 mg/rat) and/or AMI (50 mg/rat).
Uptake in kidneys and sst-expressing CA20948 tumours was calculated as % injected activity/gram (%IA/g) ± SD. n = 2-4 rats per group. The value of the saline group was set at 100%. Difference in renal uptake vs. saline group of all single agent groups was significant ($p<0.001$). Combination of Lys+GF and GF+ALBPEP showed significant additive reduction vs. single use ($p<0.01$ = *[1]), whereas Lys+GF+ALBPEP was not significantly (ns[2]) different vs. Lys+GF or GF+ALBPEP. The combination of AMI+Lys did not result in significantly less renal uptake vs. single Lys or AMI (ns[2]). No significant differences were found for CA20948 tumour uptakes in all groups.

To assess possible interference of albumin derived peptide with tumour uptake, ^{111}In-DOTA,Tyr^3-octreotate biodistribution studies in CA20948 tumour-bearing rats were performed (Figure 1). The combination of co-administration of both albumin derived peptide and Gelofusine was tested as well, resulting in a significant additive reduction of the renal retention of ^{111}In-DOTA,Tyr^3-octreotate. No effects on tumour uptake were noticed.

An overview of all obtained results on reduction of renal uptake of radiolabelled octreotate in rats after co-administration of lysine (Lys), Gelofusine (GF), ALBPEP and amifostine (AMI) (and combinations), are summarized for both renal and tumour uptake in Figure 2.

None of the administered agents affected the somatostatin receptor-mediated tumour uptake of ^{111}In-DOTA,Tyr^3-octreotate significantly, in contrast to the overall reduced renal uptake and retention. Some combinations of agents showed significant additive reduction of renal retention, whereas others did not. The observation that Lys+GF+ALBPEP did not result in further reduction compared to Lys+GF and GF+ALBPEP suggests that either the lowest achievable level of renal uptake of radiolabelled octreotate was reached, or that reabsorption via the same regions of megalin domains was inhibited. Lys+AMI/WR1065 also did not show a cumulative reduction of radioactivity in the kidneys, which could be explained by a similar mechanism of action.

The remaining level of renal uptake after maximum kidney uptake inhibition by several agents and in kidney-specific megalin-deficient mice may be due to fluid phase endocytosis. Besides active renal reabsorp-

tion this is another process by which compounds are captured from the blood and primary urine in the kidney; this process cannot be inhibited by amino acids [27]. Furthermore, in mice and men, but not in rats, sst-expression is demonstrated in the kidney, responsible for some receptor-specific retention of somatostatin analogues in the kidneys that will not be influenced by agents interfering in the megalin-mediated uptake [28, 29]. In patients this sst-expression maximally accounts for 20% of renal radioactivity. Besides proximal tubular expression of somatostatin receptors, GLP-1R and CCK2-R has been described as well [30-32].

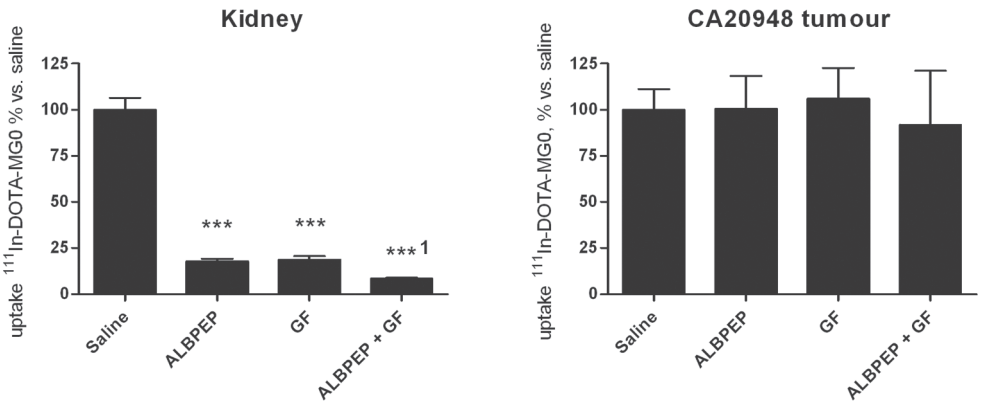


Figure 3: Biodistribution data 24 h after injection of 0.1 µg ^{111}In-DOTA-minigastrin 0 (^{111}In-DOTA-MG0) in rats with or without co-administration of ALBPEP (4 mg/rat) and/or Gelofusine (GF (80 mg/kg)).
Uptake in kidneys and sst-expressing CA20948 tumours was calculated as % injected activity/gram (%IA/g) ± SD and the value of the saline group was set at 100%. n = 4 rats per group. Difference in renal uptake of all groups vs. saline group was significant ($p<0.001$), differences between single ALBPEP and GF vs. GF+ALBPEP as well = ***[1]. No significant differences were found for CA20948 tumour uptake in all groups.

The successful results on kidney protection from radiolabelled octreotate obtained with lysine, Gelofusine and albumin derived peptide prompted us to investigate potential additive reducing effects on renal uptake of radiolabelled minigastrin analogues showing very high renal uptake values in tumour-bearing rats. A prominent reduction of more than 80% of the renal uptake of ^{111}In-DOTA-minigastrin 0 was found after single co-administration of albumin derived peptide or Gelofusine. Co-injection of their combination resulted in a significant extra reduction to 90% of the renal uptake found in rats that received saline as control (Figure 3). CA20948 tumour uptake remained unaffected in all cases. The reduced radioactivity levels in the kidneys after ALBPEP+GF without interference with tumour uptake accomplished a 2.5 and 10-fold increase of the tumour to kidney ratios after ^{111}In-DOTA,Tyr^3-octreotate and ^{111}In-DOTA-minigastrin 0 injection, respectively.

In Table 1 the results of several renal protection studies in rats from the literature have been summarized along with our recently obtained results, concerning biodistribution studies with radiolabelled somatostatin, exendin and minigastrin 0 analogues. The percentages of reduction of renal retention obtained are compared with those in non-protected animals.

Since minigastrin contains 6 anionic glutamic acid (Glu) residues, no effect on renal uptake was expected from co-administration with lysine (Lys). Indeed only marginal effects were detected [33]. When polyglutamic acids (PGA) of at least five Glu residues were used, however, a 80%-90% inhibition of renal radioactivity was achieved [34]. Co-administration of Lys or PGA with ^{111}In-exendin showed less convincing results, indicating the choice of the inhibitory amino acids should be specifically adapted to the composition of the administered radiolabelled peptide analogue. Charge seems to play an important role in this respect.

	Lys	GF	GF + Lys	PGA	GF + PGA	FRALB	ALBPEP	ALBPEP + GF
Octreotide/ octreotate	40-50	40-50	50-65	0-15	nd	30-35	30-45	65
Exendin	0-15	20-25	nd	25-35	35-45	50-55	25	nd
Minigastrin 0	0	45-80	nd	80-90	80-90	90-95	80-85	90

Table 1: Summary of renal protection studies in rats, adapted from [25, 26, 33-35] and recently obtained results. Gross percentage reduction of renal retention is given. Lys=lysine, GF=Gelofusine, PGA=polyglumatic acid, FRALB=albumin fractions, ALBPEP=albumin derived peptide, nd = not determined

Application of Gelofusine and albumin derivatives offered reduction of renal uptake of all tested radiopeptides, although the extent of reduction varied largely. Simultaneous co-administration of these two agents with radiolabelled octreotate effectuated even enhanced inhibitory effects on renal uptake, which was even more prominent when co-administered with radiolabelled minigastrin.

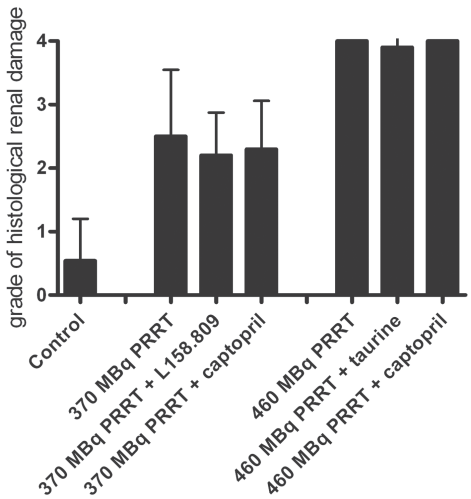


Figure 4: Extent of histological renal damage at day 146 (460 MBq ^{177}Lu-PRRT) or day 180 (370 MBq ^{177}Lu-PRRT) after therapy with or without co-treatment with taurine (10 g/l), captopril (0.5 g/l) and L158.809 (20 mg/l) added to drinking water. n = 6-8 per group.

Reduction of the risk of nephrotoxicity after PRRT can be achieved by lowering the initial uptake and retention of radiolabelled peptides in the kidneys, but also by mitigation of long-term PRRT induced renal damage, often fibrotic lesions. Based on radiation protection of the kidneys during EBRT by scavenging of active oxygen radicals using anti-oxidants or angiotensin II inhibition, we recently investigated rat kidney protection after PRRT using taurine as anti-oxidant; L158.809, an angiotensin II receptor blocker (ARB); or captopril, an angiotensin converting enzyme inhibitor (ACEI), reducing ACE dependent conversion of angiotensin I into angiotensin II [36, 37]. When the data on kidney function of these rats were compared with those of rats after PRRT without additives in the drinking water, we were not able to prove significant beneficial effects of

these agents on kidney function after PRRT. The data on histological damage are shown in Figure 4. We concluded that taurine, L158.809 and captopril are not suited as mitigating agents of nephrotoxic effects induced by ^{177}Lu-DOTA,Tyr^3-octreotate-based PRRT.

Future directions

Our current clinical protocol of ^{177}Lu-DOTA,Tyr^3-octreotate PRRT, consisting of four cycles with a total cumulative dose of 29.6 GBq and co-infusion of cationic amino acids, seems to be safe with respect to kidney function [38]. However, after ^{90}Y-DOTA,Tyr^3-octreotide PRRT, in a cumulative dose of 15.2 GBq as used in several clinical studies, kidney safety is an issue [21]. The therapeutic outcome of PRRT is favourable compared to conventional NET treatment modalities, with an objective tumour response in ~55% of the patients and occasional complete tumour regression. However, further increase of cure rates is highly warranted [7]. Therefore, there is a need of safe administration of higher cumulative therapeutic doses to obtain improved tumour responses, in which kidney protection will play a major role [39].

Options to accomplish increased tumour response include:

- Application of more cycles of ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotate [40]
- Combination of ^{90}Y-DOTA,Tyr^3-octreotide and ^{177}Lu-DOTA,Tyr^3-octreotate, either within each cycle or start with ^{90}Y-based cycles followed by ^{177}Lu-cycles or alternating ^{90}Y-and ^{177}Lu-cycles [8, 41-43]
- Combination of ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotate with radiosensitizing agents, such as capecitabine or other chemotherapeutics [11]
- Intra-arterial (locoregional) administration of ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotate instead of the commonly used systemic intravenous route [44, 45]
- Combination of anti-angiogenic agents, e.g. anti-vascular endothelial growth factor antibodies or $\alpha_v\beta_3$-integrin targeting agents such as thalidomide or RGD constructs, with ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotate [46, 47]. Nowadays these agents are combined with radioimmunotherapy.
- Research into induction of increased tumour somatostatin receptor-levels to enhance internalization of ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotate analogues [48]
- Introduction of new peptide analogues with higher affinity and/or pansomatostatin receptor specificity [49]
- Combination of different peptide analogues targeting different receptors co-expressed on tumours, such as sst2 and GLP-1R in gastrinomas [50]

The absorbed kidney radiation dose will increase when more cycles of ^{177}Lu- or ^{90}Y-labelled PRRT will be administered per patient, therefore optimal kidney protection will be essential. Moreover, the bone marrow will be the next organ at risk, because of an increased absorbed radiation dose mediated by circulating radioactivity in the blood [38].

As described in *Chapter 3.1* and by others, the combination of Gelofusine with Lys/Arg co-infusion will offer further reduction of the renal uptake of somatostatin analogues [23]. Although this combination of renal protection agents is already clinically applied with promising results, comparative data on levels of retained renal radioactivity are not available yet [8, 39]. Therefore a pilot study will be started in our institution in the near future to perform kidney and tumour dosimetry during ^{177}Lu-DOTA,Tyr^3-octreotate PRRT in which single Lys/Arg co-infusion during one cycle will be compared with combined Gelofusine and Lys/Arg co-infusion in the next cycle or vice versa. A small percentage of 0.04% of severe anaphylactic reactions and of 1.4% of mild to moderate allergic side effects after Gelofusine administration have been described, therefore vital functions such as heart rate and blood pressure will be registered [39, 51].

Compounds mitigating renal damage after irradiation may be combined with agents reducing the initial renal uptake and retention of radiopeptides, enabling safe implementation of higher therapeutic PRRT doses. In this respect the application of the anti-oxidant amifostine as kidney protector during PRRT needs more research. Previously, favourable effects on long-term kidney function and histology were described [24], which could partly be attributed to reduction of the absorbed kidney radiation dose as described in *Chapter 3.2*. New experiments in which amifostine will be co-administered either prior or post PRRT will discriminate between its short- or long term effects. Even though patients may encounter hypotension, nausea, vomiting and skin rash as side effects after amifostine administration, further research into this drug with respect to PRRT is intriguing. Especially because protection of bone marrow progenitors from harmful radiation effects has been described as well [52-54].

Besides kidney toxicity, bone marrow suppression resulting in a drop of platelets, red and white blood cells may also be encountered during PRRT [38, 55]. Haematologic toxicity is generally mild and reversible and the risk of developing myelodysplastic syndrome (MDS) or leukaemia after PRRT is low when a dose limit of 2 Gy to the bone marrow is maintained. Nevertheless several cases of bone marrow toxicity after PRRT have been described [11]. Application of higher cumulative doses of PRRT, accompanied by an optimal kidney protection regimen, will increase the risk of bone marrow toxicity and the bone marrow will become the dose-limiting organ. The absorbed radiation dose to the bone marrow is caused by cross fire from the blood circulating in the vicinity of bone marrow cells [56]. Specific uptake of radiolabelled somatostatin analogues by somatostatin receptor-expressing progenitor bone marrow cells might play a (minor) role as well [56, 57]. Therefore bone marrow protective strategies similar to kidney protection to reduce the initial uptake will not be effective, except for mitigation of radiation induced damage by amifostine e.g. Autologous bone marrow transplantation may be considered to provide bone marrow support when patients encounter severe haematological toxicity after PRRT. This procedure is already successfully being applied during radioimmunotherapy [58].

The blood clearance of radiopeptides in patients takes much longer than in rats or mice, because of the smaller total blood volume and the higher heart rate in the latter species [59]. This will result in a higher absorbed radiation dose of human bone marrow cells compared to the dose in animals. Moreover, sst-expression is not found on rat bone marrow cells in contrast

to human progenitor cells [57, 60]. Differential bone marrow cell count of terminally ill rats because of nephrotoxicity >100 days after ^{177}Lu-DOTA,Tyr^{3}-octreotate PRRT with >50 Gy on the kidneys, did not reveal aberrations in the bone marrow. Also the blood cell counts of these rats were comparable to those in control rats, indicating that haematological toxicity was not encountered in these rats. Therefore, preclinical investigations in small animals into bone marrow protection during PRRT by amifostine, other agents, or by autologous bone marrow support will not be feasible.

In summary, we demonstrated in preclinical studies that besides cationic amino acids, Gelofusine, albumin derivatives, amifostine, or combinations thereof are promising agents to reduce the renal retention of various tumour-targeting radiopeptides in patients. This is especially important when increased PRRT doses will be administered to obtain improved cure rates. First, safety issues concerning allergic reactions to Gelofusine and putative toxicity of albumin derived peptides should be studied thoroughly. Moreover, albumin derived peptides should be prepared according to good manufacturing practice regulations before they can be tested in clinical trials. Such an agent offers promising prospectives for broad application concerning kidney protection.

Our experience with reduction of radioactivity levels in the kidney may be extended to other newly developed radiolabelled biomolecules, such as neurotensin or bombesin analogues containing one or more Lys residues [61], folate derivatives, Affibody molecules or nanobodies suffering from high levels of retained radioactivity in the kidneys [62-64].

As described in this thesis, kidney radiation by radionuclides, and by X-rays as well, can cause late nephropathy. However, several agents also are known to cause nephrotoxicity as side effect after application in some cases. Renal tubular damage is reported after administration of aminoglycoside based antibiotics such as gentamicin [65], the antifungal drug amphotericin [66], the anti-folate agent methotrexate [67], used as anti-cancer drug, and Cyclosporine A [68] used to prevent rejection of transplanted organs. The latter two drugs also suppress the effects of auto-immune diseases. Co-administration of Gelofusine or albumin derived peptides might interfere in the renal handling of these substances to reduce the risk of renal damage, similar to what we described during the application of radiopeptides.

Samenvatting

De toepassing van radioactief gelabelde peptide analogen is een aantrekkelijke manier om primaire tumoren en uitzaaiingen ervan op een doelgerichte, specifieke manier in beeld te brengen. Daarnaast kunnen deze peptiden therapeutisch worden toegepast om tumorpatienten te behandelen. Tumoren die zijn ontstaan uit cellen van het neuro-endocriene systeem brengen vaak hormoonreceptoren in hoge mate tot expressie; bijv. somatostatine-, gastrine- of glucagon-like peptide-1 (GLP-1)- receptoren [1]. Deze receptoren kunnen doelgericht worden aangetoond m.b.v. respectievelijk radioactief gelabelde somatostatine, minigastrine/CCK of exendin peptidederivaten.

In 1994 werd het somatostatine analoog ^{111}In-DTPA-octreotide (Octreoscan®) goedgekeurd door de FDA voor klinische toepassing voor beeldvorming m.b.v. een γ-camera, de zgn. peptide receptor scintigrafie (PRS) voor diagnostische toepassingen om het ziekteproces in patiënten met neuro-endocriene tumoren (NET) te vervolgen. Octreoscan® wordt gekarakteriseerd door een hoge affiniteit voor de somatostatine receptoren (sst) van subtypes 2, 3 en 5 [2], terwijl de meeste NET sst2 tot over-expressie brengen [3]. Omdat ^{111}In behalve γ-straling ook therapeutische Auger- and conversie elektronen uitzendt werd ^{111}In-DTPA-octreotide getest in peptide receptor radionuclide therapie (PRRT), maar objectieve tumor respons was zeldzaam [4].

Tyr^3-octreotaat, met een hele hoge affiniteit voor sst2, werd ontwikkeld [5, 6] en door het gebruik van de DOTA-chelator was het mogelijk om een stabiele radiolabeling te verkrijgen met de therapeutische radionucliden ^{90}Y en ^{177}Lu die β^--straling uitzenden. Sinds enkele jaren wordt PRRT met ^{90}Y-DOTA,Tyr^3-octreotide of ^{177}Lu-DOTA,Tyr^3-octreotaat in verschillende medische centra toegepast [7-9], waarbij gunstige therapeutische resultaten werden verkregen in vergelijking tot historische controles [10-12]. Beoordeeld volgens SWOG criteria, resulteert ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT bij ~55% van de patiënten in een objectieve respons, terwijl bij ~30% stabiele ziekte wordt bewerkstelligd. Bij ~15% van de behandelde patiënten blijft de ziekte helaas progressief [7].

Behalve specifieke binding van de radioactief gelabelde somatostatine peptide analogen aan tumorcellen, wordt ook opname gevonden in de milt, lever en nieren. De eerste publicaties over klinische ^{90}Y-DOTA,Tyr^3-octreotide PRRT rapporteerden dat bij ongeveer 10% van de patiënten late nierschade (nefrotoxiciteit) optrad, leidend tot het eindstadium van nierfalen [13, 14]. Na deze alarmerende publicaties werden de cumulatieve geabsorbeerde stralingsdoses op de nieren tijdens PRRT gelimiteerd tot 23-27 Gy, gebaseerd op ervaringen uit de radiotherapie [15]. Ook wordt een bovengrens van 37-45 Gy toegepast op basis van het principe van de biologische equivalente dosis (BED), die corrigeert voor o.a. het effect van dosisfractionering, de nierafmeting en de niet-homogene verdeling van de radioactiviteit in de nieren [16-18]. Daarnaast worden positief geladen (kation) aminozuren gelijktijdig toegediend (co-infusie) met de radioactief gelabelde somatostatine analogen voor PRRT, waardoor de nierretentie van deze peptiden met ongeveer 40% werd verminderd met bescherming van de nieren als resultaat [13, 19, 20]. Recente overzichtsartikelen die de incidentie van late nierschade veroorzaakt door PRRT evalueren, beschrijven slechts enkele gevallen van nierschade als ^{177}Lu-DOTA,Tyr^3-

octreotaat met co-infusie van aminozuren wordt toegepast [11, 16]. Echter bij 30% van de patiënten waarbij ^{90}Y-DOTA,Tyr^{3}-octreotide PRRT met aminozuren wordt toegediend, wordt wel enige nierschade aangetroffen met stijgende serumwaardes van kreatinine en vermindering van de kreatinine klaring [16, 21]. Hiermee wordt de noodzaak van nierbescherming tijdens PRRT met ^{90}Y-DOTA,Tyr^{3}-octreotide aangetoond.

In *Hoofdstuk 1.1* worden de achtergrond en het doel van het huidige onderzoek beschreven. Na identificatie van het mechanisme van de nieropname van radioactieve peptiden, werd het mogelijk om verschillende manieren te ontwikkelen die leiden tot bescherming van de nieren tegen de schadelijke neveneffecten van PRRT.

Een uitgebreid overzicht van de huidige stand van zaken op het gebied van nierbescherming gedurende PRRT met radioactief gelabelde somatostatine analogen is beschreven in *Hoofdstuk 1.2*, dat zich voornamelijk richt op de toepassing van co-infusie met de basische aminozuren lysine en arginine en de hierdoor veroorzaakte verstoring van de terugresorptie van radioactief gelabelde somatostatine analogen in de nieren. Deze methode is succesvol ingevoerd in de huidige praktijk van klinische PRRT. Recent zijn ook Gelofusine en albumine fragmenten gedefinieerd als middelen die bij co-administratie met verschillende radiopeptiden vergelijkbare reductie van nierretentie bewerkstelligen.

Voor verder onderzoek naar de preventie van nierschade veroorzaakt door bestraling gedurende PRRT werd het opnamemechanisme verantwoordelijk voor de nieropname en nierretentie van radioactieve peptide analogen onderzocht. De conclusie dat megalin een belangrijke rol speelt in dit proces bleek de basis om nieuwe strategieën te ontwikkelen ten behoeve van nierbescherming zoals is beschreven in *Hoofdstuk 2*.

Zoals gerapporteerd in *Hoofdstuk 2.1* bleek een grote verscheidenheid te bestaan in de mate van nieropname van radioactief gelabelde somatostatine-, bombesine-, neurotensine-, minigastrine- en CCK-analogen, variërend van een erg lage (CCK) tot een heel hoge (minigastrine) opname in de nieren van ratten en muizen. De volgorde was: CCK < bombesin = neurotensin < octreotide << minigastrine, en was vergelijkbaar in beide diersoorten, maar de uitscheiding uit muizennieren was sneller dan bij ratten. Verder vonden we geen geslachtsverschillen in de nierretentie bij ratten, terwijl bij muizen de hoeveelheid radioactiviteit in de nieren vier keer hoger was in vrouwtjes t.o.v. mannetjes voor zowel gelabeld octreotide als gelabeld bombesine. Uit klinische studies zijn geen geslachtsverschillen bekend wat betreft nierretentie van radiopeptiden [17], daarom concluderen we dat de rat een beter proefdiermodel is dan de muis om de nieropname van nieuw ontwikkelde radiopeptiden te bestuderen.

De lokalisatie van de radioactiviteit in de nier, zoals bepaald m.b.v. *ex vivo* autoradiografie, was vergelijkbaar voor alle geteste radiopeptiden: de meeste radioactiviteit werd gevonden in de nierschors (cortex), minder in de buitenste laag van het merg (outer medulla), terwijl geen radioactiviteit werd gevonden in het nierbekken. Dit patroon kwam overeen met de resultaten van een immunohistochemische kleuring van niercoupes m.b.v. antilichamen gericht tegen megalin, dat voornamelijk tot expressie komt in het gekronkelde deel van de eerste buisjes na de glomeruli (proximale tubuli (PCT)) in de niercortex. Minder expressie wordt gevonden

in het rechte deel van de proximale tubuli (PST) gelegen in de outer medulla. Megalin is een groot eiwit van 600 kDa; het is een netto negatief geladen zgn. multi-ligand scavenging receptor en belangrijk voor de terugresorptie van veel eiwitten en peptiden uit primaire urine zodat deze niet verloren gaan door uitscheiding via de urine.

De suggestie dat megalin een rol speelt bij de nierretentie van radiopeptiden werd bevestigd in de studies beschreven in *Hoofdstuk 2.2*. Ten eerste werd m.b.v. micro-autoradiografie aangetoond dat de lokalisatie van radioactiviteit gelimiteerd is tot de proximale tubuli in de niercortex. Radioactiviteit werd niet gevonden in de glomeruli of distale tubuli. Vervolgens zijn drie middelen, nl. lysine, natriummaleaat en cisplatin, gelijktijdig met radioactief gelabelde somatostatine analogen toegediend aan ratten om mogelijke interventie aan te tonen in het actieve endocytose proces van terugresorptie, gemedieerd door megalin. De drie geteste middelen resulteerden in een reductie van nierretentie van radioactiviteit tot respectievelijk 60%, 15% en 70%. Door het gelijktijdig toedienen van een overmaat van het positief geladen aminozuur lysine met radioactief octreotide, dat één lysine residu bevat, wordt het terugresorptieproces van deze verbinding verstoord, resulterend in een verlaging van de hoeveelheid radioactiviteit in de nieren. Natriummaleaat remt het energie-metabolisme van cellen en de reconfiguratie van de celmembraan gedurende endocytose. De grote reductie van radioactiviteit in de nieren na voor- of gelijktijdige behandeling met maleaat kan hierdoor worden verklaard. Het chemotherapeuticum cisplatin vaak nefrotoxische neveneffecten, beperkt tot het rechte deel van de proximale tubuli (PST). Daarom is de reductie van de nieropname van ^{111}In-DTPA-octreotide door cisplatin relatief laag. Vanwege de ernstige bijverschijnselen zijn zowel maleaat als cisplatin niet geschikt voor implementatie in de kliniek ter bescherming van de nieren tijdens PRRT.

Nier-specifieke megalin-deficiënte muizen boden de mogelijkheid om de nieropname en -retentie van ^{111}In-DTPA-octreotide in deze dieren te vergelijken met die in wild-type muizen met een normale nierexpressie van megalin. De eerste resultaten zijn beschreven in *Hoofdstuk 2.3*. Moleculaire beeldvorming (imaging) 4 en 24 uur na injectie van ^{111}In-DTPA-octreotide werd uitgevoerd m.b.v. een SPECT camera speciaal geschikt voor kleine dieren. Na de laatste scan werd de hoeveelheid radioactiviteit per nier gekwantificeerd. In beide geslachten werd een opvallende 70%-85% reductie van opname in de megalin-deficiënte nieren gevonden, die werd bevestigd door *ex vivo* autoradiografie van vriescoupes van de nieren.

In de volgende studie waarin gebruik werd gemaakt van deze nier-specifieke megalin-deficiënte dieren, beschreven in *Hoofdstuk 2.4*, werd de nieropname van ^{111}In-gelabelde neurotensine, exendin, en minigastrine analogen bepaald en vergeleken met die van octreotide en octreotaat. Kwantificering van de nierretentie in megalin-deficiënte en wild-type muizen was gebaseerd op micro-SPECT analyses. Dezelfde muizen werden in opeenvolgende imaging experimenten getest met intervallen variërend van 3 tot 9 weken. Afzonderlijk werden ook biodistributiestudies uitgevoerd. Alle geteste radiopeptiden vertoonden minder nieropname in megalin-deficiënte muizen dan in wild-type controles. Daarom is de terugresorptie van radiopeptiden uit meerdere peptide families, van verschillende grootte en met variabele netto lading, tenminste deels megalin gemedieerd.

Gebaseerd op de conclusie dat megalin een belangrijke rol speelt in de terugresorptie van radiopeptiden in de nier, werd getest of toedienen van Gelofusine een reducerend effect heeft op de nieropname van radiopeptiden. Deze plasmavervanger bestaat uit negatief geladen gesuccinyleerd gelatine, met een gemiddeld molgewicht van 30 kDa. Gelofusine wordt toegediend in de kliniek om hypovolemie (onvoldoende bloedvolume) te behandelen bij patiënten die in shock of kritiek zijn vanwege veel bloedverlies. Bij deze patiënten wordt verlies van eiwitten en peptiden in de urine gedetecteerd, maar dit is van voorbijgaande aard, wijzend op een tijdelijke verstoring van het efficiënte terugresorptie-proces in de nieren [22]. Inderdaad werd in een studie bij gezonde vrijwilligers aangetoond dat co-infusie van Gelofusine met Octreoscan® resulteerde in een vergelijkbare reductie van nierretentie als co-infusie met positief geladen aminozuren [23]. In *Hoofdstuk 3.1* hebben we in ratten de optimale dosis Gelofusine bepaald die gelijktijdig met ^{111}In-DOTA,Tyr^3-octreotaat moet worden toegediend om de hoogst haalbare reductie van nieropname te verkrijgen. Een dosis van 80 mg/kg lichaamsgewicht was voldoende om 50%-60% reductie van nieropname te realiseren, wat zelfs verbeterd kon worden tot 70% reductie als Gelofusine werd gecombineerd met lysine. Daarnaast hebben we aangetoond dat de tumoropname van ^{111}In-DOTA,Tyr^3-octreotaat niet werd beïnvloed door het gelijktijdig toedienen van Gelofusine en lysine, waardoor een verdrievoudiging van de tumor/nier ratio werd bereikt.

Amifostine, een scavenger ('wegvanger') van vrije radicalen en een antioxidant, wordt toegepast als beschermend medicijn tijdens klinische radiotherapie. De neveneffecten van bestraling, geïnduceerd door vrijkomende reactieve zuurstofradicalen, worden m.b.v. amifostine in gezonde weefsels verminderd. Eerder onderzoek in ratten heeft aangetoond dat gelijktijdige toediening van amifostine tijdens ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT het ontstaan van nierschade op de lange termijn verminderde [24]. In de studie beschreven in *Hoofdstuk 3.2* was het doel om het mechanisme dat ten grondslag ligt aan deze manier van nierbescherming m.b.v. amifostine verder te onderzoeken en om mogelijke tumorbescherming voor de therapie uit te sluiten. De effecten van co-administratie van amifostine met radioactief gelabeld octreotaat werd getest in tumordragende ratten, zowel in biodistributie studies 24 uur na injectie van ^{111}In-DOTA,Tyr^3-octreotaat als in een PRRT studie met ^{177}Lu-DOTA,Tyr^3-octreotaat. De dieren werden meer dan 120 dagen na therapie vervolgd. Interferentie van amifostine met tumoropname of met het therapeutisch effect van ^{177}Lu-DOTA,Tyr^3-octreotaat werd niet gevonden. De initiële nieropname werd echter gereduceerd tot 40% van het controle niveau na co-injectie met amifostine. Dit was een onverwacht fenomeen dat werd bevestigd in een in vitro test waarbij gebruik gemaakt werd van BN-16 cellen die megalin tot expressie brengen. Co-incubatie met amifostine of de actieve metaboliet ervan, WR-1065, reduceerde de binding van ^{111}In-DOTA,Tyr^3-octreotaat aan BN-16 cellen tot respectievelijk 66% en 55% t.o.v. controlewaardes. Deze data suggereren dat de reductie van de initiële nieropname van ^{111}In-DOTA,Tyr^3-octreotaat door amifostine/WR-1065 verloopt via megalin, vergelijkbaar met de reductie zoals verkregen met lysine co-administratie. Daarom kan de nierbescherming gevonden na co-administratie van amifostine tijdens PRRT [24] veroorzaakt zijn door twee processen. Enerzijds door het wegvangen van schadelijke zuurstofradicalen, waardoor de cascade van processen leidend tot nierfibrose doorbroken wordt, anderzijds door vermindering van β^--straling uitzendende ^{177}Lu-retentie in de nieren.

De ontwikkeling van nierziekte of nefropathologie na bestraling is een langdurig proces dat in ratten enkele maanden duurt. Het vervolgen van de nierfunctie in de tijd kan worden gedaan door het bepalen van kreatinine- en ureumgehaltes in serum of het eiwitgehalte in urine. Een andere, niet-invasieve, manier om nierfalen vroegtijdig te detecteren is door m.b.v. een γ-camera de opname en uitscheiding van radioactieve tracers, specifiek voor het onderzoeken van de nierfunctie, in beeld te brengen en te kwantificeren. ^{99m}Tc-DMSA, een tracer met retentie in de corticale tubuli, is getest om de nierfunctie in ratten gedurende de follow-up van PRRT te vervolgen m.b.v. een SPECT/CT camera geschikt voor kleine dieren. De resultaten van deze studies zijn beschreven in *Hoofdstuk 4.1*. Het dosisafhankelijke niveau van nierschade, gedetecteerd meer dan 100 dagen na 278 of 555 MBq ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT, is omgekeerd evenredig met de ^{99m}Tc-DMSA opname per nier gebaseerd op statische imaging. Hoe hoger de geabsorbeerde dosis radioactiviteit in de nieren, hoe lager de ^{99m}Tc-DMSA-opname gemeten 100-150 dagen na PRRT. Bovendien liet het lokalisatiepatroon van de opgenomen ^{99m}Tc-DMSA in de nier zien dat er specifiek verlies van functie optreedt in de corticale tubuli; alleen in het gekronkelde deel (PCT) na een lage PRRT dosis, maar ook in het rechte deel (PST) na een hoge PRRT dosis.

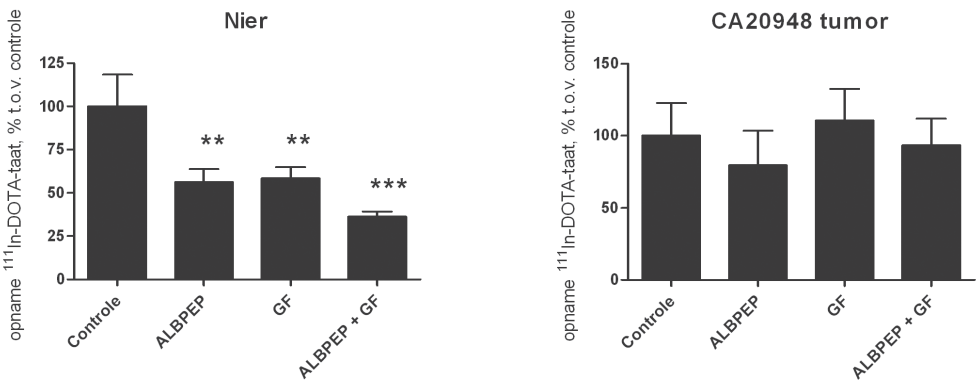
In *Hoofdstuk 4.2* wordt het vervolgen van de rattennierfunctie beschreven na 460 MBq ^{177}Lu-DOTA,Tyr^3-octreotate PRRT met of zonder lysine co-injectie, m.b.v. de radiotracers ^{111}In-DTPA, ^{99m}Tc-MAG3 en ^{99m}Tc-DMSA. ^{111}In-DTPA en ^{99m}Tc-MAG3 werden toegepast om het dynamische proces van glomerulaire filtratie (^{111}In-DTPA) en tubulaire secretie (^{99m}Tc-MAG3) in beeld te brengen. In controle ratten lieten zowel ^{111}In-DTPA als ^{99m}Tc-MAG3 tijd-activiteit curves zien met een piek een paar minuten na injectie, gevolgd door een snelle klaring via de tubuli gevolgd door uitscheiding via de urine. Als nierschade was ontstaan, na meer dan 90 dagen na PRRT, kon bij beide tracers slechts een lage piekactiviteit worden gedetecteerd. In ratten die PRRT plus lysine co-administratie hadden gekregen als nierbescherming, bleef de ^{111}In-DTPA klaring en ^{99m}Tc-DMSA-opname onaangetast, in tegenstelling tot de ^{99m}Tc-MAG3 klaring die wel een afwijkend patroon liet zien. We concludeerden dat ^{99m}Tc-MAG3 een gevoeliger manier is om vroege nierschade, geïnduceerd door PRRT, te detecteren dan ^{99m}Tc-DMSA-opname of serumchemie waarbij de kreatinine- en ureumgehaltes worden bepaald.

In *Hoofdstuk 4.3* beschrijven we dat nefrotoxische effecten werden gevonden in muizen na opeenvolgende scans waarbij ^{111}In-gelabelde radiopeptiden werden toegediend. Dit was onverwacht omdat in vroege klinische ^{111}In-DTPA-octreotide PRRT studies geen nierschade werd gevonden, zelfs niet na een geabsorbeerde stralingsdosis in de nieren van 45 Gy, een duidelijke overschrijding van de geaccepteerde veilige dosislimiet van 23 Gy. Echter, het bleek dat de hele hoge nieropname en –retentie van ^{111}In-exendin (225% IA/g nier) in wild-type en megalin-deficiënte muizen respectievelijk een nierdosis van 70 en 40 Gy opleverde, die leidde tot nierfalen in beide muizenstammen na meer dan 90 dagen na de toediening van ^{111}In-exendin. Een hoge stralingsdosis op de nieren veroorzaakt door γ-straling en Auger- en conversie elektronen uitgezonden door ^{111}In, kan dus ook lange-termijn nefrotoxiciteit veroorzaken. SPECT van kleine dieren maakt het mogelijk om ziekte- en behandelingsprocessen te vervolgen in de tijd in één dier, maar het risico van een geaccumuleerde stralingsdosis op de nieren die de veiligheidslimiet overschrijdt moet in overweging worden genomen, zoals is aangetoond in dit hoofdstuk.

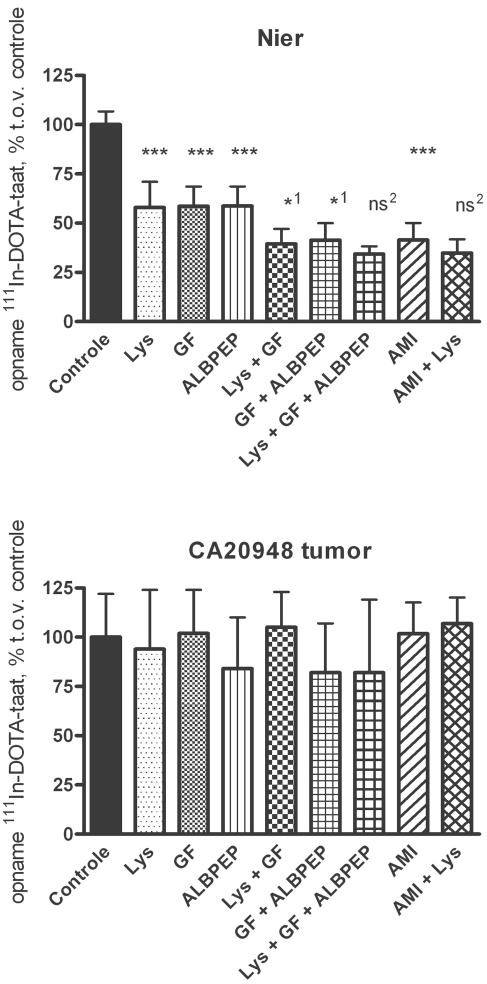
Aanvullende studies

Gebaseerd op het feit dat megalin een cruciale rol speelt in de terugresorptie in de nier van meerdere radiopeptiden, is het bestuderen van potentiële remming van dit proces door co-administratie van albuminederivaten een logische stap voor verder onderzoek omdat albumine één van de belangrijkste moleculen is die bindt aan megalin. Intact albumine (68 kDa) wordt vanwege de grootte slechts marginaal gefilterd in de glomeruli. Co-administratie van kleinere fragmenten van albumine (FRALB), verkregen door enzymatische afbraak, met ^{111}In-DTPA-octreotide resulteerde in lagere nieropname dan in controle ratten [25]. Deze waarneming bevestigde de hypothese dat co-administratie van albuminederivaten de megalin-gemedieerde terugresorptie van somatostatine-analogen reduceert. In een vervolgstudie zijn verschillende goed gedefinieerde synthetische peptiden, afgeleid van albumine, met variërende ladingen getest als specifieke remmers van nieropname. Co-administratie van een peptide bestaande uit 36 aminozuren (3.8 kDa), afgeleid van het C-terminale einde van albumine (ALBPEP), bewerkstelligde een reductie van nieropname van meerdere radiopeptiden, variërend van 26% (exendin) en 33% (octreotide) tot 88% (gastrine) remming [26].

Om mogelijke interferentie vast te stellen van ALBPEP met tumoropname van ^{111}In-DOTA,Tyr^3-octreotaat zijn biodistributiestudies uitgevoerd in CA20948 tumordragende ratten (Figuur 1). De combinatie van zowel ALBPEP als Gelofusine als co-administratie werd ook getest, resulterend in een significante additieve reductie van de nierretentie van ^{111}In-DOTA,Tyr^3-octreotaat, terwijl de tumoropname niet werd aangetast.



Figuur 1: Biodistributie gegevens 24 uur na injectie van 15 µg ^{111}In-DOTA,Tyr^3-octreotaat (^{111}In-DOTA-taat) in ratten met of zonder co-administratie van peptide, afgeleid van albumine (ALBPEP (4 mg/rat)) en/of Gelofusine (GF (80 mg/kg)).
Opname in nieren en CA20948 tumoren die sst tot expressie brengen werd berekend als % van de geïnjecteerde activiteit/gram (%IA/g) ± SD en de waarde van de fysiologisch zout controle groep werd op 100% gezet. n = 4 ratten per groep. Het verschil in nieropname t.o.v. de controle groep van groepen die alleen ALBPEP en GF kregen (** = p<0.01) en de ALBPEP+GF (*** = p<0.001) groep was significant, terwijl het verschil tussen ALBPEP+GF en ALBPEP of GF alleen ook significant verschillend was (**t.o.v. ALBPEP, *** t.o.v. GF). Geen significante verschillen werden gevonden voor CA20948 tumoropname.



Figuur 2: Biodistributie gegevens 24 uur na injectie van 15 µg ^{111}In-DOTA,Tyr^3-octreotaat (^{111}In-DOTA-taat) in ratten met of zonder co-administratie van Lys (400 mg/kg), GF (80 mg/kg), ALBPEP (4 mg/rat) en/of AMI (50 mg/rat).
Opname in nieren en CA20948 tumoren die sst tot expressie brengen werd berekend als % van de geïnjecteerde activiteit/gram (%IA/g) ± SD en de waarde van de fysiologisch zout controle groep werd op 100% gezet. n = 2-4 rats per groep. Het verschil in nieropname t.o.v. de controle groep van alle middelen die alleen werden getest was significant (***=p<0.001). Combinatie van Lys+GF en GF+ALBPEP liet significante additieve reductie t.o.v. alleen Lys, GF of ALBPEP (p<0.01 = *^{1}), terwijl Lys+GF+ALBPEP niet significant verschillend (ns^2) was t.o.v. Lys+GF of GF+ALBPEP. De combinatie van AMI+Lys resulteerde niet in significant verminderde nieropname t.o.v. alleen Lys of AMI (ns^2). Geen significante verschillen werden gevonden voor CA20948 tumoropname in alle groepen.

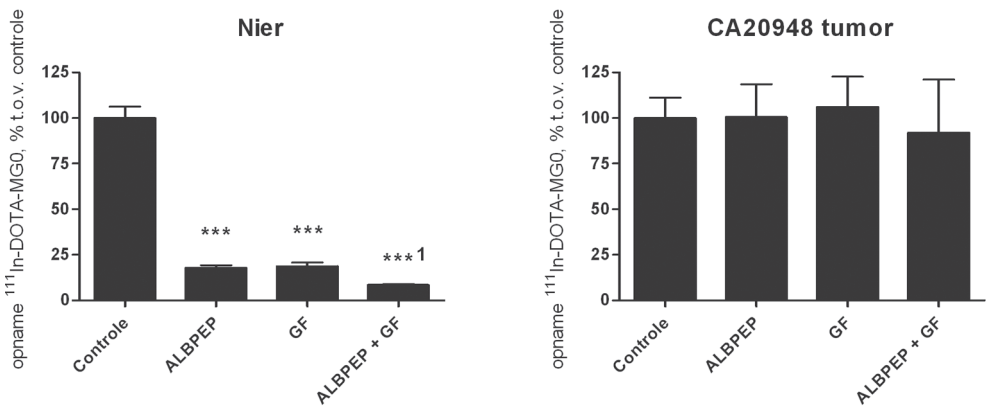
Een overzicht van alle verkregen resultaten betreffende reductie van nier- en tumoropname van radioactief gelabeld octreotaat in ratten na gelijktijdig toedienen van lysine (Lys), Gelofusine (GF), ALBPEP en amifostine (AMI) (en combinaties ervan) is samengevat in Figuur 2.

Geen van de toegediende middelen resulteerde in significante vermindering van de somatostatine receptor-gemedieerde tumoropname van ^{111}In-DOTA,Tyr^3-octreotaat, in tegenstelling tot de algemene reductie van nierretentie. Enkele combinaties van middelen vertoonden een significante additieve reductie van nieropname, terwijl andere combinaties dat niet bewerkstelligden. De waarneming dat Lys+GF+ALBPEP niet resulteerde in verdere reductie in vergelijking tot Lys+GF en GF+ALBPEP suggereert dat òf het laagst haalbare niveau van nieropname van radioactief gelabeld octreotaat was bereikt, òf dat terugresorptie via dezelfde regio's van megalin domeinen was geremd. Lys+AMI/WR1065 liet eveneens geen cumulatieve reductie van radioactiviteit in de nieren zien, hetgeen kan worden verklaard door een vergelijkbaar werkingsmechanisme.

Het resterende niveau van nieropname na maximale remming van nieropname met verschillende middelen en in nier-specifieke megalin-deficiënte muizen kan worden veroorzaakt door zgn. vloeibare fase endocytose. Naast actieve terugresorptie in de nier is dit een ander proces waarbij (afval)stoffen worden weggevangen vanuit het bloed en de primaire urine in de

nieren; dit proces kan niet worden geremd m.b.v. aminozuren [27]. Daarnaast is bij de muis en de mens, maar niet bij de rat, sst-expressie in de nier aangetoond, dat verantwoordelijk is voor enige receptor-specifieke nierretentie van somatostatine analogen dat niet beïnvloed kan worden door middelen die interfereren in de megalin-gemedieerde opname [28, 29]. In patiënten bedraagt de bijdrage van deze sst-expressie maximaal 20% van de totale radioactiviteit in de nieren. Behalve expressie van somatostatine receptoren op proximale tubuli, is ook GLP-1R en CCK2-R expressie beschreven [30-32].

De succesvolle resultaten betreffende nierbescherming voor radioactief gelabeld octreotaat, verkregen met lysine, Gelofusine en ALBPEP, stimuleerden ons om potentiële additief reducerende effecten op nieropname van radioactief gelabelde minigastrine analogen (met heel hoge nieropname) te onderzoeken in tumordragende ratten. Een hoge reductie van meer dan 80% van de nieropname van ^{111}In-DOTA-minigastrine 0 werd gevonden na co-administratie van alleen ALBPEP of Gelofusine. Co-injectie van de combinatie van deze twee middelen resulteerde in een significante extra reductie tot 90% van de nieropname t.o.v. ratten die fysiologisch zout als controle kregen (Figuur 3). CA20948 tumoropname bleef in alle gevallen gelijk. Omdat de mate van radioactiviteit in de nieren na ALBPEP+GF werd gereduceerd zonder aantasting van de tumoropname, werd een 2,5- en 10-voudige verhoging van de tumor/nier ratio bereikt na respectievelijk ^{111}In-DOTA,Tyr^3-octreotaat en ^{111}In-DOTA-minigastrine 0 toediening.



Figuur 3: Biodistributie gegevens 24 uur na injectie van 0.1 µg ^{111}In-DOTA-minigastrine 0 (^{111}In-DOTA-MG0) in ratten met of zonder co-administratie van ALBPEP (4 mg/rat) en/of Gelofusine (GF (80 mg/kg)).
Opname in nieren en CA20948 tumoren die sst tot expressie brengen werd berekend als % van de geïnjecteerde activiteit/gram (%IA/g) ± SD en de waarde van de fysiologisch zout controle groep werd op 100% gezet. n = 4 ratten per groep. Het verschil in nieropname t.o.v. de controle groep was significant (***=p<0.001), en ook de verschillen tussen alleen ALBPEP en GF t.o.v. GF+ALBPEP = ***^{1}. Geen significante verschillen werden gevonden voor CA20948 tumoropname in alle groepen.

In Tabel 1 zijn de resultaten van biodistributiestudies met radioactief gelabelde somatostatine, exendin and minigastrine 0 analogen samengevat van meerdere nierbeschermingsstudies in ratten zoals beschreven in de literatuur, aangevuld met onze recent verkregen resultaten. De waargenomen reductie van nierretentie is weergegeven als percentage t.o.v. de nierretentie in onbeschermde dieren.

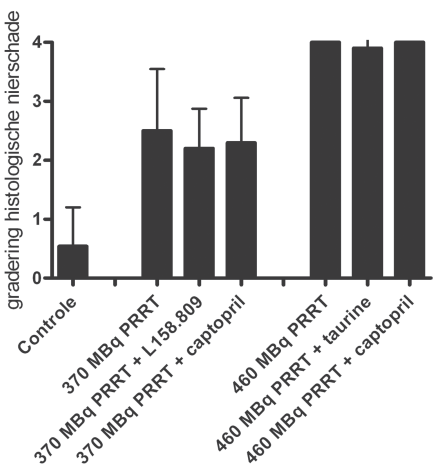
Omdat minigastrine 0 zes anionische glutaminezuur (Glu) residuen bevat, werd geen effect op de nieropname verwacht van gelijktijdige lysine (Lys) toediening. Inderdaad werden alleen marginale effecten gevonden [33]. Maar, bij toepassing van polyglutaminezuur (PGA) met tenminste vijf Glu residuen, werd een 80%-90% vermindering van radioactiviteit in de nieren verkregen [34]. Co-administratie van Lys of PGA met ^{111}In-exendin vertoonde minder overtuigende resultaten, wat er op wijst dat de keuze van de toegepaste remmende aminozuren specifiek aangepast moet zijn aan de samenstelling van het toegediende radiopeptide, waarbij lading een belangrijke rol lijkt te spelen.

	Lys	GF	GF + Lys	PGA	GF + PGA	FRALB	ALB-PEP	ALBPEP + GF
Octreotide/ octreotaat	40-50	40-50	50-65	0-15	nu	30-35	30-45	65
Exendin	0-15	20-25	nu	25-35	35-45	50-55	25	nu
Minigastrine 0	0-15	45-80	nu	80-90	80-90	90-95	80-85	90

Tabel 1: Samenvatting van nierbeschermingsstudies in ratten, overgenomen uit [25, 26, 33-35] en aangevuld met recent verkregen resultaten. Het gemiddelde percentage van reductie van nierretentie is weergegeven. Lys=lysine, GF=Gelofusine, PGA=polyglumatinezuur, FRALB=albumine fracties, ALBPEP= van albumine afgeleid peptide, nu = niet uitgevoerd.

Het gebruik van Gelofusine en albuminederivaten bood reductie van nieropname van alle geteste radiopeptiden, hoewel de mate van reductie erg varieerde. Gelijktijdige toediening van deze twee middelen met radioactief gelabeld octreotaat bewerkstelligde zelfs een toegenomen additief remmend effect op de nieropname, wat nog duidelijker was wanneer deze combinatie werd toegediend met radioactief gelabeld minigastrine.

Vermindering van het risico op nierschade na PRRT kan dus worden verkregen door verlaging van de initiële nierretentie van radioactief gelabelde peptiden, maar ook door het temperen (mitigeren) van de door PRRT veroorzaakte lange-termijn nierschade. Gebaseerd op de beschreven stralingsbescherming van nieren gedurende externe bestraling door het wegvangen van actieve zuurstofradicalen of het remmen van angiotensine II, hebben we recent in ratten het nierbeschermend effect na PRRT onderzocht van taurine als antioxidant; van L158.809, een angiotensine II receptor blokker (ARB); en van captopril, een ACE-remmer (angiotensine converting enzyme), dat de omzetting van angiotensine I naar angiotensine II remt [36,



Figuur 4: De mate van histologische schade op dag 146 (460 MBq ^{177}Lu-PRRT) of dag 180 (370 MBq ^{177}Lu-PRRT) na therapie met of zonder behandeling met taurine (10 g/l), captopril (0.5 g/l) and L158.809 (20 mg/l) toegevoegd aan het drinkwater, n = 6-8 per groep.

37]. Wanneer de gegevens over de nierfunctie van deze ratten werden vergeleken met die van ratten na PRRT zonder toevoegingen aan het drinkwater, konden we geen significante verbetering van nierfunctie aantonen. De resultaten van histologische nierschade zijn weergegeven in Figuur 4. We concludeerden dat taurine, L158.809 en captopril niet geschikt zijn om vermindering van nefrotoxische effecten veroorzaakt door PRRT m.b.v. ^{177}Lu-DOTA,Tyr^3-octreotaat te bewerkstelligen.

Richtlijnen voor de toekomst

Het huidige protocol van ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT in de kliniek, bestaande uit vier cycli met een totale cumulatieve dosis van 29.6 GBq met co-infusie van de kation aminozuren lysine en arginine, lijkt veilig te zijn wat betreft de nierfunctie [38]. Echter, na ^{90}Y-DOTA,Tyr^3-octreotide PRRT, in een cumulatieve dosis van 15.2 GBq zoals toegepast in verschillende klinische centra, kan de veiligheid van de nieren wel in het geding zijn [21]. De therapeutische resultaten van PRRT zijn gunstig vergeleken met de conventionele behandelingsmodaliteiten bij NET, met een objectieve tumorrespons bij ~55% van de patiënten met soms ook complete tumorregressie. Maar een verdere verbetering van het genezingspercentage is nadrukkelijk gewenst [7]. Daarom is veilige toediening van een hogere cumulatieve dosis om verbetering van tumorrespons te verkrijgen hoogst noodzakelijk, waarbij nierbescherming een cruciale rol speelt [39].

Opties om verhoogde tumorrespons te verkrijgen zijn:

- Toediening van meer cycli ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotaat [40]
- Combinatie van ^{90}Y-DOTA,Tyr^3-octreotide en ^{177}Lu-DOTA,Tyr^3-octreotaat, ofwel binnen een cyclus òf starten met op ^{90}Y-gebaseerde cycli gevolgd door ^{177}Lu-cycli òf alternerende ^{90}Y-en ^{177}Lu-cycli [8, 41-43]
- Combinatie van ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotaat met radiosensitiserende middelen, zoals capecitabine of andere chemotherapeutica [11]
- Intra-arteriële (locoregionale) toediening van ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotaat in plaats van de algemeen toegepaste systemische intraveneuze manier van toedienen [44, 45]
- Combinatie van anti-angiogene middelen (remmen de nieuwvorming van bloedvaten), bijv. anti-VEGF (vasculair endotheel groeifactor) antilichamen of stoffen gericht tegen $\alpha_v\beta_3$-integrine zoals thalidomide of RGD-constructen, met ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotaat [46, 47]. Recent worden deze middelen toegepast in combinatie met radioimmunotherapie.
- Onderzoek naar de inductie van verhoogde somatostatine receptor-expressie op tumoren, om de internalisatie van ^{90}Y-DOTA,Tyr^3-octreotide/^{177}Lu-DOTA,Tyr^3-octreotaat te verhogen [48]
- Introductie van nieuwe peptide analogen met hogere affiniteit en/of pansomatostatine receptor specificiteit [49]
- Combinatie van verschillende peptide analogen gericht tegen verschillende receptoren die tot co-expressie komen op tumoren, zoals sst2 en GLP-1R op gastrinoma's [50]

De geabsorbeerde stralingsdosis op de nieren zal toenemen als meer cycli van ^{177}Lu- of ^{90}Y-gelabelde PRRT zullen worden toegediend per patiënt, waardoor optimale nierbescherming essentieel is. Het beenmerg zal het volgende orgaan zijn met risico op stralingsschade, veroorzaakt vanwege een verhoogde geabsorbeerde stralingsdosis omdat meer radioactiviteit in het bloed circuleert [38].

Zoals beschreven in *Hoofdstuk 3.1* en door anderen, zal door gecombineerde co-infusie van Gelofusine en Lys/Arg verdere reductie van nieropname van somatostatine analogen kunnen worden verkregen [23]. Hoewel deze combinatie van nierbeschermende middelen al klinisch wordt toegepast met veelbelovende resultaten, zijn vergelijkende studies betreffende nierretentie van radioactiviteit nog niet beschikbaar [8, 39]. Daarom zal in de nabije toekomst in ons centrum een pilotstudie worden gestart om de nier- en tumordosimetrie te vergelijken gedurende ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT waarbij òf alleen Lys/Arg co-infusie tijdens één cyclus wordt gegeven òf gecombineerde Gelofusine en Lys/Arg co-infusie in de volgende cyclus of vice versa. Bij een klein percentage van de patiënten komen na Gelofusine toedieningen allergische neveneffecten voor; 0.04% met ernstige anafylactische reacties en 1.4% met milde tot matige. Daarom zullen vitale functies, zoals hartfrequentie en bloeddruk moeten worden geregistreerd [39, 51].

Middelen die stralingsschade op de nieren temperen (mitigeren) kunnen worden gecombineerd met stoffen die de initiële nierretentie van radiopeptiden verlagen, zodat op een veilige manier het toedienen van hogere therapeutische PRRT doses kan worden geïmplementeerd. In dit opzicht is er meer onderzoek nodig naar de toepassing van het antioxidant amifostine als nierbeschermend middel tijdens PRRT. Eerder zijn in de rat gunstige effecten op nierhistologie en nierfunctie op de lange-termijn beschreven [24], die gedeeltelijk toegeschreven kunnen worden aan de reductie van de geabsorbeerde stralingsdosis op de nier zoals beschreven in *Hoofdstuk 3.2*. Nieuwe experimenten waarbij amifostine toegediend zal worden ofwel vóór of ná PRRT moet onderscheid maken tussen de korte- en lange-termijn effecten. Hoewel patiënten na toediening van amifostine een verlaagde bloeddruk, misselijkheid, overgeven en/of huiduitslag als neveneffecten kunnen ervaren, is verder onderzoek naar de toepassing van dit medicijn m.b.t. PRRT intrigerend. Zeker omdat ook bescherming van voorloper beenmergcellen voor schadelijke bestralingseffecten is beschreven [52-54].

Naast niertoxiciteit wordt ook suppressie van beenmerg gevonden gedurende PRRT, resulterend in vermindering van bloedplaatjes en rode- en witte bloedcellen in het bloed [38, 55]. Hematologische toxiciteit is meestal mild en omkeerbaar en het risico op het ontwikkelen van myelodysplastisch syndroom (MDS) of leukemie na PRRT is laag als een dosis limiet van 2 Gy op het beenmerg wordt aangehouden. Toch zijn verschillende gevallen van beenmergtoxiciteit na PRRT beschreven [11]. De toepassing van hogere cumulatieve doses van PRRT, in een optimaal nierbeschermingsprotocol, kan het risico op beenmergtoxiciteit verhogen en daardoor zal het beenmerg het dosislimiterende orgaan worden. De geabsorbeerde stralingsdosis op het beenmerg wordt veroorzaakt door 'cross fire' vanuit het radioactieve bloed dat circuleert in de nabijheid van beenmergcellen [56]. Specifieke opname door voorloper beenmergcellen die de somatostatine receptor tot expressie brengen zou bovendien een (kleine) rol kunnen spelen [56, 57]. Daarom zullen beenmerg-beschermende strategieën zoals toegepast voor nierbescherming met reductie van de initiële nierretentie niet effectief zijn, uitgezonderd

eventuele vermindering van stralingsschade door bijv. amifostine. Autologe beenmergtransplantatie kan worden overwogen om ondersteuning van de beenmergfunctie te geven als patiënten worden geconfronteerd met ernstige hematologische toxiciteit na PRRT. Deze procedure wordt al succesvol toegepast tijdens radioimmunotherapie [58].

De bloedklaring van radiopeptiden in patiënten duurt veel langer dan in ratten en muizen, omdat deze dieren een kleiner totaal bloedvolume en een hogere hartslagfrequentie hebben dan mensen [59]. Dit resulteert in een hogere geabsorbeerde stralingsdosis in humaan beenmerg in vergelijking tot de dosis in proefdieren. Bovendien wordt geen expressie van somatostatine-receptoren gevonden op rattenbeenmergcellen in tegenstelling tot humane voorlopercellen [57, 60]. Differentiële celtellingen van beenmerg van terminaal zieke ratten vanwege nefrotoxiciteit langer dan 100 dagen na ^{177}Lu-DOTA,Tyr^3-octreotaat PRRT met >50 Gy op de nieren, liet geen afwijkingen zien in het beenmerg. Bovendien waren de aantallen van meerdere typen bloedcellen van deze ratten vergelijkbaar met die van controle ratten, wat erop wijst dat hematologische toxiciteit niet voorkwam bij deze dieren. Daarom is preklinisch onderzoek in kleine proefdieren naar bescherming van beenmerg tijdens PRRT door amifostine, andere middelen of door ondersteuning m.b.v. autoloog beenmerg niet mogelijk.

Samenvattend hebben we aangetoond in preklinische studies dat naast kation aminozuren, Gelofusine, albumine derivaten, amifostine, of combinaties hiervan veelbelovende middelen zijn om de nierretentie te verminderen van verscheidene tumorspecifieke radiopeptiden in patiënten. Dit is vooral belangrijk als verhoogde PRRT doses zullen worden toegediend om verbeterde genezingspercentages te behalen. Ten eerste zullen de veiligheidsaspecten betreffende allergische reacties op Gelofusine en mogelijke toxiciteit van peptiden afgeleid van albumine grondig worden bestudeerd. Daarnaast zal de productie van peptiden afgeleid van albumine opgezet moeten worden volgens de richtlijnen van GMP (good manufacturing practice) voordat deze getest kunnen worden in klinisch onderzoek. Deze peptiden bieden veelbelovende vooruitzichten voor brede toepassing als nierbeschermend middel.

Onze ervaring met het terugbrengen van nierretentie van radiopeptiden kan worden uitgebreid naar andere, nieuw ontwikkelde, radioactief gelabelde biomoleculen zoals neurotensine of bombesine analogen die één of meer Lys residuen bevatten [61], folaat derivaten, Affibody moleculen of nanobodies die een hoge mate van retentie van radioactiviteit in de nieren laten zien [62-64].

Zoals beschreven in dit proefschrift kan bestraling van de nier door radionucliden, maar ook door röntgenstraling, late nierschade veroorzaken. Van verscheidene andere geneesmiddelen is ook bekend dat deze na toediening nefrotoxiciteit kunnen veroorzaken. Tubulaire schade in de nieren is gerapporteerd na toediening van antibiotica gebaseerd op aminoglycoside zoals gentamicine [65], het antischimmel middel amphotericine [66], het anti-folaat methotrexaat [67], gebruikt als medicijn bij kankerpatiënten, en Cyclosporine A [68], toegepast ter voorkoming van afstoting van getransplanteerde organen. De laatste twee medicijnen onderdrukken ook de effecten van auto-immuun ziektes. Co-administratie van Gelofusine of peptiden afgeleid van albumine kunnen mogelijk interfereren met de interactie van deze medicijnen met de nier waardoor het risico op nierschade wordt verminderd, vergelijkbaar met wat we hebben beschreven voor de toepassing met radiopeptiden.

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List of abbreviations

Dankwoord

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Chapter 6

List of abbreviations

AA	afferent arteriole
ACE(I)	angiotensin converting enzyme (inhibitor)
AMI	amifostine
ANOVA	analysis of variance
ARB	angiotensin II receptor blocker
Arg	arginine
BSA	bovine serum albumin
BED	biological equivalent dose
BW	body weight
C	cortex
CCK	cholecystokinine
CE	conversion electrons
CD	collecting duct
CT	computed tomography
d	day(s)
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
^{99m}Tc-DMSA	^{99m}Tc-radiolabelled dimercaptosuccinic acid
DNA	desoxyribonuclic acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DT	distal tubule
DTPA	diethylenetriaminepentaacetic acid
EA	efferent arteriole
EBRT	external beam radiation therapy
ENETS	European Neuroendocrine Tumour Society
^{18}F	^{18}Fluoride
FCS	foetal calf serum
FDA	Food and Drug Administration
g	gram

G	glomerulus
^{68}Ga	^{68}Gallium
GBq	Gigabequerel (10^9 Bq)
GF	Gelofusine
GFR	glomerular filtration rate
GLP-1	glucagon like peptide-1
GMP	good manufacturing practice
GRP	gastrin releasing peptide
Gy	Gray
h	hour(s)
HE-staining	haematoxylin-eosin staining
HER2	human epidermal growth factor receptor 2
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
%IA/g or %ID/g	percentage of injected activity/dose per gram tissue
IC50	concentration needed to achieve 50% inhibition of binding
IgA/IgM	immunoglobulin A/M
IL-8	interleukin-8
^{111}In	^{111}Indium
i.p.	intraperitoneal
ITLC	instant thin layer chromatography
i.v.	intravenous
kBq	Kilobequerel (10^3 Bq)
kDa	kilo Dalton
keV	kilo electron volt
LD50	median lethal dose for 50% of subjects
LDL	low-density-lipoprotein
LH	lis of Henle
^{177}Lu	^{177}Lutetium
Lys	lysine
Lys/Arg	lysine/arginine

mAb	monoclonal antibody
^{99m}Tc-MAG3	^{99m}Tc–labelled mercaptoacetyltriglycine
MBq	Megabequerel (10^6 Bq)
MDS	myelodysplastic syndrome
Meg def	kidney-specific megalin-deficient mice
MeV	mega electron volt
µg	microgram
MG0	minigastrin 0
min	minute(s)
µl	microlitre (10^{-6} litre)
ml	millilitre (10^{-3} litre)
MRI	magnetic resonance imaging
MTC	medullary thyroid cancer
MW	molecular weight
NET	neuroendocrine tumour
OM	outer medulla
PAS staining	periodic acid Schiff staining
PCT	proximal convoluted tubules
PET	positron emission tomography
Phe	phenylalanine
p.i.	post injection
PRS	peptide receptor scintigraphy
PRRT	peptide receptor radionuclide therapy
PST	proximal straight tubules
p.t.	post therapy
PT	proximal tubule
R	receptor
RAAS	renin-angiotensin-aldosterone system
ROI	region of interest
ROS	reactive oxygen species
RV	renal vein
s	second

SCLC	small cell lung carcinoma
SD	standard deviation
SPECT	single photon emission computed tomography
sst2, 3 or 5	somatostatin receptor subtype 2, 3 or 5
SUVmax	maximum standard uptake value
SWOG	South West Oncology
$T_{1/2}$	half life
^{99m}Tc	^{99m}Technetium
TGF-β	transforming growth factor-β
Tyr	tyrosine
VIP	vasoactive intestinal peptide
VOI	volume of interest
WHO	World Health Organization
WT	wild-type mice
^{90}Y	^{90}Yttrium

Dankwoord

Het als laatst geschreven deel van mijn proefschrift, maar misschien wel het meest gelezen… Het is overduidelijk dat ik de inhoud van dit boekje nooit in mijn eentje tot stand kan hebben gebracht, hoewel alleen mijn naam op de omslag staat genoemd. Meer dan terecht dus dat dit dankwoord vol staat met namen van collega's en ex-collega's, andere medewerkers en natuurlijk familie en vrienden, die allemaal op hun manier hebben bijgedragen aan het verwerkelijken van dit proefschrift.

Toch wil ik niet alleen namen noemen, maar ook allerlei situaties, ontmoetingen en 'toevalligheden'. Dat is omdat ik er van overtuigd ben dat iedereen zijn of haar levensweg gaat met een doel in gedachten als richtingwijzer, maar dat meestal door onverwachte omstandigheden wordt bepaald hoe de weg feitelijk gaat. Zoals de pelgrim weet dat het gaat om onderweg zijn en dat de weg zelf het doel is; daarom kan hij/zij de vertrouwde omgeving verlaten en zich overgeven aan dat wat op zijn/haar weg komt.

"Ik ga een weg en word geleid op die weg"
Etty Hillesum

Mijn wieg heeft gestaan in een boerderij op de Walcherse kleigrond, middenin de stille, vrije natuur. Mijn ouders hebben mij, en Helma en André, opgevoed in een sfeer van niet zeuren, aanpakken en 'je best doen' om iets te bereiken. Een houding die niet altijd makkelijk is, omdat perfectionisme op de loer ligt, maar het was de basis waarop ik o.a. dit proefschrift heb kunnen afronden. Dus dank daarvoor. Helaas kan mama mijn promotie niet meer meemaken, maar ik weet hoe trots ze zou zijn geweest. Pa, je hebt net gehoord dat je ernstig ziek bent; ik hoop dat je er de 16e toch bij kan zijn.

De beslissing een vak te kiezen 'om andere mensen te helpen' was er al heel vroeg, maar wat? Geen verzorgend beroep, maar wel in de gezondheidszorg. Na wat wikken en wegen werd het geen studie geneeskunde maar de opleiding tot analist; de medische richting op de laboratoriumschool. Daar, waar Raymond en ik een stel zijn geworden, heb ik een supergezellige tijd gehad. We kregen o.a. les in Immunologie, een nieuw vak destijds wat me direct aansprak. Het bleek dat de afstudeeropdracht die ik kreeg tijdens mijn, in velerlei opzichten, leerzame stage verband hield met antilichamen; een schot in de roos. Meneer Steenhuis en Felix de Rooij, en collega docenten en laboratoriummedewerkers, bedankt dat jullie me op een positieve manier op weg hebben geholpen het vak van analist op te pakken.

Toen ik solliciteerde naar een baan bij de Klinische Genetica van de Erasmus Universiteit (erfelijkheid was mijn favoriete onderdeel van de biologielessen) kreeg ik als antwoord dat die vacature helaas vervuld was, maar dat ik werd uitgenodigd mee te solliciteren bij drie verschillende groepen van Celbiologie & Genetica. Een daarvan was bij Willem van Ewijk. Tijdens het gesprek met hem over inhoud van het beoogde project, kwam ter sprake dat er met proefdieren gewerkt zou moeten worden. Achteraf hoorde ik dat mijn vraag of ik die mocht zien, ertoe heeft geleid dat ik de baan kreeg omdat het blijk gaf van een 'gezonde nieuwsgierigheid'. Met Els van Vliet, en ook Peter van Soest, zijn we enthousiast begonnen aan het project wat

succesvol kon worden afgesloten; met ER-TR9 als 'mijn kindje'. Het leverde me werkervaring op met o.a. celkweek, ELISA, immunohistochemie en FACS in een stimulerende internationaal georiënteerde wetenschappelijke omgeving, waardoor ik o.a. in Washington en later in Marseille heb mogen werken. Na het thymuswerk, o.a. met Pieter Brekelmans, ben ik overgestapt naar de monocyt/macrofaag ontwikkeling met Pieter Leenen en Walentina Slieker. Zij hebben me geleerd dat je veel data in korte tijd kan genereren ('postzegels verzamelen'), maar dat het uitwerken en publiceren ervan een vak apart is. Zoals ik recent heb geschreven voor het boek ter gelegenheid van het 25-jarig jubileum van de afdeling Immunologie, heb ik met zowel Willem als Pieter met plezier gewerkt. De kneepjes van het researchwerk zijn me prima bijgebracht. Sleutelwoorden waren behalve nieuwsgierigheid, nauwkeurigheid, verbanden leggen, en vooral vragen stellen; een vraag is nooit dom. Leuke resultaten ging samen met een goede sfeer met heel veel collega's die ik niet allemaal bij name kan noemen. De cabarets en optredens die werden verzorgd tijdens promotiefeesten waren een hoogtepunt, zoals ook de bijdrage aan ons huwelijksfeest. Iedereen, waarbij ik Jane, Huub en Tar toch even wil noemen, hartelijk dank voor deze periode. Willem, ik vind het een eer en heel bijzonder dat je nu in mijn commissie zit.

Omdat het interessante, maar drukke researchwerk gecombineerd met de zorg voor een dochter zwaar werd, heb ik gekozen om 'lekker in het dorp' op de Willem te beginnen als TOA. Het verdiepen in de algemene biologie, omgaan met leerlingen, opzetten van nieuwe proefjes, levend model van een zwangere vrouw zijn en inzicht krijgen in het reilen en zeilen van een grote middelbare school waren heel interessant, maar gaf toch niet genoeg voldoening. Collega's van toen: bedankt voor de prettige samenwerking en misschien tot ziens, op school als docent van mijn zoon, in de kerk of op 't dorp.

Toen ik aan twijfelen was over mijn TOA-baan, belde Pieter Leenen me; hij had een baan voor 20 uur per week in de aanbieding. Alsof hij het aanvoelde. Met beide handen heb ik de kans aangegrepen en mocht, begeleid door Maarten en Pieter, zelfstandig aan de slag binnen histiocytose-projecten. Het was goed om weer aan de bench te staan en celkweek verleer je niet. In een nieuw, gezellig team (o.a. Janneke, Katarina, Femke, Annabrita, Tanja en Peter-Paul, Paul, Michel) was het lekker werken; dank daarvoor! Er was de mogelijkheid de resultaten zelf op te schrijven, maar daar kon ik naast werk en gezin geen tijd voor vinden. Gelukkig zijn de data recent gepubliceerd door Pieter en Anjali; een toegift voor mij. Pieter, ontzettend bedankt voor je vertrouwen, stimulans en vriendschap. En de wiebel zit prima hè? Het was inspirerend met je te brainstormen over stelling 6 waarin onze werkvelden bij elkaar komen.

Eigenlijk geen reden om te verkassen, maar vanbinnen kriebelde het; in plaats van fundamenteel wetenschappelijk onderzoek wilde ik graag klinisch toepasbaar onderzoek doen. Ik ben gaan solliciteren, o.a. bij de Nucleaire Geneeskunde naar een MNAA baan omdat ik niveau 5B had gehaald vanwege ^{3}H en ^{35}S werk. Het gesprek met Willem Bakker was heel prettig, maar een begintijd van 7:00 's morgens vroeg was niet te combineren met de zorg voor drie kinderen als (deels) alleenstaande moeder. Na een korte periode bij de Immunologie frontservice, ben ik bij Cardialysis gaan werken. Ook als analist, maar achter een beeldscherm i.p.v. aan de labtafel, heb ik o.a. ECG's leren analyseren: een nuttige ervaring rijker. Het was boeiend om kennis te maken met het bedrijfsleven; de strikte werktijden, hardere mentaliteit en sterke

hiërarchie waren een contrast met wat ik gewend was. Heather en Hilde, bedankt voor jullie lessen. Nol, Ellie, Annemarie (2x), Lali, Petra en Paula o.a., bedankt voor jullie collegialiteit. Daar kwam abrupt een einde aan toen de geldpot voor mijn aanstelling leeg was.

Inmiddels was er weer een vacature bij de Nucleaire. Willem herkende mijn naam en cv en belde me met de vraag of ik i.p.v. in de genoemde MNAA-vacature interesse had in een baan bij de preklinische groep omdat ik ook bevoegdheid had om met proefdieren te werken. Voor die opmerkzaamheid ben ik je super-erkentelijk, Willem. Na mijn sollicitatiegesprek met Marion en Bert zag ik tijdens de rondleiding over het lab een kweekkast en een cryostaat staan; toen kon het niet meer stuk. Arthur, je koos voor een switch van prekliniek naar de MNAA-groep. Dat gaf mij de kans om preklinisch onderzoek te gaan doen; precies wat ik zocht toen ik de Immunologie verliet.

Toen ik startte in 2003 bestond de preklinische groep uit Marion de Jong, Bert Bernard, Magda Bijster, Ria van den Berg, Astrid Capello, Suzanne Verwijnen en Monique de Visser en voor de radiochemie Wout Breeman en Erik de Blois. Marion, bedankt dat je bent ingegaan op de suggestie van Willem en me de kans hebt gegeven te werken in je groep. Voor mij ben je een inspirerende en stimulerende leidinggevende, die ruimte geeft om met eigen ideeën te komen. Arthur heeft me nog ingewijd in de kunst van autoradiografie voor zijn definitieve overstap. Bert speelde een belangrijke rol bij het inwerken op het gebied van celkweek van hechtende tumorcellijnen, het werken met radioactiviteit in veel grotere hoeveelheden dan ik gewend was, en met ratten die veel groter zijn dan muizen (met langere tanden) en die opgepakt worden om de romp i.p.v. aan de staart. Even wennen… Magda, je ruime ervaring met ratten en histologie zijn van grote waarde geweest. De gradering van nierschade op basis van histologie was essentieel. Ria, van jou heb ik de internalisatie methode tot in de puntjes geleerd. Op de werkvloer en tijdens werkbesprekingen volgde ik de promotie-onderzoeken van Astrid, Suzanne en Monique en spong bij met praktische hulp waar nodig.
Daarnaast kreeg ik de gelegenheid om zelfstandig experimenten te doen, vooral m.b.v. autoradiografie en ook micro-autoradiografie, het afstudeeronderwerp van stagiaire Katy van de Wansem. Het abstract dat over dit werk werd ingestuurd naar de EANM werd genomineerd voor de 'Marie Curie award', wat inhield dat er een artikel over geschreven moest worden. Ziedaar mijn eerste schreden op het pad van eigen publicaties schrijven, waarbij mijn vroegere buurvrouw Bea Rowlinson de correctie Engelse taal voor haar rekening nam. Het tweede artikel wat ik mocht schrijven (niet opgenomen in dit proefschrift) was n.a.v. een poster die verrassend werd gekozen als een van de beste tijdens een IRIST meeting. Uit een experiment van Astrid en Magda, bleek er sprake te zijn van verhoogde receptordichtheid op progressieve tumoren na relapse zoals gekwantificeerd m.b.v. autoradiografie.
Ondertussen kwamen er nieuwe collega's: Edgar Rolleman, arts en oude bekende van de afdeling, om zijn promotie-onderzoek over nierbescherming tijdens PRRT af te ronden. Via de zijlijn was ik af en toe betrokken bij dit onderwerp; ik wist toen nog niet dat zijn roze-paarse proefschrift niet meer van mijn bureau weg te denken zou zijn. Edgar, ik ben je veel dank verschuldigd voor alle uitleg die je me in de loop van de tijd hebt gegeven. Eerst op het lab en later vooral via de mail bij gedeelde auteurschappen, zoals je review dat ik mocht gebruiken als deel van de inleiding (Hoofdstuk 1.2).

Verder hadden we ongeveer 1,5 jaar lang Cristina Müller uit Zürich en Flavio Forrer uit Basel te gast. Besides practising English, it was inspirational to collaborate with two experienced Swiss scientists, either with medical or chemical background. I appreciated the discussions on methods concerning cell culture and in vitro assays, animal imaging with several radiopharmaca, tumour and kidney autoradiography e.g., resulting (among others) in the paper which is included with permission in this thesis as Chapter 4.1. Flavio, it's a great pleasure for me that you will be a member of the Committee during my thesis defense. Cristina, your dedication and organization of your research were an example for me. I'm looking forward to our collaboration in the near future.

Toen in 2007 geen geschikte kandidaat kon worden gevonden voor de AIO-vacature binnen het KWF-project, toegekend als vervolg op het onderzoek van Edgar, vroeg Marion of ik die functie wilde invullen, met Monique als analist. Vooral tijdens de laatste fase van Monique's promotie-onderzoek betreffende bombesine analoga, heb ik meegewerkt aan haar experimenten en nu binnen het nieuwe nierbeschermingsproject waren de rollen omgekeerd. Omdat we allebei waren ingewerkt konden we een vliegende start maken. De samenwerking was prima; we maakten gebruik van elkaars sterke kanten. Er zou een tijdschrift moeten bestaan waarin resultaten die een hypothese niet kunnen bevestigen, toch gepubliceerd kunnen worden. Ondanks het vele werk, leverde het testen van een eventuele rol voor taurine, L158.809 en Captopril ter nierbescherming tijdens PRRT, helaas geen publicabele conclusie op! Je besluit het lab achter je te laten en je volledig te wijden aan je gezin en grootste passie, het dansen, kon ik alleen maar respecteren. Toen je afscheid nam, heb ik je gevraagd of je t.z.t. mijn paranimf wilde zijn. Gelukkig was je direct positief, zodat we straks drie-vrouw sterk voor de commissie staan. Ontzettend bedankt voor je nauwgezette en handvaardige inzet bij experimenten en dataverwerking en je persoonlijke betrokkenheid.

Een aparte alinea voor de vier stagiaires die ik mocht begeleiden tijdens hun afstudeerperiode binnen de prekliniek. Na het halen van stralingshygiëne niveau 5B en aanleren van de meest gangbare technieken kregen jullie een eigen stage-opdracht. Lennert heeft optimale omstandigheden voor autoradiografie met verschillende peptiden getest. Satish werkte aan de karakterisering van een folaat analoog met Cristina. Walter heeft heel wat ratten gewogen en coupes gesneden, maar ook autoradiografie uitgevoerd met ^{68}Ga-gelabeld octreotaat. Ook Wilmer was betrokken bij de follow-up van ratten na een therapeutische dosis en bepalingen van eiwit in urine, aangevuld met kwaliteitscontrole van de autoradiografieschermen. Ik ben jullie erkentelijk voor jullie bijdrage aan genoemde onderzoeksprojecten en voor mij was de studentenbegeleiding een leerzame ervaring.

De samenstelling van de groep bleef veranderen. Stefan Pool, Rogier Schroeder, Ingrid Thuis en Marleen van de Poel startten met hun AIO onderzoek. En Jan de Swart stapte over van de klinische gamma-camera's naar de prekliniek als specialist voor de microSPECT- en microPET-camera's, met Corine als 'collega voor een dag per week'. Ingrid en mijn naamgenote twee stopten vrij snel, en ook Sandra Dieleman, die Ingrid opvolgde, bleef niet lang. Toch was het fijn elkaar te leren kennen en een Sinterklaasfeest met deels Vlaamse invloeden was heel gezellig. Door de pensionering van Bert (regelneef wat betreft bestellingen, EDC, SBE, o.a.), Ria (fijne kamergenote als we op congres gingen) en Magda (goed vrouw-tot-vrouw contact, vooral rondom het overlijden van mijn moeder) en de keus voor een ander beroep van Suzanne (bedankt voor je energieke enthousiasme en persoonlijke aandacht) en Monique kwam er ruimte voor een nieuwe generatie. Met eerst Saskia, later Stuart (bedankt voor je prachtige

illustratie bij het nierscan artikel) en Linda, als analisten, Sander als AIO, Harald als post-doc en Mark Konijnenberg als fysicus is de groep totaal vernieuwd. En door nauwere samenwerking met de preklinische groep o.l.v. Monique Bernsen van de Radiologie ook sterk vergroot. Saskia, Stuart en Linda: jullie vakkundige bijdrage aan biodistributies, scans (vooral de dual isotope dynamische) en in vitro experimenten beschreven in de recentste hoofdstukken uit dit proefschrift heb ik zeer gewaardeerd.
Voor de inbreng van hun expertise in verschillende artikelen uit dit proefschrift wil ik mijn kamergenoten van het laatste jaar, Jan en Mark, hartelijk dankzeggen. Ook voor de betrekkelijke stilte als we 'zij aan zij' of 'rug aan rug' gekluisterd waren aan het beeldscherm. En natuurlijk voor de vele kopjes heet water waar nog een theezakje door gezwengeld moest worden.
En: last but not least, in fact most important: Het is onmogelijk om onderzoek te doen bij de Nucleaire zonder radioactief gelabelde analoga! Wout en Erik, vanaf het begin kon ik bij jullie aankloppen met de vraag peptiden te labelen in allerlei specifieke activiteiten voor verschillende in vivo en in vitro doeleinden. Ook Ho-Sze draagt haar steentje bij; nu aangesteld als AIO in de groep. Met jullie is het goed discussiëren, dat houdt een mens scherp. Ontzettend bedankt voor de vele gevulde loodpotten die ik mocht ophalen.

Aansluitend aan de prekliniek wil ik iedereen van de Nucleaire Geneeskunde bedanken voor de collegialiteit en belangstelling! Tijdens afdelingsdagen, lunchmeetings, wetenschapsmiddagen, kerstborrels bijv. treffen we elkaar, en tijdens congresbezoek in kleinere groepen ontstaat vaak intensiever contact. Voor mij is het belangrijk contact te hebben met alle geledingen van de afdeling. Via Tracey zijn we sowieso met elkaar verbonden. Van de artsen wil ik Roelf met name noemen, omdat hij betrokken was bij de projectaanvraag en heeft meegewerkt aan veel van mijn artikelen. Hartelijk dank voor je uitleg en inbreng van klinische aspecten van het werk en je aanmoediging en discussie bij het publiceren. Dik, bedankt voor je inbreng wat betreft de statistiek in het amifostine stuk. En Lideke voor de contacten betreffende CCK/gastrine onderzoek; ik hoop dat we dat kunnen uitbreiden in de toekomst. Ook de MNAA groep verdient apart te worden genoemd. Als ik met een bestelbriefje kwam voor ^{177}Lu-octreotaat, ^{99m}Tc-DMSA of ^{99m}Tc-MAG3 met een 'vreemde' specificatie omdat het aan ratten of muizen toegediend zou worden, waren jullie bereid te berekenen hoe eraan kon worden voldaan. Erg bedankt voor jullie medewerking!

Omdat we veel met proefdieren hebben gewerkt, wil ik de (meeste) dierverzorgers van het EDC bij name noemen. Hun rol in dit werk is heel belangrijk. Ed en Albert, maar ook Ron, Esther, Dennis, Agnes en andere Dennis; hartelijk dank voor jullie bijdrage. Ook wil ik de verschillende art. 14 functionarissen en hun medewerkers bedanken voor hun inzet bij het voorspoedig afhandelen van vele DEC-aanvragen. Dank aan Frans, Martje, Marcel, Mathieu, Dominique, Marleen en Suzanne. Verder Lien voor het bestellen van dieren. Amélie voor haar hulp bij het invullen van werkprotocollen en kwaliteitszaken zoals screening van de muizen die uit Nijmegen werden ingevoerd. Henk, bedankt voor je hulp bij het regelen van het transport van deze dieren, waarmee de resultaten beschreven in Hoofdstuk 2.4 en 4.3 zijn behaald. En Edo als lokaal stralingsdeskundige voor de overkoepelende zaken.

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Prof. Marion de Jong, beste Marion, heel erg bedankt dat je me de mogelijkheid hebt geboden aan een eigen project te werken, alsmede voor de coaching tijdens de praktische fase van het onderzoek. Verder heb ik heel veel van je geleerd op het gebied van resultaatverwerking, schrijven en presenteren. Want leuke resultaten moeten worden vertaald naar een overzichtelijk, leesbaar artikel. Zeer erkentelijk ben ik je voor de vele, vele suggesties en correcties van manuscripten, waardoor je de inhoud van dit proefschrift waarschijnlijk wel kunt dromen. De kunst van het weglaten, essentieel voor een duidelijke presentatie en níet mijn sterkste kant, heb je me bijgebracht. Bedankt ook voor je luisterend oor als het tegenzat. Een bekende uitspraak van je is dat problemen gezien kunnen worden als uitdagingen. Dat leef je voor, en is voor mij een stimulans om op dezelfde manier met tegenslagen om te gaan.
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Een proefschrift wordt geen mooi boekje zonder goede lay-out. Ton, dankzij de kersverse samenwerking met de Radiologie mocht jij m'n teksten vormgeven. En daar ben ik superblij mee, want het was mij nooit gelukt om zo snel dit prachtige resultaat af te leveren. Gelukkig had je er ook zichtbaar lol in om er 'jouw boek, Marleen' van te maken inclusief spelen met lettertypes, kleuren en het samenstellen van de omslag.

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Al met al moge het duidelijk zijn dat ik niet het gangbare traject heb gevolgd, dat leidt tot het schrijven van een proefschrift, maar het kwam op mijn pad.

"Waar ik gelopen heb, is van nu af aan een weg"
Paul de Munnik (Vandaag ben ik gaan lopen)

Omdat ik heel goed besef dat werk (exact: linkerhersenhelft) alleen niet gelukkig maakt, beoefen ik ernaast graag activiteiten die eerder creativiteit, gevoel en spiritualiteit aanspreken (rechterhersenhelft) om tot een evenwichtige balans te komen. Hierdoor ben ik in contact gekomen met heel veel prachtige mensen, waarvan ik er een aantal bij naam wil noemen om mijn waardering te uiten.
Het contact met vele zwangere en net bevallen moeders in de tien jaar dat ik contactpersoon was voor de vereniging Borstvoeding Natuurlijk, was heel waardevol en heeft me veel geleerd zoals omgaan met groepen, luisteren en informatie overbrengen. Collega's Hanny, Jenny, Andrea, Liesbeth: bedankt voor de intensieve gesprekken.
Koorzang is al lang een grote hobby van me, omdat zingen energie geeft als je je gevoel erin kan leggen en bovendien is het gezellig. Leden van Mixed Voices (o.a. Henny, Suze, Wilna, Annet), de cantorij van (nu) de 'Open Hof' (o.a. Leo, Ida, Riet, Lies, Ed, Conny en nog veel meer) en Kioso Kyuwenda (teveel om op te noemen): dank jullie wel voor alle kippenvelmomenten! Lieve collega-Kioso-koorleden, ik wil jullie uitnodigen om te laten zien en horen dat stelling 10 waar is!
Begeleid door Sandrina zijn de cursussen 'Lichtdrager' en Lichtwerker' van grote betekenis voor me geweest. De ontstane vriendschappen eveneens (o.a. Marian, Lenie, Aad). Datzelfde geldt voor de Tarotcursus, o.l.v. Dini, het Talentenspel, o.l.v. Arnold en actief mediteren o.l.v. Hans en Connie.
Wandelen, met de verbinding naar pelgrimeren, heb ik mogen meemaken en begeleiden via 'de Wandelmaat', opgericht door pelgrim Henricus. Het ontmoeten van kloosterlingen, reisgenoten èn mezelf was bezielend en verrijkend, o.a. tijdens de mantra-weekenden met Truus. En dat geldt eveneens voor de contacten tijdens pelgrimages, de leeskring, de Vrouwenkring en andere bijeenkomsten vanuit de 'Open Hof' en NPB. Speciaal in deze context wil ik 'Creatief bezinnen' noemen o.l.v. Anna. Het schilderij op de omslag is ontstaan tijdens één van de sessies bij haar.

Andere waardevolle vriendschappen die ik hier wil noemen zijn die met José (mijn hele leven) en Johan, Connie en Hans (21 jaar, net zolang als onze dochters oud zijn), Marian en Charles (collega 'Lichtdrager' en pelgrim/klusmaat) en René en Marijke ('nieuwe' praatvrienden).
De basis van een veilig nest van familie om me heen, is van grote waarde; pa, Helma en Jan, André en Marian, bedankt. Verder wil ik mijn schoonfamilie(s) hier noemen. Pa en ma Rombout, jullie hebben me als een dochter opgenomen en tonen altijd belangstelling, samen met Maurice en Léon. Monique, bedankt voor alles wat je in de loop van de jaren voor me hebt gedaan. Ook de warme interesse die ik ondervind bij de familie van Driel stel ik erg op prijs; o.a. de gastvrijheid die ik in Canada heb mogen ervaren was hartverwarmend.

De mannen in mijn leven verdienen vanzelfsprekend een belangrijke plaats in dit dankwoord. Raymond, met jou heb ik het grootste deel van mijn leven gedeeld. Met hetzelfde diploma op zak sloegen we elk een ander vakgebied in, jij de klinische chemie en ik de research. Maar we begrepen elkaar wel en tot op de dag van vandaag toon je belangstelling voor mijn werk. We hebben samen drie prachtige kinderen op de wereld mogen zetten. Ook nu we uit elkaar zijn, kunnen we de zorg voor ze in harmonie delen en is het geen probleem als ik weer eens op congres ben. Heel erg bedankt daarvoor, waarbij ik ook Connie van harte wil betrekken.
Beste Maarten, jij steunde me tijdens één van de moeilijkste periodes in mijn leven, zowel praktisch als emotioneel. Ik kijk er met grote dankbaarheid op terug.
Lieve Anton, rots in de branding van de laatste twee jaar. We hebben elkaar écht ontmoet in een periode dat ik al druk was met werk, maar het werd nog drukker omdat ik mezelf het ultimatum had gesteld in 2010 te promoveren. Ontzettend bedankt dat je me de ruimte hebt gegeven om dat doel, mijn 'feessie', te halen, waardoor er minder tijd overbleef om samen te zijn. Je rust en luisterend oor, èn je onvoorwaardelijke liefde waren essentieel in deze drukke fase. De verbinding die er tussen ons is heeft er niet onder geleden en kan zich in de toekomst alleen nog maar verdiepen.

Lieve Nina, Onno en Wibe. Jullie zijn echt de allerbelangrijkste mensen op de hele wereld voor me. "The reason to live for." Ik geniet van jullie en jullie vrienden, want ook Hans en Jessica zijn opgenomen in de kring. Het is goed jullie te zien ontwikkelen tot zelfstandige wereldburgers, met belangstelling voor waar ik mee bezig ben. Het viel niet mee om aan klasgenoten uit te leggen welke baan jullie moeder heeft. Iets met laboratorium, ratten, kanker en radioactiviteit, tja… En als blijkt dat oma, en nu ook opa, kanker heeft, dan kan er ik er niets aan doen! Door dit soort gesprekken belandde ik wel weer met beide voeten op de grond. Wibe, jij gaat voorlopig volop voor de korfbal, maar school gaat ook goed, met toch een voorliefde voor bio… Jouw humor, imitatietalent en 538 (!) doorbreken de stilte in mijn huis; een welkome afleiding! Onno, met jou heb ik van jongs af aan kunnen oefenen met discussies voeren. Je bent kritisch, maar eerlijk en objectief, en nu een goede gesprekspartner over bedrijfskundige aspecten van het werk. Nina, ik ben er trots op dat je één van mijn paranimfen bent. Toen je hoorde dat het leek op ceremoniemeester zijn, zei je direct 'ja' toen ik het vroeg. Organiseren ligt je wel zoals blijkt uit je voortgang op de hotelschool. Toen ikzelf paranimf was bij Pieters promotie zat je overduidelijk in mijn buik. Nu, 21 jaar later, kan je mij als volwassene bijstaan bij de verdediging van mijn proefschrift. Een hele leuke bijkomstigheid van promoveren op je 50e!

En nu verder op weg. Het doel lijkt bereikt, maar het is een stap in het onderweg zijn.

"Dat je de weg mag gaan die je goed doet,
dat je opstaat wanneer je valt,
dat je mens mag worden in Gods ogen en die van anderen.
Weet dat de aarde je draagt,
dat je gaat in het licht en de wind je omgeeft.
Dat je de vruchten van je leven proeft en gaat in vrede."

19 oktober 2010

Curriculum vitae

De schrijfster van dit proefschrift, Marleen Melis, werd geboren op 31 juli 1960 te Gapinge. In 1978 behaalde ze het diploma Atheneum-b aan de Christelijke Scholengemeenschap Walcheren (CSW) te Middelburg. Van 1978 tot 1981 volgde ze aan het van 't Hoff-instituut te Rotterdam de HBO-b laboratoriumopleiding in de medisch-chemische richting. Haar stageperiode vervulde ze bij de afdeling Interne Geneeskunde II van AZR Dijkzigt o.l.v. dr. Felix de Rooij. Tijdens haar afstudeeropdracht vergeleek ze verschillende methodes om het IgM gehalte in serum te kwantificeren.

Vanaf juli 1981 werd ze aangesteld als analist bij de afdeling Celbiologie II van de Erasmus Universiteit Rotterdam. Onder leiding van dr. Willem van Ewijk werkte ze samen met Els van Vliet aan de productie van monoclonale antilichamen gericht tegen stromale thymus cellen. Deze promoveerde op dit onderzoek beschreven in het proefschrift: "Stromal cells in the mouse thymus". Vanaf 1986 tot 1991 werkte Marleen in dezelfde groep, die inmiddels onderdeel was van de afdeling Immunologie met als afdelingshoofd prof.dr. Rob Benner. Dit keer samen met Pieter Leenen aan de totstandkoming van zijn proefschrift getiteld: "Phenotypical analysis of murine macrophage differentiation".

In de jaren 1991 tot 1994 was ze technisch onderwijs assistent (TOA) biologie aan de Christelijke Scholengemeenschap (CSG) 'Willem van Oranje' in haar woonplaats Oud-Beijerland.

Van 1994 tot 2001 heeft ze weer als research-analist gewerkt op de afdeling Immunologie van het Erasmus MC Rotterdam, onder begeleiding van dr. Pieter Leenen en dr. Maarten Egeler, aan twee histiocytose-projecten: "A mouse model for malignant histiocytosis: identification of affected cells and liposome-based therapy" en "A mouse model for Langerhans cell histiocytosis: role of E-cadherin".

Vanaf eind 2001 tot voorjaar 2003 werkte ze op het CoreLab van Cardialysis te Rotterdam als analist elektrocardiografieën (ECG's) en hart-echo's.

In 2003 keerde ze terug naar het Erasmus MC. Ze werd aangesteld als research-analist op de afdeling Nucleaire Geneeskunde met als afdelingshoofd prof.dr. Eric Krenning. In de preklinische groep, geleid door dr.ir. Marion de Jong, werkte ze mee aan verschillende projecten betreffende de ontwikkeling en toepassing van radioactief gelabelde peptide-analogen, gericht op visualisatie en behandeling van tumoren.

Vanaf eind 2007 tot heden werkte Marleen aan het project: "Peptides for targeted tumour therapy and the kidney" dat werd gesubsidieerd door KWF Kankerbestrijding, in nauwe samenwerking met de afdeling Nucleaire Geneeskunde van UMC St. Radboud in Nijmegen en begeleid door prof.dr.ir. Marion de Jong, prof.dr. Otto C. Boerman en dr. Roelf Valkema. De resultaten van het onderzoek zijn beschreven in dit proefschrift.

Als post-doc zal ze verbonden blijven aan de preklinische groep van de afdeling Nucleaire Geneeskunde van het Erasmus MC.

Marleen is moeder van drie kinderen: Nina (1990), Onno (1992) en Wibe (1995).

Curriculum vitae

The author of this thesis, Marleen Melis, was born on July 31st, 1960 in Gapinge. She graduated from the high school Christelijke Scholengemeenschap Walcheren (CSW) in Middelburg (Atheneum-b) in 1978. From 1978 to 1981 she joined the Laboratory School 'van 't Hoff-instituut' in Rotterdam to follow the HBO-b course in the medical-chemical direction. During her practical training period she was employed at the Department of Internal Medicine II of AZR Dijkzigt, supervised by Dr. Felix de Rooij. The subject of her graduation thesis was the comparison of different methods to quantify IgM content in serum.

From July 1981 she worked as a technician at the Department of Cellbiology II at the Erasmus University Rotterdam. Supervised by Dr. Willem van Ewijk she was involved in the production of monoclonal antibodies directed against thymic stromal cells. This work was performed with Els van Vliet who obtained her PhD on the thesis entitled: "Stromal cells in the mouse thymus". Marleen continued her work in the same group, part of the Department of Immunology headed by Prof.dr. Rob Benner. Between 1986 and 1991 she participated in the research of Pieter Leenen, described in his PhD thesis: "Phenotypical analysis of murine macrophage differentiation".

Between 1991 and 1994 she organized and supervised) during the practical training in biology at the high school Christelijke Scholengemeenschap (CSG) 'Willem van Oranje' in her residence Oud-Beijerland.

From 1994 until 2001 she again joined the Department of Immunology of the Erasmus MC Rotterdam as research-technician to participate in two projects on histiocytosis under supervision of Dr. Pieter Leenen and Dr. Maarten Egeler: "A mouse model for malignant histiocytosis: identification of affected cells and liposome-based therapy" and "A mouse model for Langerhans cell histiocytosis: role of E-cadherin".

From the end of 2001 until spring 2003 she analysed electrocardiography registrations (ECG) and cardiac ultrasound images at the CoreLab of Cardialysis in Rotterdam.

In 2003 she again got a position at the Erasmus MC as research-technician at the Department of Nuclear Medicine headed by Prof.dr. Eric Krenning. In the Preclinical Group, supervised by Dr.ir. Marion de Jong, she cooperated in several projects concerning the development of various tumour-targeting radiolabelled peptide analogues for diagnostic or therapeutic applications.

Since December 2007 she is involved as researcher in a project granted by the Dutch Cancer Society entitled: "Peptides for targeted tumour therapy and the kidney" in close cooperation with de Department of Nuclear Medicine of UMCN St. Radboud in Nijmegen. Marleen has been supervised by Prof.dr.ir. Marion de Jong, Prof.dr. Otto C. Boerman and Dr. Roelf Valkema. The results are described in this thesis.

As a post-doc she will continue her work in the Preclinical Group of the Department of Nuclear Medicine at the Erasmus MC.

Marleen is the mother of three children: Nina (1990), Onno (1992) and Wibe (1995).

List of publications

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Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody.
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PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Marleen Melis (M.L.)
Erasmus MC Department: Nuclear Medicine
Research School: Molecular Medicine

PhD period: 1-12-2007 – 1-1-2011
Promotor(s): Prof. M. de Jong, Prof. E.P. Krenning and Prof. O.C. Boerman
Supervisor: Prof. M. de Jong

1. PhD training

	Year	Workload (Hours/ ECTS)
General courses		
- Biomedical English Writing and Communication	2009	4
- Laboratory animal science	2007	4
- Rapporteren in het Engels	2007	1
Specific courses (e.g. Research school, Medical Training)		
- Course Biomedical Research Techniques	2007	1
- Introductie tot de Klinische en Fundamentele Oncologie	2008	1
Seminars and workshops		
- 2nd Small animal imaging workshop Tübingen	2007	1
Presentations		
- COST B12 Final conference Warsaw, 1 oral and 1 poster	2005	
- IRIST London, 2 posters	2006	
- EANM Copenhagen, 1 oral	2007	1
- COST/IRIST Krakow, 2 orals	2008	
- EANM München, 1 oral	2008	1
- MolMed day, 2 posters	2009	
- SNM Toronto, 1 oral and 1 poster	2009	1
- EANM Barcelona, 2 orals and 1 poster	2009	1
- MolMed day, 2 posters	2010	
- WFNMB Cape town, 2 orals	2010	1
- EANM Vienna, 1 oral	2010	

(Inter)national conferences		
- COST B12 Final conference	2005	
- 18th IRIST meeting London	2006	
- 20th meeting of EANM Copenhagen	2007	1
- Work group meeting of COST BM0607 action, Krakow	2008	
- 19th IRIST meeting Krakow	2008	
- 21th meeting of EANM München	2008	1
- SNM Toronto	2009	1
- 22th meeting of EANM Barcelona	2009	1
- WFNMB Cape Town	2010	
- 23th meeting of EANM Vienna	2010	1
Other		

2. Teaching

	Year	**Workload (Hours/ ECTS)**
Lecturing		
- 'Animal models' during European Radiopharmacy Course block 2	2009	1
- 'Refereeravond' dept. of Nuclear Medicine	2008	
- Lunchmeeting dept. of Nuclear Medicine	2008	} 1
- Journal club dept. of Nuclear Medicine	2010	
Supervising practicals and excursions, Tutoring		
- Practicals AMIE course	2007/2008/ 2009	1
- Junior science students and 'keuzeonderwijs tweedejaars'	2009, 2010	1
Supervising Master's theses		
Students HLO:		
- Lennert Aarts	2007	2
- Satish Boedhoe	2007	2
- Walter Spreeuwenberg	2008	2
- Wilmer Wamsteeker	2009	2
Other		

Bij de omslag:

"Energie die straalt"

Tijdens het verval van radionucliden wordt vanuit de kern straling uitgezonden. Bijvoorbeeld γ-straling; zonder massa met een groot bereik of β-straling, bestaande uit deeltjes met een kort bereik. In de nucleaire geneeskunde wordt van de hoge energie van deze straling gebruik gemaakt tijdens diagnostiek (γ) of therapie (β). Tumortherapie beoogt het beschadigen van tumorcellen, maar straling op gezond weefsel wordt zo goed mogelijk geblokkeerd.

Leven vanuit je kern geeft energie, die uitgestraald wil worden. Dichtbij als bron van liefde voor jezelf of verder weg als liefde en licht voor anderen. Blokkeren van energieverlies is echter noodzakelijk om te overleven.

De stralende zonnebloem staat symbool voor het zoeken naar de zon (le tournesol); een bron van licht en warmte, net als vuur.

"Als alles duister is, ontsteek dan een lichtend vuur dat nooit meer dooft".
Taizé