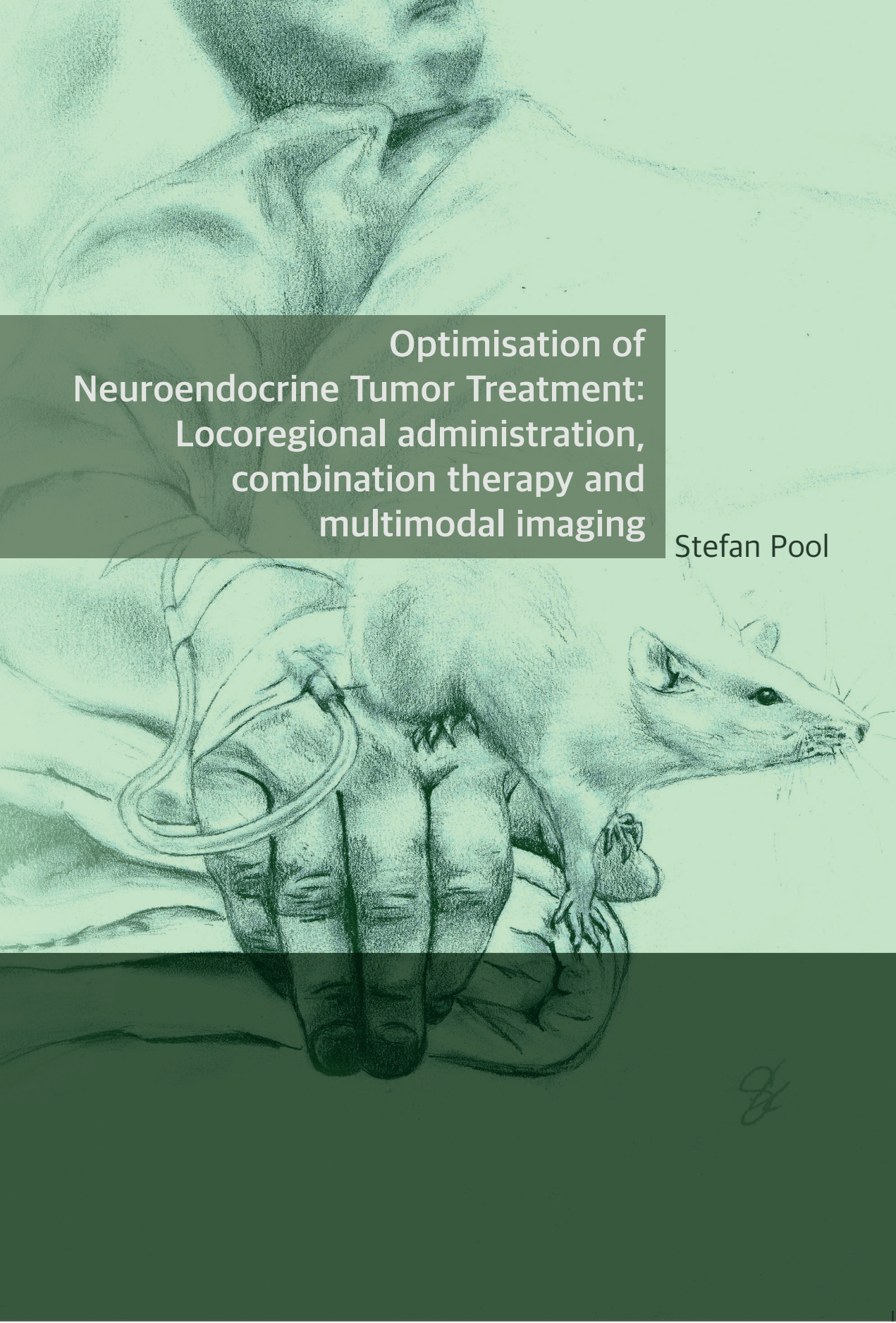

Optimisation of Neuroendocrine Tumor Treatment: Locoregional administration, combination therapy and multimodal imaging

Stefan Pool

OPTIMISATION OF NEUROENDOCRINE TUMOR TREATMENT: LOCOREGIONAL ADMINISTRATION, COMBINATION THERAPY AND MULTIMODAL IMAGING

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Optimalisatie van de behandeling van neuro endocriene tumoren: locoregionale toediening, combinatietherapie en multimodale visualisatie

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1 INTRODUCTION

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General introduction

There are many treatment options for neuroendocrine tumors, including surgical resection, chemotherapy, (radio) embolization, radiofrequency ablation (RFA), targeted drug therapy and peptide receptor radionuclide therapy. This thesis focuses on attempts to combine some of these methods in order to improve treatment outcome in patients with neuroendocrine tumors (NETs).

In this Introduction the following topics will be discussed:

Neuroendocrine Tumors

Neuroendocrine tumors belong to a family of rare neoplasms that are mostly found in gastro enteric, pancreatic, and pulmonary tissues, but that may be present virtually in any organ of the body. Neuroendocrine tumors originate from cells of the neuroendocrine system. Some of these neuroendocrine tumors produce hormones (functioning NETs) resulting in distinct clinical symptoms like in Cushing's syndrome (ectopic ACTH production), Zollinger–Ellison syndrome (ectopic gastrin secretion), Verner–Morrison syndrome (vasoactive intestinal peptide production) and Carcinoid syndrome (ectopic serotonin production)[1,2]. in a large epidemiological study in the USA the majority of NETs were located in the gastrointestinal tract (69.7%) (13,715 NET patients), whereas

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24.5% of the NETs were located in the tracheobronchopulmonary tract. Ovary, pancreas, thymus and gallbladder were the remaining localizations.[3] This study excluded patients with functioning or nonfunctioning pancreatic NETs (pNETs) because the then common indication 'carcinoid' was used as search term. These pNETs account for approximately 1.3-2% of all pancreatic cancers with regard to incidence[4,5] but due to their slow-growing nature they account for almost 10 % of pancreatic cancers in prevalence[4]. Nonfunctioning pancreatic pNETs are more common compared to functioning pNETs.[6] Most of NETs overexpress the somatostatin 2 receptor (sst_2), a G-protein coupled receptor that inhibits the secretion of a wide range of hormones upon activation by binding of the hormone somatostatin. Five sst subtypes have been identified (sst_2) which all have different roles[7]. The sst_2 overexpression on NETs enables imaging with for example [^{111}Indium-DTPA^0]-octreotide (Octreoscan®) or [^{68}Ga-DOTA^0-Tyr^3]-octreotate and/or treatment by Peptide Receptor Radionuclide Therapy.

Epidemiology

The incidence of NETs (pancreatic NETs not included) in the Netherlands was 1.8 per 100,000 inhabitants for men and 1.9 per 100,000 inhabitants for women in the period 1989-1996[8]. The highest incidence of NETs occurs in the seventh decade of life [8,9]. A rise in incidence of NETs has been reported by several authors[3,4,9]. This rise in incidence could partly be explained by the increased use and improved techniques of diagnostic modalities. Also the introduction of the World Health Organization classification for NETs of the gastroenteropancreatic tract in the year 2000 may have resulted in an increased awareness of the existence of these tumors and in more intelligibility for the nomenclature and categorization of gastroenteropancreatic neuroendocrine tumors (GEP-NETs).

Diagnosis

As mentioned before, part of the NETs is hormone producing, resulting in specific hormone-related symptoms that are quite often the first signs of (functioning) NET presence. Non-specific tumor-related symptoms are for example unexplained weight loss, pain and anorexia. Because of the specific hormone-related symptoms, functioning NETs are most of the time diagnosed earlier in the course of disease compared to non-functioning NETs. The carcinoid syndrome, consisting of secretory diarrhea, flushing, wheezing, and right-sided valvular heart disease, is caused by serotonin excretion by liver

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metastases. When the carcinoid syndrome is present, most of the times the tumor has already metastasized to the liver or retroperitoneal lymph nodes with drainage through the caval vein instead of the portal vein. Also localization of a primary tumor in the testis or ovary may result in the carcinoid syndrome. In these cases there is no hepatic breakdown of serotonin resulting in serotonin availability in the systemic circulation.

Diagnostic Procedures

Several laboratory tests can be used in de diagnostic process like chromogranine A (CgA), neuron-specific enolase (NSE), specific hormones in case of functioning NETs, general blood tests, such as liver function tests in the case of liver metastasis. Also the 5-HIAA (serotonin metabolite) urine test is commonly used.

Imaging can be performed by anatomical and functional imaging. Examples of anatomical imaging of NETs are conventional radiography, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) and angiography.

Examples of functional imaging are somatostatin receptor scintigraphy using [^{111}Indium-DTPA0]-octreotide (Octreoscan®)[10] and positron emission tomography-computed tomography (PET-CT) with for example ^{68}Ga-DOTA^{0}-Tyr^{3}-octreotide[11] or ^{68}Ga-DOTA-Tyr3-octreotate[12]. These imaging techniques can screen the total body and provide information about the presence of stt on the tumor. Other functional imaging techniques for the detection of NETs, not based on sst receptor targeting, include PET imaging with 6-^{18}F-fluoro-L-DOPA[13] or ^{11}C-5-hydroxytryptophan[14]. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET imaging, reflecting glucose metabolism, may be of value in NETs with a high proliferation index[15].

By endoscopy of the gastrointestinal tract, e.g. upper gastrointestinal endoscopy or colonoscopy, primary NETs can be diagnosed. Endoscopic ultrasonography can be used for diagnosis of pancreatic NETs including assessment of tumor relation with surrounding structures and the presence of pathological lymph nodes[16].

For a conclusive diagnosis a pathological analysis must be performed on tumor tissue derived by biopsy from the primary tumor or metastasis. Hematotoxylin and eosin staining, immunostaining for chromogranine A and synaptophysin, assessment of the

mitotic index and the Ki67 proliferative index should be performed[17–19]. Furthermore immunohistochemical staining for sst_2, insulin, gastrin, glucagon, or vasoactive intestinal peptide is optional[17–19].

Treatment

Chemotherapy

Several studies show a limited effect of chemotherapy in patients with well-differentiated NETs of non-pancreatic origin. Some objective responses have been reported on mostly poorly differentiated NETs with different chemotherapies like streptozocin in combination with doxorubicin[20], fluorouracil in combination with doxorubicin and streptozocin[21], cisplatin in combination with etoposide[22], capecitabine in combination with temozolomide[23], 5-fluorouracil and leucovorin in combination with irinotecan[24], temozolomide in combination with thalidomide[25] and temozolomide in combination with bevacuzimab[26]. The use of most of these chemotherapeutic regimens is hampered by the mostly short duration of response and significant (hematological) toxicity.

Radiotherapy

Radiotherapy is mainly applied for local treatment in case of brain metastases, spinal cord compression due to bone metastases or painful bone metastases. In case of localized bronchial NETs radiotherapy is being applied, especially if surgery is not an option anymore[27]. Partial responses and clinical benefit experienced by the patients were reported by Saif et al. after chemoradiation on the primary pancreatic neuroendocrine tumor (bed)[28]. Prospective studies to investigate the role of radiation and chemoradiation in NET treatment are warranted.

Surgery

Resection of the primary tumor is currently the only curative treatment option in NET patients, provided there is no advanced disease. In patients with locally advanced

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disease, neo-adjuvant treatment can sometimes be considered as an option to make surgery feasible. Unfortunately most NET patients suffer from metastasized disease already at the time of diagnosis, most often to the liver. However, even in patients with liver metastasis surgery can play a role. Kleine et al. reported in a rectrospective non-randomized study that extended surgery (partial liver resection/portal vein resection/partial gastric resection/liver transplantation) is feasible in highly selected patients; disease-specific survival of patients who had a liver resection was similar to patients without liver metastasis[29]. Another group reported, although also in a retrospective, non-randomized study, an encouraging 5-year survival rate of 82% after major surgery in patients with liver metastases from carcinoids and gastrinomas[30]. In a different study the same group reported a 5-year survival rate of 80% after extended (hepatic) surgery in patients with pancreatic or duodenal NETs metastasized to the liver[31]. Also symptom-control surgery can play a role as described in a study by Sarmiento et al.[32]. In this study hepatic resection was associated with a partial or complete response with respect to hormonal symptoms in 104/108 GEP-NET patients. The median time to recurrence was 46 months. Resection of the primary tumor was associated with a better survival in several non-randomized retrospective studies[33,34]. However selection bias could have played a role here, as younger and healthier patients are probably more likely to get surgery.

Liver-Directed Therapies

For patients with metastasized disease primarily localized in the liver, several non-surgical liver-directed interventional therapies are available: hepatic artery/transarterial (chemo-) embolization (H/TA(C)E), radiofrequency ablation (RFA), cryoablation and laser-induced thermotherapy (LITT). Intrahepatic malignancies mainly depend on the hepatic artery for their blood supply in contrast to normal liver parenchyma, which mainly relies on the portal vein. Therefore these intrahepatic malignancies can be selectively treated by H/TA(C)E. With HAE/TAE, objective responses were achieved in 40-75% of patients with a large variability in duration of the effect (3 - 88 months). With HACE/TACE objective responses were reported in 8-100%, again with variable duration (6-63 months, means 14-42 months)[35,36]. RFA has a high rate of local tumor control with limited local recurrence. Unfortunately these interventions only affect large tumor lesions within the liver with almost certain disease recurrence at other microscopic tumor sites in the liver.

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Intra-arterial radio embolization with yttrium-90 microspheres is an increasingly applied treatment option for patients with unresectable primary or secondary hepatic malignancies refractory to systemic therapies[37]. The high-energy beta-radiation emitting microspheres subsequently strand in the arterioles (mainly) of the tumor, and a tumoricidal radiation absorbed dose is delivered. The clinical results of this form of internal radiation therapy are promising[38,39]. Recently ^{166}Ho-loaded poly(L-lactic acid) microspheres (^{166}Ho-PLLAMS) have been developed which like ^{90}Y-microspheres emit high-energy beta particles to eradicate tumor cells whereas ^{166}Ho in addition also emits low-energy (81 keV) gamma photons which allows for nuclear imaging. This facilitates pre-therapeutic administration of a small scout dose, predicting the distribution of the therapeutic dose. Secondly post therapy imaging can be used for dosimetry calculations[40].

Intra-arterial administration of sst targeted PRRT is used by several groups for increasing the radionuclide tumor uptake and thereby the therapeutic radiation effect[41-44].

Medical Therapy

Binding of somatostatin analogs such as octreotide and lanreotide to the sst2 can reduce hormonal overproduction by a NET and may result in symptomatic relief in most patients with metastasized disease[45-47]. The long-acting somatostatin analog octreotide LAR (Sandostatin LAR®; Novartis Basel, Switzerland) also showed a positive effect on time to tumor progression compared to placebo in patients with functionally active and inactive metastatic midgut NETs[48]. This anti-tumor activity was suggested to be enhanced by combination of a somatostatin analog with recombinant interferon alpha in some retrospective studies[49,50]. However in a prospective, randomized clinical trial, no significant difference in overall survival was found between patients with midgut NETs treated with the combination of octreotide and interferon alpha versus octreotide alone[51].

Molecular Targeted Agents

Recently the results of two phase III trials investigating the efficacy of Everolimus and Sunitinib, both recently developed targeted therapies, were presented[52,53]. Treatment of pNET patients with Everolimus (Affinitor, RAD001; Novartis Pharmaceuticals; Basel; Switzerland), an inhibitor of mammalian target of rapamycin (mTOR), resulted in a longer median progression free survival (PFS) of 11.0 months compared to 4.6 months with placebo[53]. Also in PNET patients, treatment with Sunitinib (Sutent; Pfizer Inc., New

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York, NY), a tyrosine kinase inhibitor, resulted in a progression free survival of 11.4 months versus 5.5 months with placebo[52]. Everolimus combined with Octreotide LAR treatment in progressive PNET patients also resulted in a longer median PFS of 16.4 months versus 11.3 months with placebo combined with Octreotide LAR[54].

Peptide Receptor Radionuclide Therapy

Peptide Receptor Radionuclide Therapy (PRRT) is a promising targeted therapy for NETs using radiolabeled somatostatin analogs and is reviewed in Chapter 2. In summary, PRRT is and has been performed with several somatostatin analogs labeled with different radionuclides, such as [^{111}Indium-DTPA^{0}]-octreotide (Octreoscan®), [^{90}Y-DOTA^{0},Tyr^{3}] octreotide (^{90}Y-DOTATOC) and [^{117}Lu-DOTA^{0},Tyr^{3}]octreotate (^{117}Lu-DOTATATE). PRRT is discussed in more detail in Chapter 2.

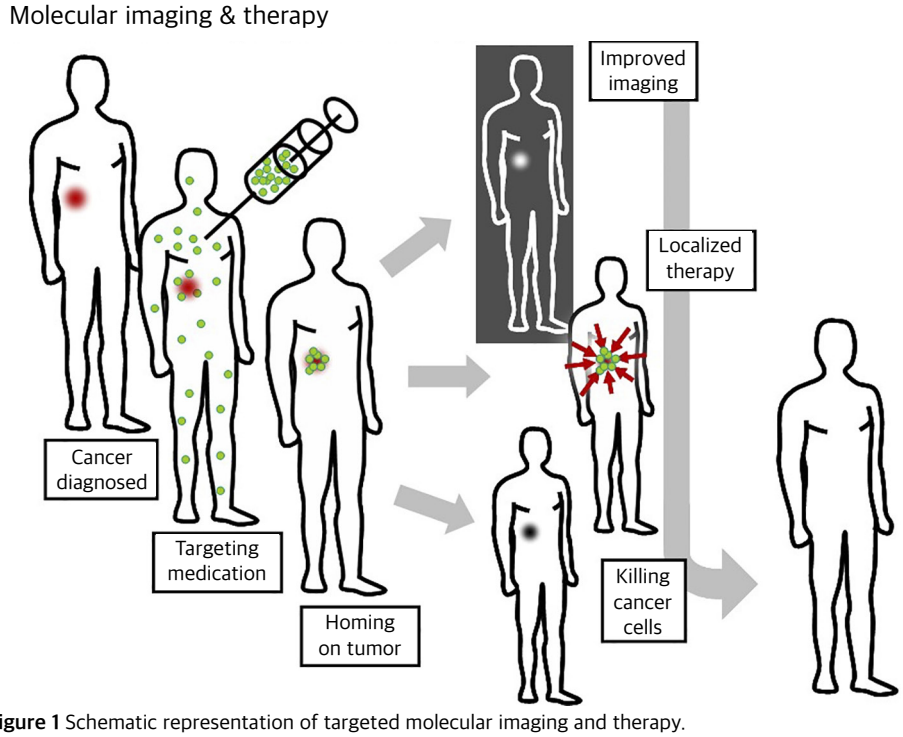

Figure 1 Schematic representation of targeted molecular imaging and therapy.

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Molecular imaging is a multidisciplinary field, which has emerged as a discipline at the intersection of molecular biology and in vivo imaging. It is used for non-invasively visualizing cellular function/localization and the follow-up of the molecular process in living organisms without the necessity to sacrifice them. Molecular imaging originates from nuclear medicine since from early on this discipline has applied radiolabeled tracers to show uptake or metabolism in specific organs/pathology. The most well known example is visualization of well-differentiated thyroid carcinoma using radioactive iodine (^{123}I and ^{131}I, both gamma ray emitters). Besides visualization of the tumor, treatment is also possible with ^{131}I, which next to gamma ray emission, used for imaging, emits high-energy beta particles, inducing damage to the cancer cells. Based on the same principle is the visualization of somatostatin receptors on GEPNETs using [^{111}Indium-DTPA^{0}]-octreotide and treatment of these tumors by PRRT targeting the overexpressed somatostatin receptor. One targeting moiety that can be applied for diagnosis, treatment selection, and treatment is known as a theranostic.

Tracers used for molecular imaging can be labeled with different radionuclides to be used for single photon emission computer tomography (SPECT) or positron emission tomography (PET) imaging, with contrast agents with high relaxivity for magnetic resonance imaging (MRI), and fluorophores for optical imaging. Especially preclinical bioluminescence and fluorescence imaging is increasingly used from intracellular imaging for example by confocal microscopy to in vivo imaging for tumor imaging/follow-up.

Translational research: preclinical-animal models

Animal NET model

In this thesis, most of the animal experiments have been performed in an established tumor model in Lewis rats[55]. In these rats, CA20948 tumor cells have been inoculated subcutaneously or subscapsularly in the liver. CA20948 cells are derived from a sst_2-positive pancreatic tumor of acinar origin that was originally induced by azaserine and that is transplantable in syngeneic Lewis rats[55]. The CA20948 tumor has shown to be very useful, both in culture as in vivo, as a model for peptide receptor radionuclide scintigraphy and/or therapy[55,57].

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Preclinical Imaging

Molecular imaging of the animals in the studies described in this thesis has been performed using a dedicated camera platform for (small) animal imaging. (Micro) SPECT/CT scanning was performed with a NanoSPECT/CT (Bioscan Inc., Washington, DC), a multiple pinhole, helical SPECT/CT camera (Fig. 3) that can visualize and gamma emitting radionuclides in vivo with high sensitivity and in sub-millimeter resolution. Bioluminescence imaging (BLI) was performed with the IVIS camera system (Xenogen, Hopkinton, MA).

Aims and outline of this thesis

The aims of the studies presented in this thesis are to improve PRRT by different interventions:

1 Evaluate the effect of intra-arterial versus intravenous administration on [^{111}Indium-DTPA^{0}]-octreotide tumor uptake in NET liver metastases in the rat model and in patients.

2 Evaluate the effects of combining ^{177}Lu-DOTATATE PRRT with the mTOR inhibitor RAD001 (Everolimus, Affinitor®) in the rat tumor model.

3 Transfection of the sst_2 overexpressing CA20948 rat tumor cell line to facilitate tumor follow-up by bioluminescence imaging in preclinical studies.

Chapter 2 gives an overview of preclinical and clinical PRRT studies. In Chapter 3 the effect of intra-arterial administration on [^{111}Indium-DTPA^{0}]-octreotide tumor uptake in NET liver metastases is described in a pre-clinical rat model and in three GEPNET patients. Based on the results derived from one patient, pharmacokinetic modeling and ^{177}Lu dosimetry has been performed. Chapter 4 and 5 depict several animal experiments in which ^{177}Lu-DOTATATE PRRT has been combined with the mTOR inhibitor RAD001 (Affinitor®). The therapeutic effect of this combination therapy is described, but more

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importantly the unexpected development of metastasis in this tumor model after RAD001 treatment has been studied. In chapter 6 the development and in vivo application of the luciferase-transfected CA20948-luc tumor cell line has been described. Chapter 7 and 8 provide a summary of the presented data and a general discussion.

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2 PRECLINICAL AND CLINICAL STUDIES OF PEPTIDE RECEPTOR RADIONUCLIDE THERAPY

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Abstract

In the 1980s, the ^{111}In-labeled somatostatin analog OctreoScan (Covidien, Hazelwood, MO) was developed for imaging of somatostatin receptor subtype 2 (sst_2) overexpressing tumors. On the basis of this success, peptide receptor radionuclide therapy (PRRT) was developed using similar somatostatin analogs with different therapeutic radionuclides. Clinical application of PRRT demonstrated impressive results on tumor response, overall survival, and quality of life in patients with gastroenteropancreatic neuroendocrine tumors. The peptides 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), Tyr^3-octreotate (DOTATATE) and DOTA, Tyr^3-octreotide (DOTATOC) (brand name Onalta), predominantly targeting sst_2, have been granted Orphan Drug status by the European Medicines Agency and the US Food and Drug Administration for application in PRRT. Besides somatostatin receptor-targeting peptides, multiple other radiopeptide analogs were developed targeting several other receptors overexpressed on various tumors. Some of these peptide analogs, including cholecystokinin, gastrin, gastrin-releasing peptide, arginine-glycine-aspartate (RGD)-peptides, and glucagon-like peptide 1 analogs appeared very promising in preclinical and clinical imaging and PRRT studies. Although the success of PRRT with radiolabeled somatostatin analogs has been established, there is still room for improvement. The therapeutic window of PRRT could be enlarged by the use of new and improved targeting compounds, of which new antagonists with excellent tumor to background ratios are very promising. Furthermore, locoregional administration, improved healthy tissue protection, and combination treatment can be applied to increase the effectiveness of PRRT. Combination treatment might include cocktails of different peptide analogs of different therapeutic radionuclides and of radiolabeled peptides with chemo-therapeutic or radiosensitizing agents. This review summarizes results of PRRT and describes clinical and preclinical studies regarding PRRT optimizing strategies.

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Introduction

Neuroendocrine cells are regulated by various hormones acting through specific receptors on the membrane surface, mostly G protein–coupled receptors. Tumors derived from neuroendocrine tissues usually express high levels of these receptors on their cell surface, which can be used as a target for tumor cell–specific therapy. The principle of high affinity targeting of such receptors for imaging or radionuclide therapy purposes has led to the development of a multitude of radiopharmaceuticals, most importantly radiopeptides [1,2]

A well-known example is the development in the late 1980s of the somatostatin analog ^{111}In-diethylene triamine pentaacetic acid (DTPA)-octreotide (OctreoScan, Covidien, Hazelwood, MO) for imaging of gastroenteropancreatic neuroendocrine tumors (GEP-NETs).[3] These tumors overexpress somatostatin receptors, predominantly receptor subtype 2 (sst_2), to which octreotide binds with high affinity.[4] In the past decade, several somatostatin analogs labeled with therapeutic radionuclides, such as ^{111}In, ^{90}Y, and ^{177}Lu, were developed and have been applied in peptide receptor radionuclide therapy (PRRT) studies with very impressive results,[5–22] as summarized in Table 1 and reviewed in detail in Reference 9.

Besides sst_2 targeting with somatostatin analogs, many other peptides have been developed for targeting of other peptide receptors, including cholecystokinin-2/gastrin receptors (CCK-2r), gastrin-releasing peptide receptors (GRP-r), vasoactive intestinal peptide receptors-1 (VPAC1-r), melanocortin-1 receptors (MCR-1r), neurotensin receptors-1 (NTR-1), neuropeptide Y-Y1 receptors (NP-Y Y1r), $\alpha v\beta 3$ integrins, gonadotropin-releasing hormone receptors (GnRHr-I), and glucagon-like peptide-1 receptors (GLP-1r). These receptors are overexpressed on various tumor types and can be targeted with peptide analogs with high affinity.[1]

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Despite the success of sst_2-targeted PRRT, there is room for improvement. For instance, development of more stable and/or antagonistic peptides, locoregional administration, combination with (radiosensitizing) chemotherapeutics, and healthy tissue protection are possible ways of enlarging the therapeutic window of PRRT. This article, aiming to review the current status and future potential of PRRT, consists of 3 parts:

1. current clinical PRRT studies using radiolabeled somatostatin analogs
2. promising new peptide candidates for PRRT
3. strategies to improve PRRT

Current Clinical PRRT Studies Using Radiolabeled Somatostatin Analogs

The most widely used and known ligands with therapeutic efficacy in GEP-NET in clinical practice, including nuclear medicine, are somatostatin analogs. Five somatostatin receptor subtypes have been characterized, the role of which is still not completely elucidated. Agonist binding to somatostatin receptors induces internalization of the ligand-receptor complex into endosomes and activation of postreceptor mechanisms. After internalization, the receptor is either recycled to the membrane surface or routed to a lysosomal degradation pathway.[23] This process of internalization has been considered a crucial step in PRRT. However, this paradigm has recently been challenged, as will be discussed in the section "Promising New Peptide Candidates for PRRT, Somatostatin Analogs" (sst_2-antagonists).

Tumors overexpressing somatostatin receptors typically include pituitary adenomas, gastrointestinal and pancreatic endocrine carcinomas, paragangliomas, pheochromocytomas, small cell lung cancers, medullary thyroid carcinomas, breast cancers, and malignant lymphomas. Most of the tumors listed express multiple receptor subtypes simultaneously, with sst_2 being the subtype most frequently detected.[2] In the late 1980s, ^{111}In-DTPA-octreotide (OctreoScan) was developed, and was approved in 1994 by the Food and Drug Administration for imaging of sst_2-overexpressing tumors.

After the successful application of radiolabeled octreotide for diagnostic imaging of

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tumor lesions overexpressing somatostatin receptors, the next logical step was to develop PRRT using ^{111}In-DTPA-octreotide. The aim of this therapy was to bring radioactivity into the tumor cell through internalization of the somatostatin receptor–radiolabeled analog complex. Besides the gamma radiation that is used for imaging, ^{111}In also emits Auger and conversion electrons with a medium-to-short tissue penetration (0.02–10 and 200–500 μm, respectively). In vitro PRRT experiments with ^{111}In-DTPA-octreotide indeed showed the therapeutic effect to be dependent on internalization, enabling the Auger electrons to reach the nucleus.[24]

Table 1 Clinical PRRT Studies With ^{111}In, ^{90}Y, and ^{177}Lu Labeled Somatostatin Analogs in Patients With GEP-NETs

Authors (yr of publication)	No. of patients	CR*	PR*	MR†	CR+PR
^{111}In-DTPAOC					
Valkema et al[5] (2002)	26	0	0	8	0
Anthony et al[10] (2002)	26	0	8	n.i.	8
Buscombe et al[14] (2003)	12	0	17	n.i.	17
Delpassand et al[15] (2008)	18‡	0	11	n.i.	11
Limouris et al[18] (2008) (locoregional)	11	9	45	n.i.	54
^{90}Y-DOTATOC					
Otte et al[6] (1999)	16	0	6	n.i.	6
Waldherr et al[22] (2001)	37	3	24	n.i.	27
Waldherr et al[8] (2002)	37	3	19	n.i.	22
Waldherr et al[21] (2002)	35	6	29	n.i.	34
Bodei et al[13] (2003)	21	0	29	n.i.	29
Valkema et al[19] (2006)	54	0	7	13	7
Frilling et al[16] (2006)§	19	0	21	n.i.	21
^{90}Y-DOTALAN					
Virgolini et al[20] (2002)	39	0	0	20	0
90Y-DOTATATE					
Baum et al[11,12] (2004)	75	0	37	n.i.	37
^{177}Lu-DOTATATE					
Kwekkeboom et al[17] (2008)	310	2	28	16	46

Table has been adapted from Teunissen et al [7] and more recent data have been added.
*Criteria of tumor response (SWOG/WHO criteria): CR (complete remission), no evidence of disease; PR (partial remission), >50% reduction of tumor size.
†Modification of the criteria: MR (minor remission), between 25% and 50% reduction of tumor size; n.i., not indicated.
‡One of 18 patients had a pheochromocytoma (response n.i.).
§Some of the patients received ^{177}Lu-DOTATOC after initial ^{90}Y-DOTATOC therapy.

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Clinical PRRT trials with high doses of ^{111}In-DTPA- octreotide showed promising therapeutic effects (Table 1),[5,10,14,15,18] but partial responses were seldom achieved. Preclinical PRRT experiments with ^{111}In-DTPA-octreotide in rats bearing small (≤1 cm^2) and larger (>8 cm^2) sst_2-overexpressing subcutaneous tumors showed significantly more therapeutic effects in animals bearing small tumors. The lower efficacy in larger tumors was most likely because of the lack of crossfire of the Auger electrons.[25] In this context the use of radionuclides, such as ^{90}Y and ^{177}Lu that emit β-particles with higher energy and longer particle ranges exceeding the tumor cell diameter, could have a greater therapeutic potential. Indeed, preclinical and clinical studies with ^{90}Y and ^{177}Lu labeled to the somatostatin analogs DOTATOC and DOTATATE showed more impressive tumor response rates compared with those obtained with ^{111}In-labeled octreotide.[5-11,13-20,22]

DOTA-coupled analogs can be labeled with either therapeutic radiometals, such as ^{90}Y or ^{177}Lu, or with positron or gamma radiation emitters, such as ^{68}Ga for positron emission tomography (PET) and ^{111}In for single-photon emission computed tomography (SPECT) imaging. Peptide receptor imaging and PRRT can therefore be performed by using the same peptide, which is named a theranostic. To select patients who are likely to benefit from PRRT, a scan using a peptide labeled with a diagnostic radionuclide can be made. Upon a positive outcome, selected patients can then be treated using the same or a similar peptide labeled with a therapeutic radionuclide. The development of such theranostics could greatly advance the development of personalized treatments.

In addition to patient selection for PRRT, other imaging applications of targeted radiopeptides include localization of primary tumors, detection of metastatic disease (staging/restaging), dosimetry, prediction of response and radiotoxicity, and monitoring effects of surgery, PRRT, or chemotherapy.

The radiopeptides for PRRT that have been studied most extensively are ^{90}Y-DOTATOC and ^{177}Lu-DOTATATE. Published results of ^{90}Y-DOTATOC and ^{177}Lu-DOTATATE derived from phase I-II trials, however, were not consistent with regard to patient selection, inclusion criteria, treatment schemes, and dosages. Therefore, an inter-study comparison is, in fact, not possible. Nevertheless, despite differences in the protocols applied in various centers, complete, partial, and minor remissions were registered in a

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maximum of 46% of patients with GEP-NETs.[17] Also, a clear survival benefit was reported after both ^{90}Y-DOTATOC[19] and ^{177}Lu- DOTATATE.[17] Patient self-assessed global health status improved significantly after therapy with ^{177}Lu-DOTATATE PRRT.[26,27]

From preclinical studies it was concluded that DOTATATE is a more suitable somatostatin analog than DOTATOC for PRRT, because of the higher affinity of DOTATATE for the sst_2 than that of DOTATOC, leading to a higher tumor uptake and resulting in a significantly higher tumor radiation dose.[28-31]

To compare these 2 analogs in patients, Forrer et al[32] used ^{111}In as a surrogate for ^{90}Y and ^{177}Lu and examined whether one of the ^{111}In-labeled DOTA-peptides had a more favorable biodistribution and tumor-targeting profile using diagnostic peptide amounts. ^{111}In-DOTATOC showed a higher tumor-to-kidney absorbed dose ratio in 7 of 9 evaluated tumors. On the basis of these results, the authors concluded that there were advantages for ^{111}In-DOTATOC over ^{111}In-DOTATATE, and therefore continued to use ^{90}Y-DOTATOC for PRRT.

By contrast, we compared the 2 analogs under PRRT conditions (with much higher peptide amounts) in a group of GEP-NET patients.[33] Comparing ^{177}Lu-DOTATATE with ^{177}Lu-DOTATOC, the mean residence time ratios of TATE to TOC-peptide were 2.1 for tumor, 1.5 for spleen, and 1.4 for kidneys. ^{177}Lu-DOTATATE had a longer tumor residence time than ^{177}Lu-DOTATOC. Therefore, we concluded that DOTATATE is the better peptide for use in PRRT.

The data on PRRT compare favorably with the limited number of alternative treatment options, such as chemotherapy.[9] Therefore, PRRT might become the therapy of first choice in patients with metastasized or inoperable GEP-NETs. Also, the role of PRRT in somatostatin receptor-expressing non-GEP-NETs, like metastasized paraganglioma/pheochromocytoma and nonradioiodine avid differentiated thyroid carcinoma, might become more important.[34-36]

Nevertheless, several research questions remain, including the optimal timing of sequential PRRT treatments. Most PRRT results thus far derive from phase I-II studies and many studies were carried out in patients with relatively advanced stages of disease, whereas recent data indicated a possible higher efficacy of PRRT when applied in an earlier phase of the disease.[17]

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Promising New Peptide Candidates for PRRT

Somatostatin Analogs

Several new somatostatin analogs have been introduced for therapeutic and diagnostic purposes, including the agonists DOTA-(1-Nal^3)octreotide (DOTANOC) and DOTA-(BzThi^3)octreotide (DOTABOC).[37,38] These compounds have a broader somatostatin receptor affinity profile than DOTATATE and DOTATOC because of a higher affinity for sst_3 and sst_5 in addition to their high affinity for sst_2. This could increase the number of tumors that could benefit from PRRT in the future. Promising preliminary PET imaging results have been obtained in favor of DOTANOC vs DOTATATE.[39] Recently, peptides targeting all the sst receptors (pansomatostatins) were studied by Ginj et al[40] demonstrating high affinity of ^{90}Y-DOTA-cyclo(D-diaminobutyric acid-Arg-Phe-Phe-D-Trp-Lys-Thr-Phe) (^{90}Y-KE88) for all 5 sst receptors. Surprisingly, sst_2-dependent internalization was demonstrated to be very low. Sst_3-expressing tumors had high and persistent uptake in mice biodistribution studies. The further development of these pansomatostatins could improve the therapeutic potential of sst-targeted PRRT in future.

So far, the studies in patients have been performed with somatostatin receptor agonists because agonists are internalized in (tumor) cells after which the radioactivity is retained in the cell. Most antagonists do not internalize[41,42] and block the downstream intracellular cascade.

However, Ginj et al[43] recently demonstrated in a preclinical study almost twice as high tumor retention of a radiolabeled sst_2 antagonist (^{111}In-DOTA-sst_2-ANT) compared with the agonist ^{111}In-DTPATATE, despite a somewhat lower receptor affinity of the antagonist for the sst_2. Tumor to kidney ratios 4 hours postinjection of the sst_2 antagonist and sst_2 agonist were 2.7 and 1.4, respectively. The higher tumor uptake was thought to be caused by binding of the antagonist to a larger variety of receptor conformations. This preclinical finding of a superior tumor targeting by noninternalizing somatostatin receptor antagonists is revolutionizing the current paradigm of the internalization of the receptor-ligand complex as the basis for PRRT. If these findings can be translated to the patient situation, antagonists can be applied to increase

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tumor radioactivity retention during imaging and PRRT. In Table 2, an overview of the affinity profiles of some radiolabeled somatostatin analogs is given.

Table 2 Affinity Profiles (IC50 values) of Different sst Targeting Radiopeptides

Radiopeptide	Abbreviation (Brand Name)	Targeted Receptor Subtype*	IC_{50} (nM, mean± SE)
Agonists			
Somatostatin-28	SS-28	sst_1	5.2 ± 0.3[4]
		sst_2	2.7 ± 0.3[4]
		sst_3	7.7 ± 0.9[4]
		sst_4	5.6 ± 0.4[4]
		sst_5	4.0 ± 0.3[4]
^{111}In-DTPA-octreotide	^{111}In-DTPAOC (OctreoScan)	sst_2	22 ± 3.6[4]
^{90}Y-DOTA,Tyr^3-octreotide	^{90}Y-DOTATOC (Onalta)	sst_2	11 ± 1.7[4]
DOTA,Tyr^3-octreotate	DOTATATE	sst_2	1.5 ± 0.4[4]
^{90}Y-DOTA-lanreotide	^{90}Y-DOTALAN	sst_2	23 ± 5[4]
		sst_5	16 ± 3.4[4]
^{111}In-DOTA-1-Nal^3-octreotide	^{111}In-DOTANOC	sst_2	3.3 ± 0.2[38]†
		sst_3	45 ± 3.3[38]†
		sst_5	12.2 ±1.9[38]†
^{111}In-DOTA BzThi^3-octreotide	^{111}In-DOTABOC	sst_2	3.1 ± 0.3[38]†
		sst_3	21 ± 1.8[38]†
		sst_5	7 2.1[38]†
^{90}Y-DOTA-cyclo (D-diaminobutyric acid-Arg-Phe-Phe-D-Trp-Lys-Thr-Phe)	^{90}Y-KE88	sst_1	10 ± 2[40]†
		sst_2	12 ± 0.5[40]†
		sst_3	5.4 ±1.1[40]†
		sst_4	2.8 ±2.3[40]†
		sst_5	2.8 ±1.12[40]†
Antagonist			
^{111}In-DOTA-[4- NO_2-Phe-c (Cys-Tyr-D- Trp-Lys-Thr-Cys)-DTyr-NH_2]	^{111}In-DOTA-sst_2-ANT	sst_2	11 ± 0.5[43]†

*sst subtype receptor to which the peptide has an IC50 value <50 nM. †IC50 values corrected for differences in the SS-28 IC50 values reported by Reubi et al in References 4 vs 38 or 40 or 43: (SS28 IC50 [Reference 4/ SS-28 IC50 [Reference 38 or 40 or 43]) / IC50 of the tested compound for the same sst.

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Glucagon-like Peptide 1 Receptor Targeting Peptides

The GLP-1r is highly expressed in the majority of insulinomas,[44] opening opportunities for imaging and radionuclide therapy by radiolabeled exendin analogs that bind with high affinity to GLP-1r. Also, pheochromocytomas[45] and gastrinomas[2] have been shown to demonstrate elevated GLP-1r expression. Preclinical experiments in mice bearing sub- cutaneous insulinomas showed specific uptake and internalization of ^{123}I-labeled GLP-1 amide and the GLP-1r analog ^{123}I-exendin-3,[46] enabling tumor scintigraphy. Preclinical experiments with the further optimized ^{111}In-DTPA- conjugated exendin analogs ^{111}In-DTPA-Lys^{40}-exendin-4[47] and (Lys^{40}[Ahx-DTPA-^{111}In]NH$_2$) exendin-4[48] showed high tumor to background ratios in animal tumor models. PRRT with (Lys^{40}[Ahx-DTPA-^{111}In]NH$_2$)exendin-4 showed to be feasible in repressing insulinoma growth in mice.[49] However, in these studies high radiopeptide renal uptake resulted in long-term kidney toxicity after PRRT. The fact that coinfusion of albumin fragments, lysine and/or gelofusin, reduces renal uptake of both targeting peptides in animal experiments[50] opens the way for PRRT studies with (Lys^{40}[Ahx- DTPA-^{111}In]NH$_2$)exendin-4 in the clinical setting. Wild et al[51] showed excellent antitumor effects in mice using this exendin analog in a lower dose and combined with the oral angiogenesis inhibitor PTK. Recently, a prospective clinical pilot study evaluating ^{111}In-DOTA-exendin-4 in SPECT/CT (computed tomography) localization of insulinomas showed impressive results demonstrating the high potential of targeting the GLP-1r [52]

Gastrin-Releasing Peptide Receptor Targeting Peptides

On both prostate and breast tumors, which are among the major causes of death worldwide, GRPr overexpression has been shown.[53] Targeting of the GRPr with radiolabeled bombesin analogs has been studied in the past decade with many combinations of radionuclides and analogs.[44,54-59] The most promising bombesin analog studied in the preclinical setting is ^{99m}Tc-Demobesin1, which demonstrated the highest absolute tumor uptake in animals in combination with high stability in vivo and rapid clearance from the (GRPr-positive) pancreas.[44,60] To date, few radiolabeled bombesin analogs have been tested in the clinic[61] and only 1 analog was used for PRRT.[62] Van de Wiele et al[55] published imaging data on ^{99m}Tc-RP527 in 4 androgen-independent prostate cancer (PC) patients with metastatic bone lesions. Another GRP analog, ^{99m}Tc-

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(Leu^{13})bombesin, was described in different studies.[63-66] Scopinaro et al[66] evaluated this analog in 8 PC patients and reported all 8 primary PCs to be visualized by SPECT, whereas 2 patients with benign adenomas did not show uptake. De Vincentis et al[65] reported SPECT-detected PC in all 12 patients with androgen-dependent PC, and locoregional lymph node visualization in 4 patients.

Only a very few PET studies have been reported for visualization and quantification of GRPr expression in PC patients. A clinical study by Hofmann et al[67] using ^{68}Ga-DOTABOM for PET imaging delineated malignant PC lesions in 13 of 15 patients.

In a phase I PRRT study in hormone refractory PC patients using the ^{177}Lu-labeled bombesin agonist AMBA, SPECT imaging revealed lesions in 5 of 7 patients.[62] Froberg et al[68] reported high initial uptake of both the radiolabeled agonist MP2248 and the antagonist Demobesin-1 in the pancreatic region of 4 PC patients, but retention of radioactivity in the pancreas after injection of MP2248 was much longer than after Demobesin-1 injection. Because of this slower decrease of pancreatic radioactivity after radio-agonist injection, a higher radiation dose will be given to this organ during PRRT. In addition, side effects can be expected at higher peptide amounts of the agonists, indicating the clear advantage of antagonists over agonists for PRRT using bombesin analogs.

CCK2 Receptor Targeting

Medullary thyroid cancer (MTC) has a rather low sst_2 expression compared with other neuroendocrine tumors, and sst_2 expression is even absent in clinically aggressive forms of the disease.[69,70] The CCK-2/ or gastrin receptor has been shown to be overexpressed in more than 90% of MTCs, and in a high percentage of small-cell lung cancers, stromal ovarian cancers, astrocytomas, and several other tumor types.[71] ^{111}In- DTPA-CCK$_8$ was able to visualize advanced metastatic MTC in patients.[72] Laverman et al[73] reported promising levels of tumor uptake and low levels of kidney uptake of the sulfated ^{111}In-DOTA-sCCK$_8$ in tumor-bearing mice, an ideal situation for radionuclide imaging and therapy. Beside CCK analogs, radiolabeled analogs of gastrin and minigastrin also showed suitable targeting affinity for the CCK$_2$-receptor. Preclinical studies by Behr et al[74] showed promising tumor uptake and therapeutic efficacy using ^{131}I-labeled

gastrin-1. Tumor targeting of this compound was also shown in a metastatic MTC patient. More recently, von Guggenberg et al[75] were able to image tumors in mice using the cyclized minigastrin analog ^{99m}Tc-EDDA-HYNIC-cyclo-MG1. In patients, ^{111}In-DTPA-minigastrin could visualize most tumor sites in MTC pa- tients.[74,76] Nock et al[77] synthesized ^{99m}Tc-labeled N_4-derivatized analogs of minigastrin. N°—1, Gly^0, $(D)Glu^1$-minigastrin (Demogastrin 2) was selected as the most promising after evaluation in preclinical studies. In a clinical study, the qual- ity of Demogastrin 2 could be confirmed by clearly delineating tumor deposits in metastatic MTC patients.[78] Gotthardt et al[79] compared the results of CCK_2 gastrin receptor scintigra- phy (GRS) in metastatic MTC patients, using ^{111}In-$(D)Glu^1$- minigastrin, with somatostatin receptor scintigraphy, CT, and ^{18}F-fluorodeoxyglucose PET. The combination of GRS with CT was the most effective in detecting metastatic MTC. The authors concluded that GRS may become the scintigraphic imaging modality of choice in MTC patients. Clinical CCK_2-targeted PRRT studies have not started yet, but its future is promising.

$\alpha_V\beta_3$ Integrin Targeting

The $\alpha_V\beta_3$ integrin is upregulated and accessible on proliferating endothelial cells, whereas it is not on quiescent endothelial cells. For their growth, solid tumors depend on angiogenesis, a process requiring endothelial cell proliferation. This makes $\alpha_V\beta_3$ integrins an interesting target for receptor-mediated tumor imaging and therapy for a large number of different tumors. The arginine-glycine-aspartic acid (RGD) peptide sequence was found to be responsible for extracellular matrix proteins binding to the $\alpha_V\beta_3$ receptor.[80] Cyclic RGD analogs conjugated to DOTA and DTPA have been developed enabling SPECT and PET imaging and PRRT when labeled with ^{111}In, ^{68}Ga, ^{64}Cu, ^{90}Y, and ^{177}Lu.[81-83] ^{18}F-labeled cyclic RGD analogs were also developed for PET imaging.[82,84,85] ^{18}F-galacto-RGD could effectively demonstrate the level of $\alpha_V\beta_3$ expression in human beings.[86,87] Dijkgraaf et al[89] synthesized DOTA-linked mono- and multimeric RGD pep- tides[88] as well as multimeric RGD peptides as dendrimers: macromolecules consisting of multiple perfectly branched monomers. The tetrameric RGD peptide and the tetrameric RGD dendrimer had the highest affinity and tumor uptake. However, kidney retention was also increased. In another study, Dijkgraaf et al[90] found in an intraperitoneal (i.p.) tumor model that i.p. administration resulted in better tumor-to- kidney ratios and a significant tumor growth inhibition during PRRT compared with intravenous injection, showing the therapeutic potential of RGD peptides.

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Epidermal Growth Factor Receptor Targeting

Overexpression of the epidermal growth factor receptor (EGFr) has been found in a variety of cancers like breast, bladder, gastric, and non-small-cell lung cancer, generally indicating a more aggressive behavior compared with normal or low expression.[91] A preclinical study by Chen et al[92] showed an antitumor effect of PRRT with ^{111}In-DTPA-EGF on EGFr-overexpressing breast carcinoma xenografts in mice. In a clinical study in 9 squamous carcinoma patients, ^{131}I-labeled EGF was able to visualize all tumors. A drawback was formed by the frequently observed adverse effects probably caused by the agonistic effect of EGF (nausea, vomiting, diarrhea, hypotension, fever, and chills), recorded during the dose escalating studies.[93] Radiolabeled antibodies that target against the EGFr (family) could be an interesting alternative for imaging or PRRT. However, a known disadvantage is the size of the antibodies (about 150 kDa), which causes increased systemic retention and poor tissue penetration. To overcome this problem, Tolmachev et al[94] applied EGFr- mediated uptake and imaging using ^{111}In-labeled affibody molecules (6-7 kDa) in EGFr-expressing A431 xenografts. However, the high uptake in healthy tissue expressing the EGFr, for example, the liver, remains a problem in radionuclide imaging and PRRT targeting the EGFr.

Strategies to Improve PRRT

Tumor Mass and Choice of Radionuclide

Tumor radiation dose does not only depend on the administered dose of radioactivity and the uptake vs time, but also on the tumor mass. Smaller masses have higher chances of mass reduction, as confirmed by clinical data showing that tumor remission was among other things positively correlated with a limited number of liver metastases, whereas disease progression was significantly more frequent in patients with a low performance status and a high tumor load.[95] Considering the use of 2 different radionuclides for PRRT using the 2 most commonly used radiopeptides, ^{90}Y-DOTATOC and ^{177}Lu- DOTATATE, in a mathematical model showed that ^{177}Lu would perform better in small tumors (optimal diameter of 2 mm), whereas ^{90}Y potentially had better tumor responses in larger tumors (optimal diameter 34 mm).[96] Very small tumors will not absorb all the energy deposited in the tumor by ^{90}Y, whereas larger

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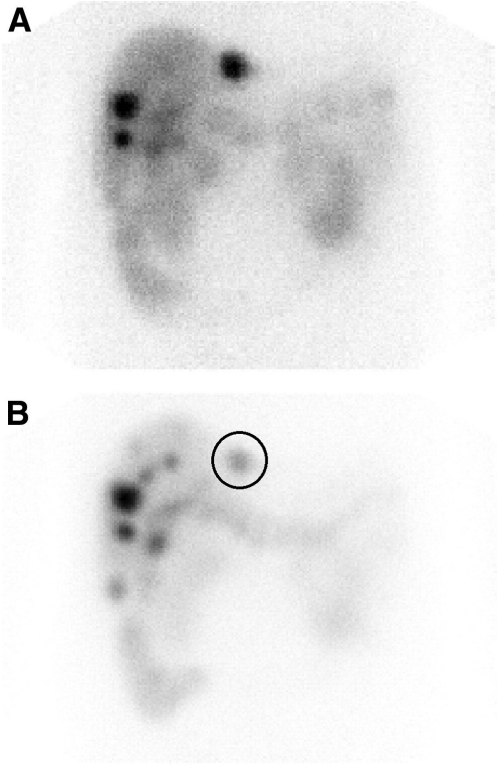

Figure 1 Anterior spot view images of the upper abdomen, 24 hours after injection of 111In-DTPA-octreotide (230 MBq/10 µg) in the same patient with a 2-week interval; (A) i.v. injection, (B) locoregional injection. catheter was placed in the right hepatic artery. The encircled metastasis, situated in the left part of the liver, therefore did not receive 111In-DTPA-octreotide locoregionally.

tumors will suffer from the possible lack of homogeneous distribution of ^{177}Lu throughout the tumor. In addition, the longer physical half-life of ^{177}Lu requires a longer exposure to deliver the same radiation dose to a tumor in comparison with the use of ^{90}Y. Therefore, we hypothesized that a combination therapy with ^{90}Y and ^{177}Lu labeled peptides, either given simultaneously or in separate sessions, might overcome the difficulties of treatment of lesions with different sizes in a single patient.[97] Preclinical studies indeed showed ^{177}Lu-DOTATATE to be more effective in small tumors (<1 cm^2), whereas ^{90}Y-DOTATOC was more effective in larger tumors (>1 cm^2).[98,99] A combination of ^{177}Lu-DOTATATE and ^{90}Y-DOTATOC in rats bearing both smaller (<0.5 cm^2) and large (7-9 cm^2) tumors led to a better survival compared with that after single dose of ^{177}Lu-DOTATATE or ^{90}Y-DOTATOC.[97]

Kunikowska et al[100] showed higher overall survival in 36 patients with diffuse neuroendocrine tumors treated with a combined regimen of ^{177}Lu/^{90}Y-DOTATATE (mixed dose 1:1) compared non-randomly to ^{177}Lu-DOTATATE alone. However, in

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the clinical setting randomized controlled trials comparing PRRT with ^{90}Y, ^{177}Lu or combined regimens are still lacking. Of high interest for PRRT is also the application of alpha emitters, as described in the article of Norenberg et al in this issue.

Locoregional Administration

To increase the therapeutic window of PRRT with radiolabeled somatostatin analogs, several groups have studied the possibility of locoregional administration for treatment of GEP-NET liver metastasis. McStay et al[101] prospectively evaluated the safety and effectiveness of hepatic arterial injection of the somatostatin analog ^{90}Y-DOTA-lanreotide with or without embolization as a treatment for patients with pro- gressive, large-volume GEP-NET liver metastasis. This study showed hepatic arterial injection of ^{90}Y-DOTA-lanreotide, with or without embolization, to be safe and as effective as, or even more effective than, the systemic administration in the Mauritius trial.[20] The added value of locoregional compared with systemic administration could not be shown, probably because a limited number of patients[23] were included, locoregional administration was performed with or without embolization, and a (systemically administered) control group was lacking. Limouris et al[18] reported high tumor to liver ratios after hepatic arterial infusion of ^{111}In-DTPA-octreotide in patients with inoperable liver-metastasized GEP-NETs. Also, a (systemically administered) control group to compare intra- hepatic tumor uptake and treatment effectiveness was lacking in this study. Seventeen patients underwent selective hepatic artery catheterization (180 in total) up to 15 times with no severe side effects. In another clinical study, 17 patients with primary or metastasized NETs were treated by locoregional and systemic administration of ^{131}I-MiBG. Locoregional administration resulted in a 69% mean increase in tumor to whole body ratio.[102] These studies all show that locoregional administration of radionuclide therapy can be regarded to be safe. However, in both studies targeting the sst_2 with radiolabeled somatostatin analogs, an intravenously administered control group was lacking.

We recently demonstrated doubling of the ^{111}In-DTPA- octreotide uptake in a liver metastasis rat model after locoregional injection through the hepatic artery compared

with systemic injection.[103] Direct intratumoral injections of ^{111}In-DTPA-octreotide resulted in even >10 times higher tumor uptake compared with systemic injection in preclinical mice experiments.[104]

Currently, we are performing a clinical pilot study to investigate GEP-NET liver metastasis uptake of ^{111}In-DTPA-octreotide after systemic and hepatic artery injection in the same patient with a 2-week interval. Preliminary results from this study are promising. In the first patient locoregional administration resulted in 2.4 times higher tumor uptake in GEP-NET liver metastasis.[103] In another patient, besides higher tumor uptake, more lesions could be visualized (Fig. 1). In this patient, the catheter was placed in the right hepatic artery resulting in locoregional administration in the right part of the liver. One metastasis, situated in the left part of the liver (encircled in Fig. 1B), therefore did not receive ^{111}In-DTPA-octreotide locoregionally. Recently, Beauregard et al[105] described up to 4 times higher uptake in GEP-NET liver metastasis after hepatic artery infusion of ^{177}Lu-DOTATATE in patients. Further experiments will have to be performed to be able to conclude on the benefit in terms of tumor uptake and therapy efficacy after locoregional vs systemic administration in PRRT.

Combination Treatment of PRRT

In external radiation therapy, radiosensitizing agents are commonly used. In several preclinical and clinical studies, combination of these agents with PRRT have been evaluated. In preclinical studies with tumor-bearing mice combinations of ^{177}Lu-DOTATOC with doxorubicin or cisplatin during a 4-week period, respectively, showed to be 14% or 23% more effective than single treatment.[106] In 21 patients, the radio- sensitizing agent 5-fluorouracil (5-FU) was combined with high-dose ^{111}In-labeled octreotide. This PRRT combined with 5-FU showed to be safe and therapeutic response rates obtained were at least comparable to those reported for ^{111}In-DTPA-octreotide treatment alone.[107] Two years ago our group started a multicenter, 2-armed, random- ized, prospective study investigating the combination of

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^{177}Lu-DOTATATE PRRT with the oral prodrug of 5-FU, capecitabine, vs ^{177}Lu-DOTATATE PRRT alone, after evaluation of the feasibility of this combination treatment in patients in a phase II trial.[108] As mentioned earlier, Wild et al[51] showed excellent antitumor effects of (Lys^{40}(Ahx-DTPA-^{111}In)NH_2)exendin-4 in a low dose combined with the oral angiogenesis inhibitor PTK.

Kidney Protection

In PRRT with somatostatin analogs, the kidney is one of the dose-limiting organs as was shown by renal toxicity after PRRT in clinical studies.[109-111] Reducing the uptake of radiopeptides in the kidney would therefore strongly contribute to enlarging the therapeutic window of PRRT. In rats, radiolabeled somatostatin analogs were shown to be filtered and reabsorbed in the proximal tubules of the kidneys.[112] In the human kidney, radioactivity was also found to be mostly located in the cortex.[113] In preclinical animal studies, megalin receptor-negative mice had 70%-85% less renal uptake compared with wildtype mice. This showed that the megalin/cubulin system is essential for a major part of the uptake of radiolabeled somatostatin analogs in the proximal tubules.[114]

Sst_2-specific uptake was shown to be responsible for 18% of renal uptake in human beings.[115] In our institution, renal uptake during PRRT in patients is reduced by a 4 hour coadministration of lysine and arginine together with ^{177}Lu- DOTATATE.[17] In a preclinical study in rats, we demonstrated that orally administered lysine also reduced renal uptake by 40% comparable to that of i.v.-administered lysine.[116] Further protection of the kidneys during PRRT with different peptides can be achieved using gelofusin and albumin-derived peptides.[50] The cytoprotective drug amifostine also protected the kidneys during PRRT with ^{177}Lu-DOTATATE given in high doses to rats.[117]

Conclusions

Several different somatostatin receptor-binding analogs have now been described that proved to be an excellent tool for PRRT of patients with GEP-NETs. PPRT showed few serious adverse effects and important tumor responses, long progression-free survival rates, and considerable improvement in quality of life. This field is rapidly growing; new

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agonist and antagonist peptides have been described that are or can soon be tested in clinical trials.

As tumors can also overexpress other receptors that may bind peptide analogs of naturally occurring hormones, for example, CCK-2, bombesin, neuropeptide Y, or vasoactive intestinal peptide receptors, even simultaneously, radioanalogs of these peptides will allow (multi)receptor PRRT in the future.

Future perspectives include studies exploring the effects of the combined use of PRRT with other drugs, such as radiosensitizing chemotherapeutic agents and the effect of locoregional administration of peptides, intra-arterially or even intratumorally injected, on PRRT efficacy.

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[^{111}IN-DTPA]OCTREOTIDE TUMOR UPTAKE IN GEPNET LIVER METASTASES AFTER INTRA-ARTERIAL ADMINISTRATION: AN OVERVIEW OF PRECLINICAL AND CLINICAL OBSERVATIONS AND IMPLICATIONS FOR TUMOR RADIATION DOSE AFTER PEPTIDE RADIONUCLIDE THERAPY

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Aims:

With the aim to improve peptide receptor radionuclide therapy effects in patients with gastroenteropancreatic neuroendocrine tumor (GEPNET) liver metastases we explored the effect of intra-arterial (IA) administration of [^{111}In-DTPA]octreotide (^{111}In-DTPAOC) on tumor uptake in an animal model and in a patient study.

Methods:

Preclinical study: After administering ^{111}In-DTPAOC intra-venously (IV) or IA, biodistribution studies were performed in rats with a hepatic somatostatin receptor subtype 2 (sst_2)-positive tumor. Clinical study: 3 patients with neuroendocrine liver metastases were injected twice with ^{111}In-DTPAOC. The first injection was given IV, and 2 weeks later, the second was injected IA (hepatic artery). Planar images of the abdomen were made up to 72 hours after injection. Blood samples were taken and urine was collected. Pharmacokinetic modeling was performed on the IV and IA data of the same patient. Based on this model, additional ^{177}Lu dosimetry calculations for IV and IA administrations were performed.

Results:

The preclinical study showed a two-fold higher ^{111}In-DTPAOC tumor uptake after IA administration than after IV injection. Patient data showed a large variability in radioactivity increment in liver metastases after IA administration compared with IV administration. Renal radioactivity was not significantly lower after IA administration;^{177}Lu dosimetry simulations in 1 patient using a maximum kidney radiation dose of 23 Gy showed IA administration resulted in a mean increase in tumor radiation dose of 2.9-fold.

Conclusion:

Preclinical and clinical data both indicate that IA administration of radiolabeled somatostatin analogs via the hepatic artery can significantly increase radionuclide uptake in GEPNET, sst_2-positive, liver metastases up to 72 hours postinjection, although the effect of IA administration can differ between patients.

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Introduction:

Gastroenteropancreatic neuroendocrine tumors (GEPNETs) are usually slow growing tumors that are often metastasized at time of diagnosis. In these cases curative treatment by surgery is most often not an option anymore. Various chemotherapeutic agents like streptozotocin, doxorubicin, 5-fluorouracil, chlorozotocin, etoposide, and cisplatin have been and are still being used alone or in combination for treatment of GEPNETs. Variable objective response rates and considerable toxicity were encountered though.[1] Recent studies show encouraging results in terms of tumor growth control by inhibition of growth factor receptors like vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and C-kit by sunitinib maleate.[2] In addition, inhibition of the mammalian target of rapamycin signal transduction pathway in GEPNETs by Everolimus (RAD001) most recently demonstrated a significantly improved progression-free survival of 11 months compared with 4.6 months observed in the placebo-treated patients.[3] Unfortunately, the affect on overall survival has not been shown yet. Despite these promising developments the standard biotherapy treatment at present is by somatostatin analogs like octreotide (short acting or long acting release). Octreotide treatment mainly aims at prevention of carcinoid syndrome and has been described to inhibit tumor growth to some extent.[4] Overexpression of the somatostatin 2 receptor (sst_2) on GEPNETs resulted in the 1980s in the development of radiolabeled somatostatin analogs like [^{111}In-DTPA]octreotide (^{111}In-DTPAOC) for visualization of sst_2-expressing NETs. In the past decade, several radiolabeled somatostatin analogs have not only been applied for visualization of NETs but also for peptide receptor radionuclide therapy (PRRT).

Initial PRRT studies were performed with high doses of the Auger electrons and γ-emitting ^{111}In-DTPAOC[5] and later with the β- and γ-emitting radiopeptide [^{177}Lu-DOTA,Tyr^3]octreotate (^{177}Lu-DOTATATE) and the β-emitting [^{90}Y-DOTA,Tyr^3]octreotide (^{90}Y-DOTATOC), both being applied now for treatment of GEPNETs. ^{177}Lu-DOTATATE and ^{90}Y-DOTATOC studies have shown very convincing results with regard to tumor response, overall survival, and quality of life.[6,7] Few side effects have been reported. Dose limiting organs due to radiotoxic effects are bone marrow[8] and the kidneys, the organs of excretion in PRRT. Co-infusion of amino acids reduces kidney uptake and the renal radiation dose.[9,10] The maximum administered activity is usually 29.6 GBq for ^{177}Lu-DOTATATE and 22.2 GBq/m^2 for ^{90}Y-DOTATOC. Complete responses are still rare though. We hypothesized that a higher tumor uptake of the radiopharmaceutical

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would improve the currently suboptimal tumor response.

Up to 75% of GEPNET patients have liver metastasis at time of diagnosis.[11] The aim of this study was to use intra-arterial (IA) administration of the radioligand via the common hepatic artery to increase tumor uptake of ^{111}In-DTPAOC. McStay et al. demonstrated IA PRRT with ^{90}Y-lanreotide to be safe and at least as effective as intravenous (IV) administration.[12] Limouris et al. also showed encouraging results with IA PRRT with ^{111}In-DTPAOC, ^{90}Y-DOTATOC, and/or ^{177}Lu-DOTATATE.[13,14] There was little proof of increased radiolabeled somatostatin analogue tumor uptake, until a 2009 study by Beauregard et al. reported that 3 patients had a 72% increased tumor to kidney ratio after IA ^{177}Lu-DOTATATE, in comparison with IV ^{177}Lu-DOTATATE PRRT. Intra-individually in 1 patient IV and IA administered ^{68}Ga-DOTATATE was quantified by PET imaging at 30 minutes postinjection (pi), showing a five-fold increase in tumor uptake after IA administration.[15] Kratochwil et al. showed IA administration resulted in a 1.4-7.8-fold (mean 3.75) higher tumor uptake of ^{68}Ga-DOTATOC as quantified by PET imaging at 40 minutes pi.[16] The same group also reported on the pharmacokinetics after IA and IV 20 minutes infusion of ^{111}In-DOTATOC intra-individual in patients with GEPNETs. At the end of IA infusion tumor uptake was 3.5-fold higher compared with IV. This tumor uptake showed a decrease to a 2-fold ratio at 4 hours pi and a 1.3-fold ratio at 72 hours pi. Additionally, impressive objective responses were reported after IA administration of ^{90}Y- and/or ^{177}Lu-DOTATOC in 15 patients with GEPNET liver metastasis.[16] In our study, we explored the effect of IV versus IA administration of ^{111}In-DTPAOC on tumor uptake in an sst_2-expressing liver metastasis model in the rat and intra-individually in 3 GEPNET patients with liver metastasis up to 72 hours pi. Based on the longitudinal measurements with ^{111}In-DTPAOC we performed pharmacokinetic modeling and simulated ^{177}Lu dosimetry on tumor tissue and healthy organs. Here, we describe major differences between 3 patients with regard to the ratio of tumor radioactivity up to 72 hours pi after IV versus IA injection in the same patient.

Materials and Methods

Radionuclides, peptide, chemicals

For the animal experiments $^{111}InCl_2$ was purchased from Covidien (Petten, The Netherlands). DTPAOC (Octreoscan®) was obtained from Tyco Health Care (Petten, The Netherlands). Radiolabeling was performed according to previously published procedures.[17] The labeling efficiency exceeded 99%, as confirmed by thin-layer

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chromatography. The specific activity of ^{111}In-DTPAOC was 3 MBq/0.5 µg peptide.

For the human study the commercially available Octreoscan kit (^{111}In-DTPAOC) was used in a specific activity of 220 MBq/10 µg peptide.

Liver metastasis model in the rat

The animal studies were in accordance with the Animal Welfare Committee requirements of our institution and were conducted following generally accepted guidelines. For the experiments, male Lewis rats (Harlan, Horst, The Netherlands) bearing an intra-hepatical CA20948 tumor[18] were used (n=6 per group, 2 groups). Mean bodyweight at the time of tumor inoculation was 300 g. All surgical and injection procedures were performed under isoflurane/O_2anesthesia and using a microsurgery microscope. During surgery, animals were kept warm with a heating pad.

After laparotomy of the rat's upper abdomen, the main liver lobe was fixated between two swabs and 1.5×10^{6}CA20948 tumor cells suspended in 100 µL matrigel basement membrane matrix (BD Biosciences, San Jose, CA) were injected subcapsularly via a 27-gauge needle. The abdomen was closed by absorbable sutures.

On day 14 after inoculation, laparotomy of the abdomen was performed again by a 3.5 cm incision along the linea alba. Silicon tubing (inner diameter 0.012 inch and outer diameter 0.025 inch) was placed in the gastroduodenal artery with the tip just in front

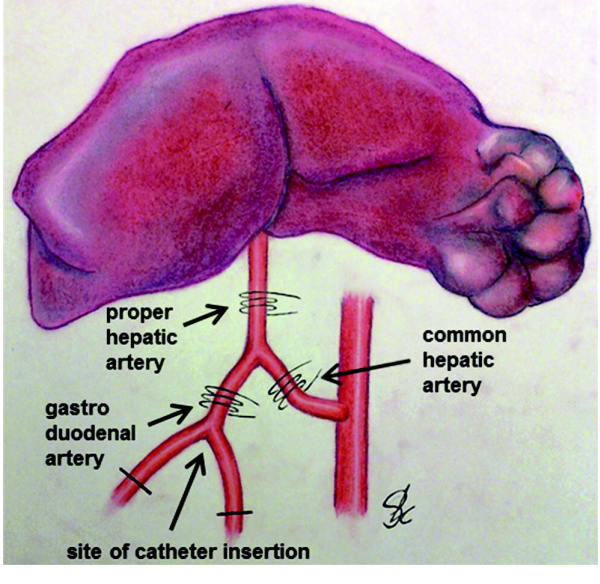

Figure 1. Schematic representation of the surgical technique used in rats to provide an intra-arterial (IA) route of administration to the liver. Before injection of [^{111}In-DTPA]octreotide (^{111}In-DTPAOC) the ligatures around the common and proper hepatic artery were removed to restore normal blood flow towards the liver.

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of the bifurcation of the common and proper hepatic arteries. A sham laparotomy was performed on all animals that received IV ^{111}In-DTPAOC administration. A schematic representation of the surgical technique used to provide an IA route of administration to the liver is shown in Figure 1.

Before injection, the blood supply to the liver was restored by removing the ligatures around the proper and common hepatic arteries, necessary for placement of the silicon tubing without major blood loss. One hundred fifty microliters of 3 MBq/0.5 µg ^{111}In-DTPAOC was injected in about 3 seconds. After injection the catheter was flushed with saline and removed.

After euthanasia at 24 hours pi normal organs and tumors were dissected and blood samples were taken. Organs and tumors were weighed and radioactivity was measured with a gamma counter (Wallac, 1480 Wizard 3"; PerkinElmer, Turku, Finland). The uptake of radioactivity was expressed as the percentage of injected activity per gram tissue (%IA/g).

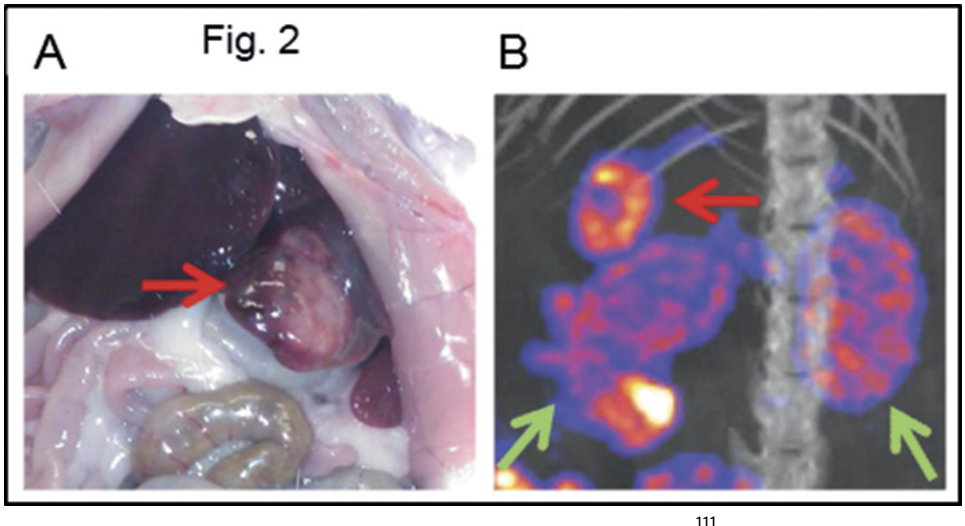

Figure 2. **(A)** Intrahepatic CA20948 tumor 10 days after inoculation. **(B)** ^{111}In-DTPAOC uptake in an intrahepatic CA20948 tumor visualized by micro-SPECT/CT. Red arrow, intrahepatic CA 20948 tumor; green arrows, kidneys.

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MicroSPECT/CT imaging

One additional rat with a subcapsular CA20948 tumor in the liver was imaged by microSPECT/CT imaging. Twenty-four hours before scanning, the rat was injected IV with 30 MBq/0.5 µg ^{111}In-DTPAOC. Scanning was performed with a four-headed multi-pinhole NanoSPECT/CT camera (Bioscan, Inc., Washington, DC). Nine pinhole-apertures with a diameter of 2.5 mm were used with 24 projections (1 minute per projection). The ^{111}In energy peaks were set at 171 and 245 keV. Guided by the CT topogram, the upper abdomen was scanned for 60 seconds per projection. The whole procedure was performed under Isoflurane/O_2 anesthesia. SPECT scans were reconstructed iteratively using InVivoScope software version 1.32 (Bioscan, Inc.) with medium noise reduction, a voxel size of 0.3 mm^3, and standard reconstruction settings.

Patient study

Three patients (age 32, 54, and 64 years) with metastatic nonresectable pancreatic NETs were enrolled to receive two injections of ^{111}In-DTPAOC; one IV injection and one IA injection with a 2 week-interval. From previous imaging it was known that these patients had hepatic metastasis enabling dosimetry measurements. All patients were on short acting octreotide treatment (Sandostatin; Novartis, Basel, Switzerland), which was discontinued 24 hours prior to both injections. This 24 hours discontinuation was chosen because before standard ^{177}Lu-DOTATATE treatment the same period of short acting octreotide treatment discontinuation is used. The study was performed after written informed consent from the patient to participate in this study, which was approved by the Erasmus MC Medical Ethical Committee.

IA administration

IA administration was performed via a catheter placed angiographically through Seldinger's technique via the femoral artery with the tip into the common hepatic artery. Immediately after this procedure the patient was placed on the gamma camera bed in a supine position. The ^{111}In-DTPAOC was injected in about 10 seconds. The catheter was flushed with 10 mL of 0.9% saline. The same injection protocol was used for IV injected ^{111}In-DTPAOC.

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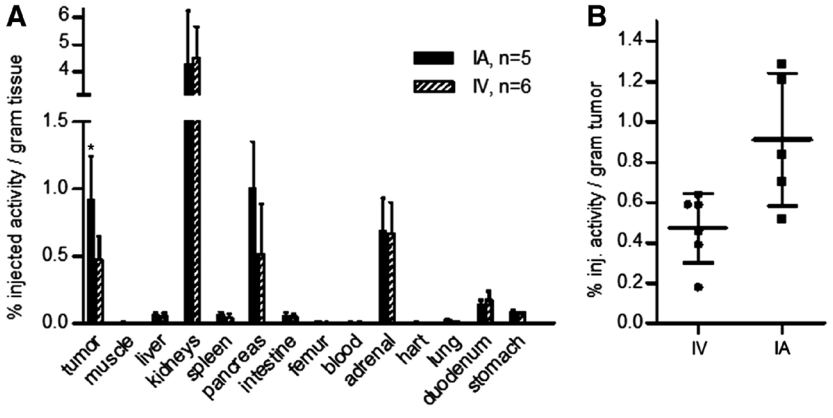

Figure 3 (A) Percentage injected ^{111}n-DTPAOC activity per gram tissue in tumor and several organs after intravenous (IV) and IA administration in CA20948 intrahepatic tumor bearing rats (mean±standard deviation [SD]) *p<0.05. **(B)** Dotplot of ^{111}In-DTPAOC uptake in the intrahepatic tumor after IV and IA administration (mean,±SD), p<0.05.

Imaging

All images were acquired with a dual-head gamma camera Picker Prism 2000 XP (Philips, Eindhoven, The Netherlands). The windows were centered over both ^{111}In photon peaks (245 and 171 keV) with a width of 20%. Parallel-hole, medium-energy general-purpose collimators were used. After each injection method, the same scan protocol was followed: dynamic imaging up to 30 minutes pi with a field of view over the kidneys and liver for the anterior and posterior projections (120 images, 15 seconds per image). Upper abdomen anterior and posterior scans were obtained at 1, 4, 24, 48, and 72 hours after injection. The acquisition time for all scans was 20 minutes. The accumulated radioactivity in tumor and organs was quantified by drawing regions of interest (ROIs) in Phillips odyssey LX software.

Measurement of radioactivity in blood and urine

Blood samples were drawn at 1 minute before and 2, 5, 10, 15, 20, 30 minutes and 1, 4, 24, 48, and 72 hours pi. Urine was collected in four intervals: 0-1, 1-4, 4-24, and 24-48 hours after pi. Radioactivity in blood samples was quantified using a gamma counter (Cobra II Autogamma, Packard, a Canberra Company). Radioactivity in urine samples was quantified using a dose calibrator (VCD-404; Veenstra Instruments, Joure, The Netherlands).

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Pharmacokinetics and dosimetry

ROIs were drawn manually on the anterior and posterior spot views of the upper abdomen around tumor lesions, liver, spleen, and kidneys. The background region was placed close to the ROIs for background correction. The geometric mean value, derived from the anterior and posterior scans, was taken and corrected for attenuation and physical decay. The activity in the syringe before injection minus the remaining activity in the syringe after injection was defined as 100% of the injected activity. A compartmental pharmacokinetic model was used to fit double-exponential curves through the uptake data. ^{177}Lu dosimetry calculations were performed on tumor and organs based on a supposed 23 Gy radiation dose to the kidneys after IV administration. Tumors were modeled as spheres. Actual tumor diameters were measured by CT and MRI. The organ and tumor residence times were used as input into the Olinda/EXM radiation dosimetry code.[19] The bone marrow residence time was calculated from the plasma activity

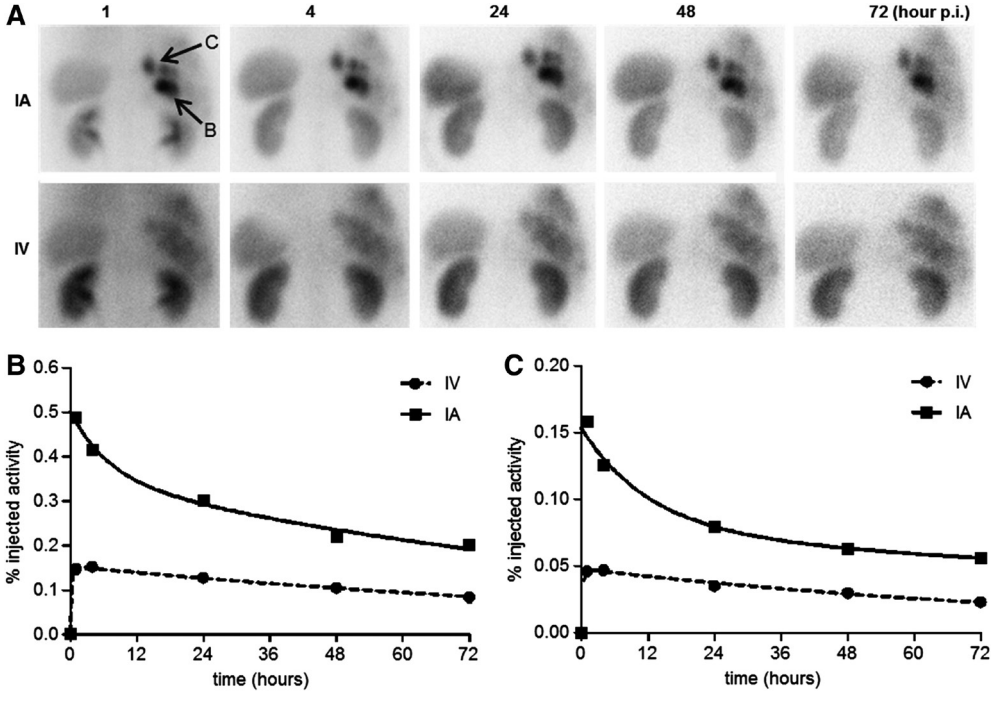

Figure 4. Patient 1; **(A)** planar posterior upper abdomen images showing higher ^{111}In-DTPAOC uptake in neuroendocrine liver metastasis at 1, 4, 24, 48, and 72 hours after IA and IV administration of ^{111}In-DTPAOC. **(B, C)** Quantification of ^{111}In uptake in liver metastasis B and C (see first picture A) after IA and IV administration. IA administration resulted in both liver metastases in a 2.4-fold increase of the area under the curve (AUC) compared with IV administration.

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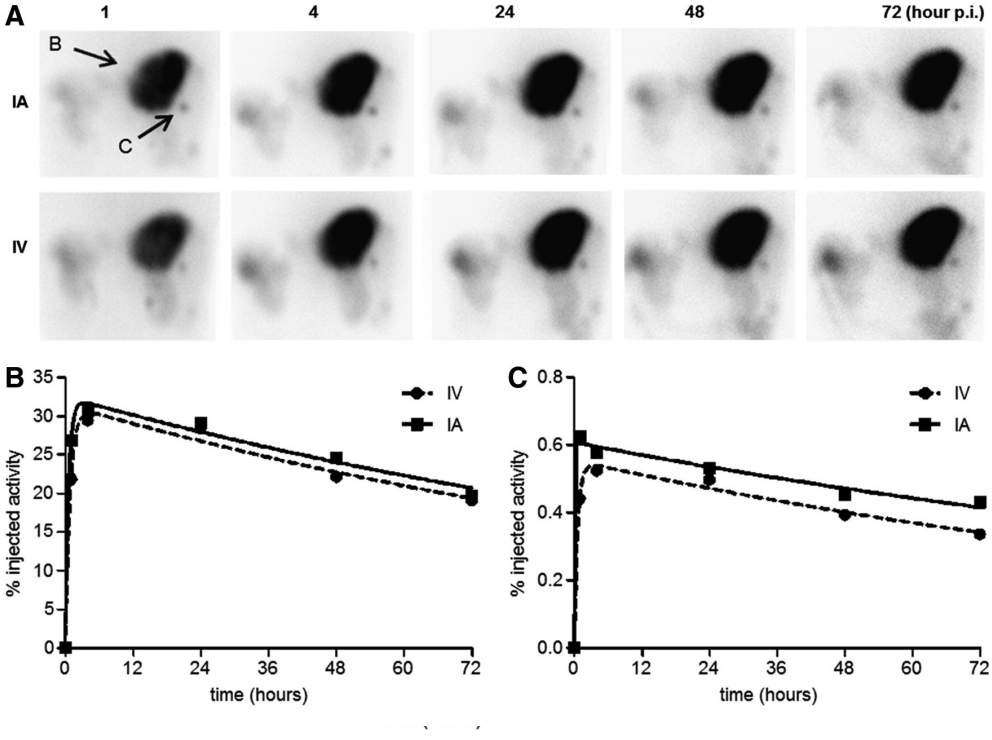

Figure 5. Patient 2; **(A)** planar posterior upper abdomen images showing almost comparable ^{111}In-DTPAOC uptake in neuroendocrine liver metastasis at 1, 4, 24, 48, and 72 hours after IA and IV administration of ^{111}In-DTPAOC. **(B, C)**Quantification of ^{111}In uptake in liver metastasis B and C (see first picture A)after IA and IV administration. IA administration resulted in liver metastasis B in a 1.06-fold increase of the AUC and in liver metastasis C in a 1.14-fold increase of the AUC compared with IV administration. **(D)** Digital subtraction angiography illustrating the arterial blood supply and positioning of the catheters tip during IA administration.

concentration curve.[8] The dosimetry output was not corrected for the actual volumes of the organs. The dose to the tumors was calculated by the spherical node option within the Olinda/EXM code.

Statistics

Data were expressed as mean±standard deviation. Statistical analysis was performed using the unpaired Student's t-test.

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Results

Liver metastasis model in the rat

Inoculation of CA20948 tumor cells from in vitro cultures, mixed with matrigel, resulted in a palpable solid tumor (Fig. 2A) 10 days later. The tumor could clearly be visualized by micro-SPECT scanning 24 hours pi of ^{111}In-DTPAOC (Fig. 2B). Ex vivo biodistribution at 24 hours after injection revealed the tumor uptake of ^{111}In-DTPAOC administered via the common hepatic artery to be twofold higher ($p<0.05$) than the uptake after systemic (IV) administration (Fig. 3A, B). Uptake in kidney, liver, stomach, duodenum, adrenals, blood, and muscle did not significantly differ after both injection methods. Surprisingly, after IA administration the radioactivity in the pancreas was higher than after IV administration.

Patient study

Low ^{111}In-DTPAOC uptake (iso-intense compared to the liver) was seen in the liver metastases of the first patient after IV administration on all images made at 1, 4, 24, 48, and 72 hours pi. After IA administration in this patient clear visualization of the liver metastases was obtained (Fig. 4A). Quantification data of tumor uptake after IA administration showed a 2.4-fold higher ^{111}In-DTPAOC uptake (Fig. 4B, C) in these liver metastases in comparison with that after IV administration. Kidney uptake and urine radioactivity was not significantly different after either route of administration.

Strikingly, in the second patient IA administration did not result in significantly higher ^{111}In-DTPAOC uptake in the liver metastases. Quantification showed a 1.06- and 1.14-fold increase of ^{111}In-DTPAOC uptake after IA versus IV administration for metastasis B and C (Fig. 5A), respectively. When compared with the first patient, the tumor uptake was exceptionally high in patient 2. At 4 hours pi after IV and IA administration around 30% of the injected activity was located in the very large liver metastasis B (Fig. 5A), whereas for the first patient the maximum uptake was only 0.5% of the injected activity in liver metastasis (Fig. 4A, metastasis B). So, tumor uptake in the second patient was already exceptionally high after IV administration.

In a third patient three liver metastases were clearly visualized at 1, 4, 24, 48, and 72 hours post IV administration. The equivalent scans after IA administration revealed several additional liver metastatic lesions (Fig. 6A), whereas the lesions that were visible on the IV scan showed higher uptake after IA injection. Surprisingly, one metastatic

nodule (hotspot 8, Fig. 6A) also shown on the scan after IV injection and located in liver segment 5 did not show an increased ^{111}In-DTPAOC uptake after IA administration. Analysis of the dynamic scans made during the injection phase, showed that the tip of the catheter in this patient was not situated in the common hepatic artery, but in the right hepatic artery (Fig. 6A, picture in the right upper corner). As a result, ^{111}n-DTPAOC was administered IA to the right part of the liver, whereas the left part received the ^{111}In-DTPAOC after first pass through the body via systemic administration.

Urine sample data, blood sample data, and region of interest (tumors and kidneys, Fig. 6B) quantification data of this patient were fitted in a compartmental pharmacokinetic model (Fig. 6C) for IA and IV administration in Figure 6D and E, respectively. Quantification of ^{111}In-DTPAOC uptake in liver metastasis 4 (in the right part of the liver) over 72 hours showed a mean 2.3-fold increase after IA versus IV administration (Fig. 6D, E). ^{111}In-DTPAOC uptake in the metastasis (hotspot 8) located in the left part of the liver showed to be comparable after IA and IV administration.^{111}In-DTPAOC kidney uptake was 13% lower after IA administration compared with IV administration. The percentage injected activity in plasma only differed significantly between IA and IV administration at 2 minutes pi. Five minutes pi, IA and IV plasma values were almost comparable (Fig. 6F).

Based on the compartmental pharmacokinetic model ^{177}Lu dosimetry calculations were performed (Fig. 6G) for all liver metastasis after IA and IV administration assuming similar pharmacokinetics for ^{111}In-DTPAOC and ^{177}Lu-DOTATATE. Radiation dose with ^{177}Lu-DOTATATE PRRT was calculated for all metastases based on a radiation dose to the kidneys of 23 Gy after IA and IV administration. These calculations showed for all metastases located in the right part of the liver an increase of the radiation dose by a factor 1.9-4.5 after IA administration. The calculated radiation dose for hotspot 8 in Figure 6A was not significantly different after IA or IV administration. The estimated radiation dose to several organs is shown in Figure 6H after an injected activity leading to a renal radiation dose of 23 Gy. Please note the relative large (30%) renal radiation dose reduction after IA administration. These calculations are performed on the data collected from only 1 patient and definitely have no statistical significance in predicting the results of IA administration in a group of GEPNET patients.

As biodistribution study in rats showed increase of ^{111}In-DTPAOC in the pancreas after IA administration we looked for pancreas uptake on the patient scans. But none of the

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scans showed ^{111}In-DTPAOC uptake in the pancreas. Therefore, a possible increase of uptake in the pancreas after IA administration via the hepatic artery could not be shown.

Discussion

Both the preclinical and clinical studies indicated that IA hepatic administration of ^{111}In-DTPAOC can result in a significantly increased ^{111}In-DTPAOC tumor uptake compared with that after IV administration. This increased uptake in the hepatic NET metastases will, when applying a therapeutic radiolabeled analogue such as ^{177}Lu-DOTATATE or ^{90}Y-DOTATOC, result in a higher tumor absorbed radiation dose.

In the animal study IA administration resulted in a doubling of the ^{111}In-DTPAOC uptake in both tumor and pancreas. This unexpected increase in pancreas (a sst_2-positive organ in the rat) uptake is most likely explained by backflow of ^{111}In-DTPAOC via the common hepatic artery and coeliac trunk into the aorta during injection.

In patient 2, IA administration did not result in significantly higher tumor uptake. The large tumor volume in combination with the high tumor sst_2 expression (grade 4) and the limited amount of peptide, only 10 µg DTPAOC was used, could have played a role here. Interesting is the fact that in patient 2 within the time frame of 1-4 hours pi after IA administration the uptake in the liver metastases was still increasing while in both other patients the slope of the curve was already declining at 1 hours pi. We cannot fully explain this phenomenon right now, but we assume all receptors in the liver metastases in patient 1 and 3 to be saturated after IA administration, whereas in patient 2 binding of ^{111}In-DTPAOC was still possible at relatively low ^{111}In-DTPAOC plasma concentrations at later time points.

In patient 3, IA administration resulted in a 2.3-fold increase in ^{111}In-DTPAOC uptake in the metastasis located in the right part of the liver. The fact that the one metastasis located in the left part of the liver showed to have equal uptake as measured after systemic administration suggests that ^{111}In-DTPAOC uptake in tumor lesions outside the liver compartment, which was supplied by the IA-route, was apparently not affected

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by the IA administration route. The ^{111}In-DTPAOC plasma values showed significant difference between IA and IV administration at 2 minutes pi (Fig. 6F) and were almost equal at 5 minutes pi. Apparently, there was sufficient radiopharmaceutical left to enter the systemic circulation and reach other tumors. Therefore, an unsuspected metastasis outside the liver (if this patient had one) would probably also have taken up the same

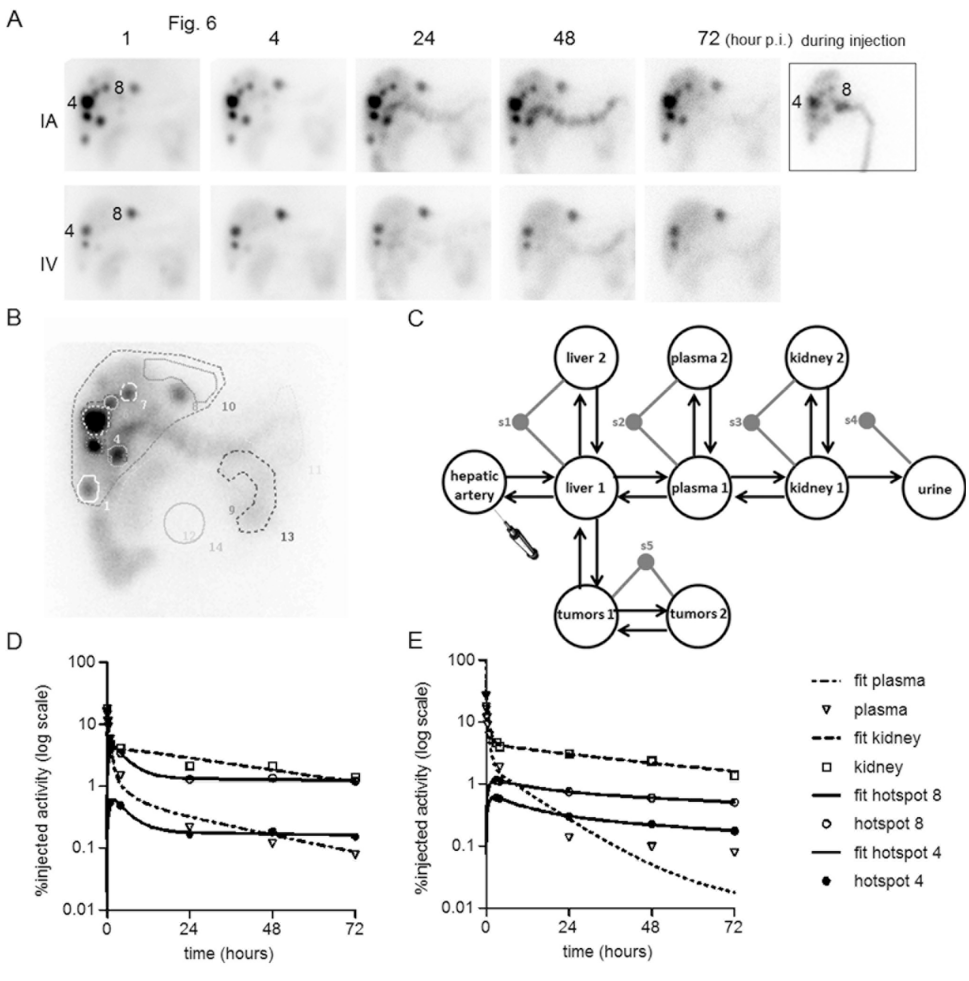

Figure 6. first part.

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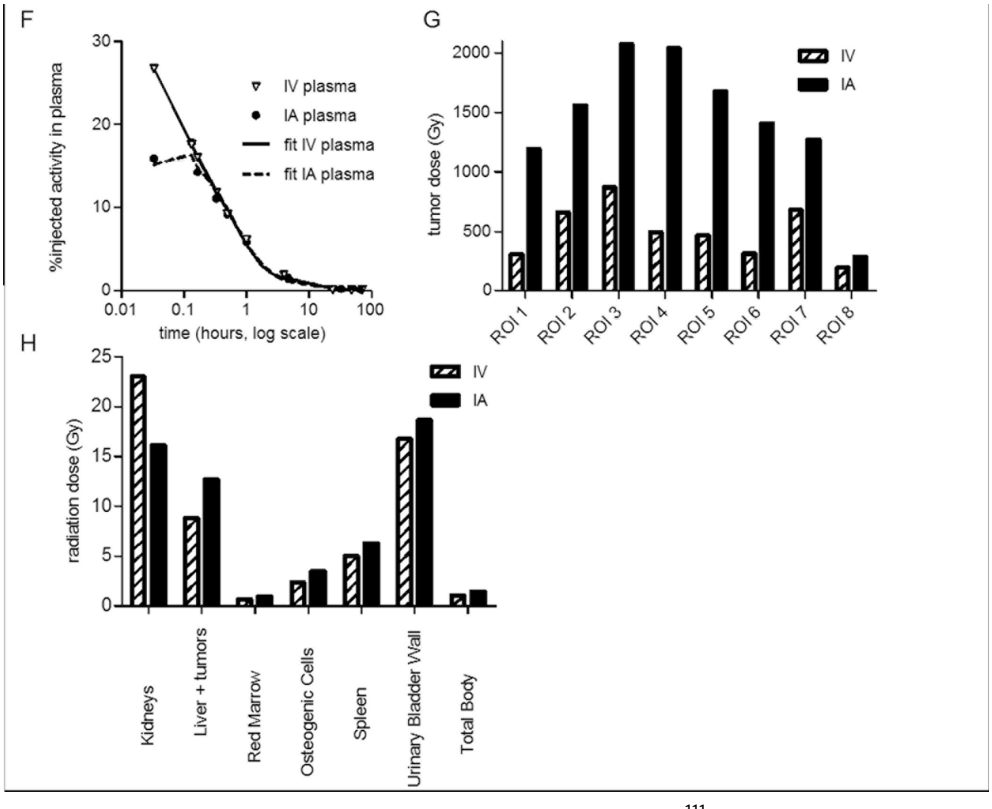

Figure 6. Patient 3; (A) planar anterior upper abdomen images showing ^{111}In localization, 1, 4, 24, 48, and 72 hours after IA and IV administration of ^{111}In-DTPAOC. Notice the higher uptake after IA administration in comparison with IV administration in hotspot 4 (2.3-fold increase of the AUC), which is not seen for hotspot 8. An additional image taken during IA injection is shown. Notice only half the liver is receiving IA administration resulting in ^{111}In-DTPAOC uptake comparable to systemic administration in the metastasis located in segment 5 of the liver (hotspot 8). (B) Regions of interest (ROI) as used for calculating accumulated activity in tumors and organs. (C) Simplified compartmental model as used in the SAAM II software. Organs and tumors were modeled as two compartmental. (D, E) Post IA (D) and IV (E) administration data of patient 3 fitted in a pharmacokinetic model. (F) Plasma values and fits after IA and IV administration. Notice the only difference in plasma values between IA and IV administration is only in the early time point (2 minutes postinjection).(G) ^{177}Lu tumor dosimetry after IV and IA administration. Radiation dose on all liver metastasis in the right part of the liver (ROI 1-7) would be significantly increased by IA administration while the dose on the liver metastasis in the left part of the liver (ROI 8) would not be significantly higher or lower compared with IV administration. (H) ^{177}Lu organ dosimetry after IV and IA administration.

amount of ^{111}In-DTPAOC after IA administration as after IV administration. Certainly, this assumption can only be made in this patient. Probably a higher hepatic tumor mass and/or sst_2 density could even result in a lower uptake in extra hepatic tumor lesions after IA administration compared with IV administration. Despite kidney uptake and excretion was minimally decreased by IA administration,^{177}Lu dosimetry calculations in patient 3 showed a 30% dose reduction to the kidneys by IA administration. This reduced kidney and higher intrahepatic tumor radiation dose resulted in a significant increase of the therapeutic index. IA ^{177}Lu-DOTATATE PRRT would administer a 1.9-4.5 times higher estimated tumor radiation dose when the kidney radiation dose would be 23 Gy. The radiation dose to the bone marrow did not show a significant increase after IA administration. For these calculations we assumed the pharmacokinetics for ^{177}In-DTPAOC and ^{177}Lu-DOTATATE to be equal, but the fact that ^{177}Lu-DOTATATE has a four-fold higher affinity for the sst_2 and also shows some affinity for the sst_2 indicates that our dosimetry calculations are a rough estimation.

In the studies described here, the procedure differed from the routine PRRT treatment with ^{177}Lu-DOTATATE in our institution. PRRT with ^{177}Lu-DOTATATE is administered in a 30 minutes infusion whereas in the current study ^{111}In-DTPAOC was injected as a bolus in 10 seconds. Second, the amount of peptide used in a PRRT setting is 20 times the amount of peptide we used in this study (200 µg DOTATATE versus 10 µg DTPAOC). In addition, DTPAOC has a four-fold lower affinity for the sst_2 compared DOTATATE. In our study, DTPAOC and not DOTATATE was used because the scans were included in the standard clinical workup for PRRT and not for research purposes only. Future experiments with DOTATATE as ligand using a therapeutic peptide dose and injection protocol will be performed to show the additional effect of locoregional administration in a therapeutic setting. In 2008 Limouris et al. showed a relatively high tumor response rate after IA PRRT with ^{111}In-DTPAOC in patients with GEPNET liver metastasis.[13] This relatively high tumor response could be (partially) caused by an increased ^{111}In uptake after the IA administration. Recently, Kratochwil et al. showed a mean 3.75-fold increase of ^{68}Ga-DOTATOC uptake at 40 minutes pi after selective IA administration in GEPNETs.[16] Considering our observations at later time points obtained with ^{111}In-DTPAOC we feel this mean increase of 3.75-fold cannot be translated to the tumor radiation dose. At 1 hours pi IA administration the slope in the curve showing the % injected activity present in the liver metastasis is still quite steep (Fig. 4B, C). Probably a measurement at 24 hours pi, after the curve has a more stable slope, would be more

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predictive in estimating the increase in tumor uptake after IA administration. In this study, we demonstrated that IA administration resulted in the same (1 patient) or an increased ^{111}In-DTPAOC uptake in NET liver metastasis up to 2.4-fold compared with IV administration over a period of 72 hours. The increase in uptake after IA administration is probably depending on sst_2 density, tumor load, and tumor perfusion. We therefore conclude that locoregional IA administration should be considered as the optimal route of administration in patients in which the GEPNET tumor load is mainly localized in the liver. Though, an increase in radionuclide tumor uptake after IA administration in comparison to IV administration is not guaranteed, as in one of our patients tumor uptake was high, but similar after IV and IA administration. If IA PRRT will be applied, the positioning of the catheter should be well planned using contrast enhanced CT for imaging of possible hepatic arterial vasculature abnormalities.

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CHAPTER 3

4

MTOR INHIBITOR RAD001 PROMOTES METASTASIS IN A RAT MODEL OF PANCREATIC NEUROENDOCRINE CANCER

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Cancer Research 2013;73:12–18.

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Abstract

Inhibition of mTOR is commonly considered a valid target in cancer treatment, but this assertion does not address effects on the immune microenvironment that may be detrimental to cancer treatment. Here we show how administration of the mTOR inhibitor RAD001 (everolimus) results in the occurrence of distant metastasis in a rat model of pancreatic cancer. RAD001 was administered twice weekly for 4.5 weeks as a single treatment or combined with [^{177}Lu-DOTA,Tyr3]octreotate (^{177}Lu-DOTATATE), where the latter targets the somatostatin receptor-2. The hypothesized synergistic therapeutic effect of RAD001 combined with ^{177}Lu-DOTATATE was, however, not observed in our experiments. The combination was shown to be less effective than ^{177}Lu- DOTATATE alone. Unexpectedly, tumor metastasis was observed in 77% of the subjects treated with RAD001, either alone or as part of the combination treatment. This was a striking effect, because metastasis did not occur in control or ^{177}Lu-DOTATATE-treated animals, including those where the primary tumor was surgically removed. These findings may be important clinically among noncompliant patients or patients that discontinue RAD001 therapy because of adverse effects. Cancer Res; 73(1); 12-18. ©2012 AACR.

Introduction

Gastroenteropancreatic neuroendocrine tumors (GEPNET) are usually slow growing and most patients present with already metastasized disease at time of diagnosis. As published by Missiaglia and colleagues (1) in many pancreatic neuroendocrine tumors (PNET), the mTOR signal transduction pathway is upregulated. This pathway plays a key role in regulating cell growth, metabolism, proliferation, and angiogenesis. Inhibition of the mTOR signal transduction pathway in PNETs by RAD001 has shown promising results with a clinical benefit of 76%, when given as a single treatment (n = 115), and of 82%, when given in combination with octreotide-LAR (n = 45; ref. 2). The RADIANT-3 study, a randomized, double-blind, placebo-controlled, multicenter phase III trial of 10 mg RAD001 daily in PNET-patients (n = 410) has been completed recently. Median progression-free survival for RAD001 plus best supportive care was 11 months compared with 4.6 months in the placebo group (P < 0.001) with acceptable toxicity (3). Most recently, the U.S. Food and Drug Administration approved RAD001 for the treatment of PNET patients.

From the late 1990s, clinical peptide receptor radionuclide therapy (PRRT) studies have been carried out with radiolabeled somatostatin analogs, such as ^{177}Lu-DOTATATE and [^{90}Y-DOTA,Tyr^{3}] octreotide (^{90}Y-DOTATOC; Onalta), targeting sst_2 overexpressed on most GEPNETs. These studies

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have shown very promising results with regard to tumor response, overall survival, and quality of life (4, 5).

Considering the promising results of both RAD001 and PRRT for the treatment of GEPNETs, we combined these 2 therapies in the CA20948 syngeneic pancreatic tumor model in the rat. This radiosensitive model is commonly used for preclinical PRRT experiments and also shown to be susceptible to RAD001 treatment (6). For comparison, studies were also carried out in the xenograft H69 tumor model in nude mice. The aim of the current study was to investigate the therapeutic effects of combined treatment with RAD001 and ^{177}Lu-DOTATATE compared with those of the 2 monotherapies.

Materials and Methods

Cell lines

The CA20948 rat pancreatic tumor cell line (derived from a rat pancreas at our institution) is of acinar origin (7), has high sst_2 expression, and was cultured as reported previously (8, 11). The cells were passaged for a maximum of 20 times and checked for mycoplasma infection and sst_2 expression every 3 months.

The H69 tumor cell line is a human small-cell lung carcinoma (the American Tissue Culture Collection, Wesel, Germany), has high sst_2 expression, was passaged for a maximum of 10 times, and was cultured according to the supplier's protocol.

Tumor models

The animal studies were in agreement with the Animal Welfare Committee requirements of our institution and conducted in accordance with generally accepted guidelines. For the first 2 experiments, male Lewis rats (Harlan; Horst, the Netherlands) with a mean body weight of 275 g were used. For tumor induction, 10^7 CA20948 tumor cells in 0.5 mL ice-cold PBS per animal were injected subcutaneously in the lower flank. Monitoring of body weight and tumor size by caliper measurements was carried out by a technician blinded for the treatment groups. Tumor volume was calculated according to 0.4 × length × width × height. In the first study (Fig. 1), animals were euthanized when

tumor size had reached a volume of more than 4 cm^3 or when a tumor was bleeding due to skin penetration. In the second study, the primary subcutaneous tumors were surgically removed when tumor volume exceeded 4 cm^3 or when the tumor was bleeding due to skin penetration, thus saving the animals for follow-up. Animals were euthanized when more than 10% loss of body weight was detected.

For the third experiment, 24 male NMRI Nu/Nu mice with a mean body weight of 35 g xenotransplanted sst_2-expressing H69 cells were used. Animals were euthanized when tumor size reached a volume of more than 2 cm^3, when a tumor was bleeding due to skin penetration, or when more than 10% loss of body weight was detected.

RAD001

In the first experiment, RAD001 (kind gift from Novartis, Basel, Switzerland) and placebo was prepared according to the manufacturer's protocol. In the second and third experiment, RAD001 powder (LC laboratories, Woburn, USA) was dissolved in 2 mL ethanol and further diluted to the appropriate concentration in 5% (w/v) glucose solution. RAD001 was administered orally by gavage with a blunt needle.

Radionuclides and peptides

DOTA,Tyr^3-octreotate was obtained from Mallinckrodt, St Louis, Missouri. $^{177}LuCl_3$ was obtained from NRG, Petten and was distributed by IDB-Holland, Baarle-Nassau, the Netherlands. ^{177}Lu-DOTA,Tyr^3-octreotate was locally prepared as described previously (9) in a specific activity of 125 MBq/3.4 mg peptide. Labeling of ^{111}In-DTPA-octreotide (Octreoscan; Tyco Health Care, Petten, the Netherlands) in a specific activity of 30 MBq $^{111}InCl_3$/0.5 mg DTPA-octreotide was carried out as previously described (10). For the mouse experiments, a specific activity of 30 MBq $^{111}InCl_3$/0.1 mg DTPA-octreotide was used.

In vitro autoradiography and hematoxylin and eosin staining

These procedures were carried out as previously described in detail (11).

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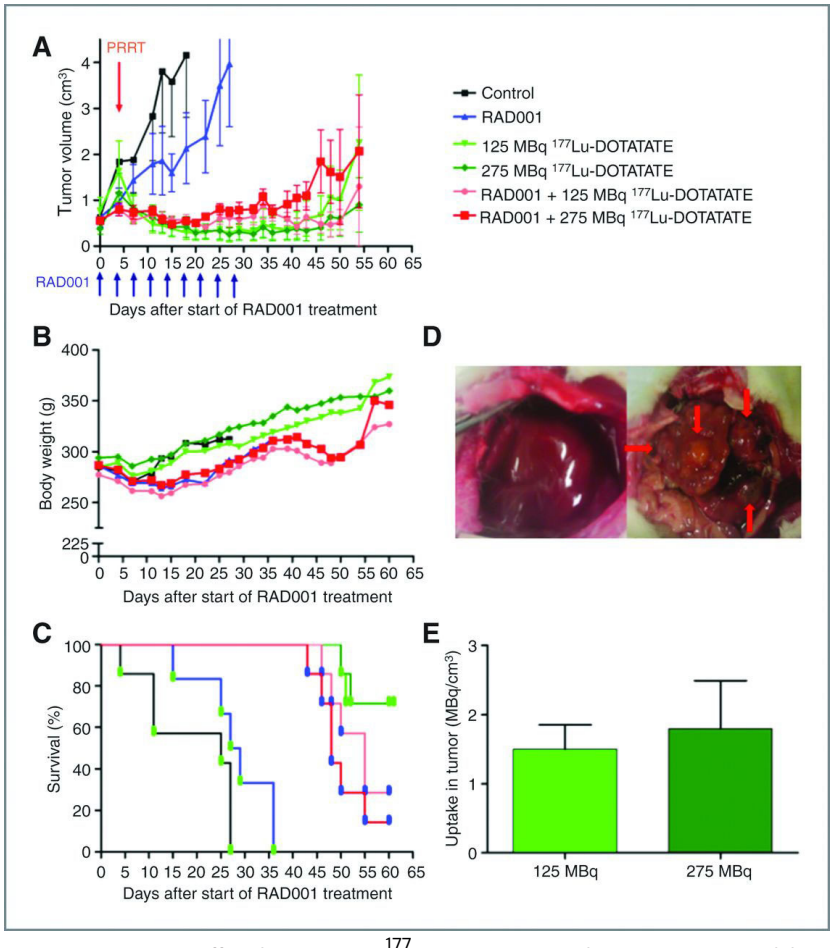

Figure 1. Antitumor effect by RAD001 +^{177}Lu-DOTATATE combination treatment did not lead to better antitumor effects compared with^{177}Lu-DOTATATE alone and unexpectedly resulted in distant metastasis. A, subcutaneous CA20948 tumor size after treatment with vehicle of RAD001 (control), RAD001 (5 mg/kg),^{177}Lu-DOTATATE (125 MBq or 275 MBq), or a combination of RAD001 plus ^{177}Lu-DOTATATE (Table 1). The red arrow depicts the administration of ^{177}Lu-DOTATATE. The blue arrows depict RAD001 administrations. Data are presented as mean ± SEM. B, mean body weight per treatment group from start of RAD001 treatment. Both combination treatment groups showed loss of body weight beyond day 40. Data are presented as mean. C, survival curves of the different treatment groups, a green dot represents sacrifice of at least 1 animal because of subcutaneous tumor size >4 cm^3 or a ruptured tumor. A blue dot represents sacrifice of at least 1 animal because of loss of body weight or poor condition, correlating with metastasis. D, animals with (right) and without (left) metastases in the liver as indicated by the red arrows. E, ^{177}Lu-DOTATATE uptake in subcutaneous primary CA20948 tumors after different doses of ^{177}Lu-DOTATATE (125 MBq/3.4 µg and 275 MBq/7.5 ug) as quantified by SPECT.

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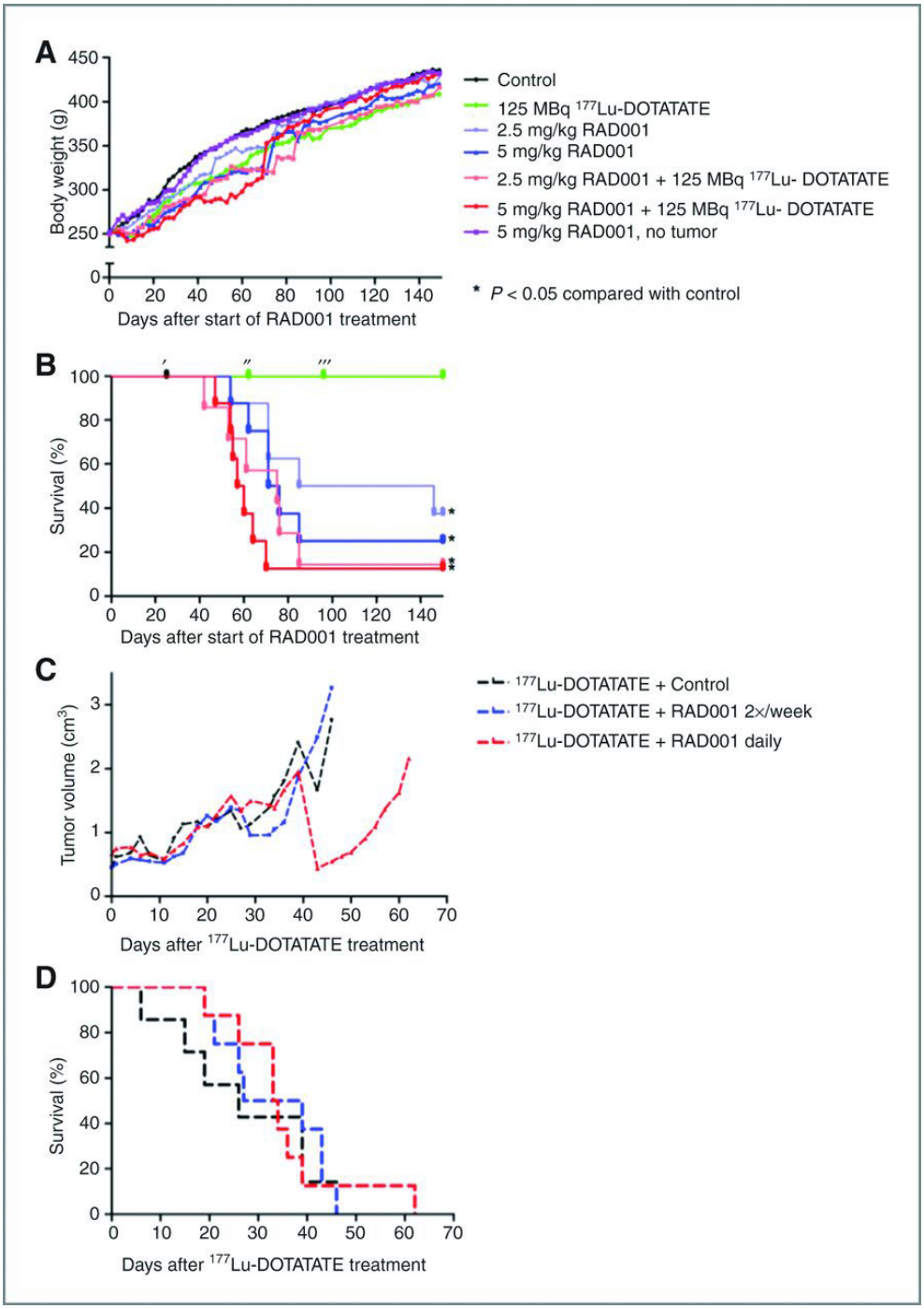
A
Body weight (g)
Days after start of RAD001 treatment
Control
125 MBq 177Lu-DOTATATE
2.5 mg/kg RAD001
5 mg/kg RAD001
2.5 mg/kg RAD001 + 125 MBq 177Lu- DOTATATE
5 mg/kg RAD001 + 125 MBq 177Lu- DOTATATE
5 mg/kg RAD001, no tumor
* P < 0.05 compared with control
B
Survival (%)
Days after start of RAD001 treatment
C
Tumor volume (cm3)
Days after 177Lu-DOTATATE treatment
177Lu-DOTATATE + Control
177Lu-DOTATATE + RAD001 2×/week
177Lu-DOTATATE + RAD001 daily
D
Survival (%)
Days after 177Lu-DOTATATE treatment

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Figure 2. RAD001 treatment (with or without ^{177}Lu-DOTATATE) resulted in the occurrence of distant metastasis. A, mean body weight per group from start of RAD001 treatment. Again, the dip in body weight in the RAD001-treated animals was observed from day 40 onward in the high-dose RAD001 group and from day 60 onward in the low-dose RAD001 group. B, survival curve, censored for metastasis-unrelated death. All RAD001 treatment groups had significantly lower survival rate compared with control and PRRT-only groups. ′, animal found dead in cage; no metastases on autopsy. ″, animal did not survive subcutaneous tumor surgical resection; no metastasis on autopsy. ‴ , animal with macroscopically visible tumor spill during subcutaneous tumor surgical resection. C, subcutaneous H69 tumor size in NMRI Nu/Nu mice was monitored after treatment with ^{177}Lu-DOTATATE in combination with vehicle of RAD001(control), RAD001 (5 mg/kg, twice a week), and RAD001 (5 mg/kg, daily). On day 21, a second ^{177}Lu-DOTATATE treatment was given for reduction of subcutaneous H69 tumor size, facilitating a longer follow-up. No additional therapeutic effect of RAD001 on ^{177}Lu-DOTATATE treatment is seen in this tumor model. Data are presented as mean. D, survival curve showing no significant difference between combination treatment of ^{177}Lu-DOTATATE with vehicle RAD001 (control), RAD001 administered twice weekly, or RAD001 administered daily.

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Figure 3

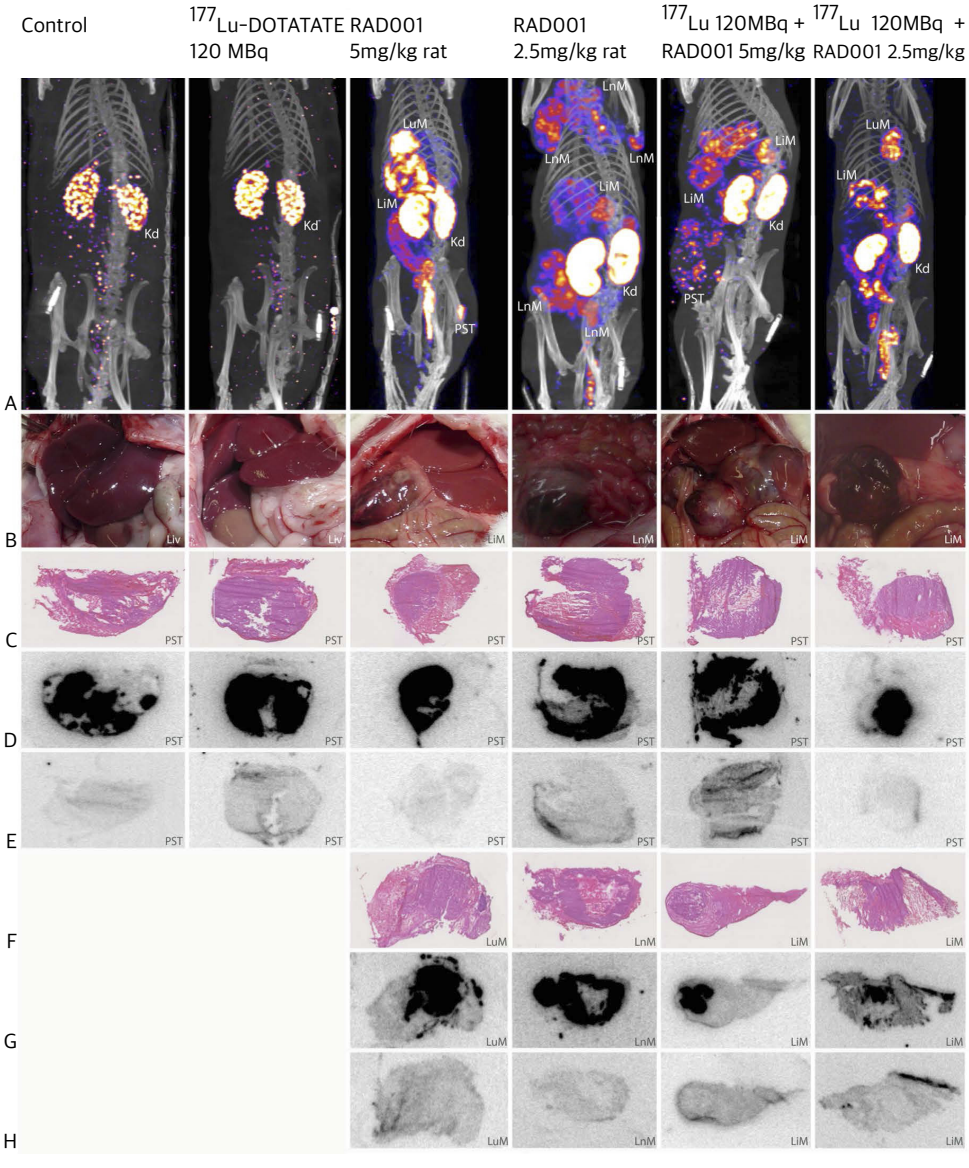

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Figure 3. RAD001 treatment resulted in the occurrence of distant metastasis, shown by ^{111}In-DTPA-octreotide SPECT/CT and in vitro autoradiography. A, SPECT/CT of animals representing all treatment groups. The SPECT/CT of control and ^{177}Lu-DOTATATE–treated animals were carried out at the end of the experiment at day 150 and only showed uptake in the kidneys. The SPECT/CTs of the other animals were made just before euthanasia because of tumor growth and showed clear uptake in tumor tissue and kidneys. Kd, kidney; LuM, lung metastasis; LiM, liver metastasis; LnM, lymph node metastasis; and PST, primary subcutaneous tumor. B, autopsy images. Healthy livers (Liv) in control and ^{177}Lu-DOTATATE–treated animals. Distant metastasis in the liver (LvM) in RAD001–treated animals. C, hematoxylin and eosin–stained sections of primary subcutaneous tumors (PST). D, corresponding 1^{111}In-DTPA-octreotide in vitro autoradiography images. E, corresponding ^{111}In-DTPA-octreotide in vitro autoradiography images with addition of a 1000× excess of unlabeled DTPA-octreotide. F, hematoxylin and eosin–stained sections of distant metastasis in RAD001–treated animals. G, corresponding ^{111}In-DTPA-octreotide in vitro autoradiography images. H, corresponding ^{111}In-DTPA-octreotide in vitro autoradiography images with block.

Experimental setup

In experiment 1, RAD001 treatment was started 10 days after tumor inoculation, followed by ^{177}Lu-DOTATATE injection. Animals were randomized into matching treatment groups with regard to tumor size at the start of treatment. Six treatment groups were created using 1 dose of RAD001 (5 mg/kg body weight twice a week) and 2 doses of ^{177}Lu-DOTATATE, 125 or 278 MBq, either as monotherapy or in combination (Table 1). Experiment 1 ended at day 60. To keep animal discomfort as low as possible and for practical reasons, RAD001 was administered twice weekly.

Experiment 2 was essentially similar as experiment 1; variations were 2.5 next to 5 mg/kg body weight dose of RAD001, alone or combined with 125 MBq ^{177}Lu-DOTATATE (Table 1). The most important difference in comparison with experiment 1 was the resection of the subcutaneous tumor when tumor size increased to more than 4 cm^3. Experiment 2 was terminated at day 150. All animals still alive at the end of the experiment were checked for distant metastases by ^{111}In-DTPA-octreotide single-photon emission computed tomography (SPECT) or computed tomography (CT) and autopsy.

In experiment 3, NMRI Nu/Nu mice bearing subcutaneous H69 tumors were first treated with 28 MBq ^{177}Lu-DOTATATE injected intraperitoneally. Four days later, 3 combination treatment groups were created at random. The first group received RAD001 (5 mg/kg body weight) daily, the second group received RAD001 (5 mg/kg body weight) twice a week with placebo treatment daily for the rest of the week, and the third group received placebo treatment daily. RAD001/placebo treatment was given for a total of 4 weeks. Three weeks after the first ^{177}Lu-DOTATATE treatment, a second 25 MBq ^{177}Lu-DOTATATE treatment was given to all animals as an alternative for surgical removal of the primary tumor. When subcutaneous tumor size exceeded 2 cm^3, the animal was checked for distant metastases by ^{111}In-DTPA-octreotide SPECT/CT and autopsy.

Surgical procedure

During all surgical procedures, isoflurane/O_2 anesthesia was applied and animals were kept warm using a heating pad. After shaving and disinfection, an incision was made just

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Table 1. Characteristics of treatment groups studies in 1 and 2

Group	Treatment	n	Tumor V >4 cm^3
Study 1			
1 Control	Vehicle only	7	†
2 RAD001	RAD001 (2×/week, 5 mg/kg bw)	6	†
3 Combination of low-dose ^{177}Lu-DOTATATE with RAD001	RAD001 (2×/week, 5 mg/kg bw) and 125 MBq ^{177}Lu-DOTATATE	7	†
4 Combination of high-dose ^{177}Lu-DOTATATE with RAD001	RAD001 (2×/week, 5 mg/kg bw) and 275 MBq ^{177}Lu-DOTATATE	7	†
5 Low-dose ^{177}Lu-DOTATATE	125 MBq ^{177}Lu-DOTATATE	6	†
6 High-dose ^{177}Lu-DOTATATE	275 MBq ^{177}Lu-DOTATATE	7	†
Study 2			
7 Control	Vehicle only	8	Surgery
8 Low-dose RAD001	RAD001 (2×/week, 2,5 mg/kg bw)	8	Surgery
9 High-dose RAD001	RAD001 (2×/week, 5 mg/kg bw)	8	Surgery
10 Combination of low-dose RAD001 with ^{177}Lu-DOTATATE	RAD001 (2×/week, 2,5 mg/kg bw) + 125 MBq ^{177}Lu-DOTATATE	7	Surgery
11 Combination of high-dose RAD001 with ^{177}Lu-DOTATATE	RAD001 (2×/week, 5 mg/kg bw) + 125 MBq ^{177}Lu-DOTATATE	8	Surgery
12 ^{177}Lu-DOTATATE	125 MBq ^{177}Lu-DOTATATE	8	Surgery
13 RAD001, no tumor	RAD001 (2×/week, 5 mg/kg bw)	7	n.a.

NOTE: All animals were bearing CA20948 tumors, except for animals in group 13.
Abbreviations: BW, body weight; n.a., not applicable.
†, euthanasia.

adjacent to the tumor. The capsule surrounding the tumor was carefully dissected from the surrounding tissue. The wound was closed by using absorbable sutures.

SPECT/CT scanning

After 24 (experiment 1) or 48 hours (experiment 2) of intra-venous (i.v.) injection of ^{177}Lu-DOTATATE, a helical SPECT scan was acquired covering the tumor region using the 4-headed NanoSPECT/CT system (Bioscan) using Nucline software (v2.01, Mediso) for the quantification of ^{177}Lu-DOTATATE tumor uptake. Just before euthanizing an animal, a whole-body SPECT/CT scan was carried out 4 to 24 hours after i.v. injection of ^{111}In-DTPA-octreotide for detection of distant metastasis.

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Results and Discussion

Our first study, described in Table 1 (groups 1 - 6), showed inhibition of CA20948 tumor growth by RAD001 monotherapy as was previously found by Boulay and colleagues as well (6). In addition, in clinical studies mTOR inhibition generally seems to elicit a cytostatic, rather than a cytotoxic, response (12, 13). Boulay and colleagues also showed twice weekly administration of RAD001 to be as effective as daily administration in this tumor model (6). All animals in the control and the RAD001-only group had to be euthanized within 36 days after start of treatment because of tumor growth beyond 4 cm^3 or a ruptured tumor. As expected, all animals receiving ^{177}Lu-DOTATATE with or without RAD001 exhibited significant antitumor response compared with controls ($P \leq 0.05$). However, ^{177}Lu-DOTATATE in combination with RAD001 did not show evidence of being more effective than ^{177}Lu-DOTATATE alone (Fig. 1A). A dose-effect relationship for the low- and high-dose ^{177}Lu-DOTATATE was not found, which is in agreement with similar levels of ^{177}Lu-DOTATATE tumor uptake in these 2 groups 24 hours after injection, as quantified on the basis of SPECT (Fig. 1E). Partial saturation of the sst_2 receptors on the tumor cells in the high-dose group could play a role in this regard.

Strikingly, from day 40 after start of treatment, most animals in the combination therapy groups showed unexpected decrease in body weight (Fig. 1B). Tumor metastasis to the liver and occasionally to the lung was found in these animals on autopsy (Fig. 1D). In the combination treatment groups, 11 out of 14 animals had to be euthanized because of loss of body weight and apparent metastasis (Fig. 1C). Metastases could be visualized after injection of ^{111}In-DTPA-octreotide by SPECT/ CT, indicating sst_2 expression. The ^{177}Lu-DOTATATE-only-treated animals did not show loss of body weight and were all free of metastasis, as confirmed by negative ^{111}In-DTPA- octreotide SPECT/CT scans and negative autopsies (Fig. 1D). The occurrence of spontaneous metastasis in the subcutaneous CA20948 tumor model has not been described previously, despite the fact that this model has often been used in PRRT studies with long follow-up periods (14). We, therefore, hypothesize that RAD001, alone or in combination with ^{177}Lu-DOTATATE, or the discontinuation of RAD001 treatment might be the cause of the metastasis. Unfortunately, the RAD001 monotherapy treatment group in this study did not survive long enough to develop or to be tested for metastases by SPECT.

For further investigation, a second study was carried out (Table 1; groups 7-13) in which a subcutaneous tumor reaching a volume of 4 cm^3 was surgically removed

to allow long-term follow-up. Furthermore, in this experiment also a 2.5 mg/kg body weight dose of RAD001 was applied next to the earlier-used 5 mg/kg body weight, combined with 125 MBq ^{177}Lu-DOTATATE (Table 1). In an additional group (group 13), 5 mg/kg body weight of RAD001 was given to control rats without tumor.

The therapeutic effects obtained in this second study were in agreement with the first study, without significant difference with the use of the lower RAD001 dose. PRRT resulted in complete response of the CA20948 tumor in 3 out of 7 animals, whereas in all other animals the subcutaneous tumor had to be removed surgically. In 1 animal treated with ^{177}Lu-DOTATATE, clear tumor spill into the wound bed was observed during the surgical procedure, resulting in tumor regrowth in the wound bed and metastasis in an ipsilateral lymph node in the groin. Therefore, this animal was not included in our analysis. The decrease in body weight seen in the RAD001 + PRRT animals in study 1 was less explicit in study 2 (Fig. 2B), probably because of earlier intervention. Again, distant metastasis developed in animals receiving RAD001, either in high or low dose, or in combination with ^{177}Lu-DOTATATE. (Fig. 2A), whereas control and ^{177}Lu-DOTATATE-treated animals remained metastasis free. When development of distant metastasis was suspected on the basis of loss of body weight, ^{111}In-DTPA-octreotide was injected and, 4 to 24 hours later, a SPECT/CT scan (Fig. 3A) was acquired. When a distant metastasis could be visualized, the animal was euthanized, followed by autopsy as illustrated by photographic images (Fig. 3B). From the tumor tissue collected, frozen sections were prepared and used for hematoxylin and eosin staining (Fig. 3C and F) and ^{111}In-DTPA-octreotide in vitro autoradiography with (Fig. 3E and H) or without (Fig. 3D and G) a 1000× excess of unlabeled octreotide (block). All metastasis appeared to be sst$_2$-positive, in agreement with the receptor status of the primary subcutaneous tumors. In a third study, RAD001 administered daily/twice weekly combined with PRRT in a suboptimal dose did again not show any significant additional therapeutic effect on subcutaneous human H69 xenografts in nude mice compared with placebo combined with PRRT (Fig. 2C + D). In this experiment, no distant metastasis was found after RAD001 therapy. A possible explanation could be the slow growth rate of the H69 tumor; longer follow-up and surgical removal of the primary subcutaneous tumor as carried out in the rat experiment 2 was preferred, although this was not allowed according to the animal ethical protocol for this study.

An explanation of the mechanism of action leading to the unexpected metastases in lung, liver, and lymph nodes in the rat model cannot be given on the basis of

these studies. Understanding the pathways involved and how they are interconnected is needed to explain the current findings. As recently discussed by Ebos and colleagues (15), sustained suppression of the VEGF pathway may lead to a rebound in tumor growth, after it is discontinued. Comparable with our findings with RAD001, acceleration of metastasis was found in preclinical models after short-term treatment with the vascular endothelial/platelet derived growth factor receptor (VEGFR/PDGFR) kinase inhibitor sunitinib (16). The fact that, in our studies, RAD001 treatment was discontinued after 4.5 weeks may have resulted in such rebound effect via VEGFR as well. The twice-weekly administration of RAD001 could also have resulted in an incomplete inhibition resulting in an (twice-weekly repeated) upregulation of growth pathways. Discontinued mTOR inhibition could also have stimulated glucose uptake, glycolysis, and de novo lipid biosynthesis, which are considered hallmarks of cancer and cancer metastasis. A possible explanation for the higher therapeutic effects found for ^{177}Lu-DOTATATE-only treatment compared with the combination treatment of RAD001 and ^{177}Lu-DOTATATE could be the fact that, in the combination treatment, the tumor cell proliferation rate is decreased by RAD001, resulting in decreased radiosensitivity. The acceleration of metastasis could be caused by an effect on the immune system that could be unique to this particular preclinical CA20948 tumor model. Therefore, comparable experiments with other preclinical tumor models, such as the H69 mice model with surgical removal of the primary subcutaneous tumor, will have to be conducted. If the results of our studies can be translated to humans, mTOR inhibition treatment should be closely watched, especially after discontinuation of this therapy, because of adverse effects or in noncompliant patients, despite the positive therapeutic results of mTOR inhibition in different types of tumors.

CHAPTER 4

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5

PEPTIDE RECEPTOR RADIONUCLIDE THERAPY (PRRT) WITH [^{177}LU-DOTA^{0},TYR^{3}] OCTREOTATE IN COMBINATION WITH RAD001 TREATMENT; FURTHER INVESTIGATIONS ON TUMOR METASTASIS AND RESPONSE IN THE RAT PANCREATIC CA20948 TUMOR MODEL

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CHAPTER 5

Abstract

Purpose

Previously we reported on the unexpected development of distant metastases in the subcutaneous rat pancreas CA20948 tumor model after 4.5 weeks treatment with RAD001-only or in combination with [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate (^{177}Lu-DOTATATE)[1]. Moreover, the combination therapy was less effective compared to ^{177}Lu-DOTATATE-only. In the current study we address the following questions; 1) Why was the combination therapy less effective? Is ^{177}Lu-DOTATATE tumor uptake affected by pretreatment with RAD001? 2) Could sudden cessation of RAD001 therapy cause the development of distant metastases? 3) Is ^{177}Lu-DOTATATE an effective treatment option for these metastases?

Methods

Lewis rats (HanHsd or SsNHsd substrain with a slight difference in immune response) bearing subcutaneous CA20948 tumors were treated with either 125 or 275 MBq ^{177}Lu-DOTATATE, RAD001 or their combination. RAD001 was given twice a week for 4.5 or 12 weeks, ^{177}Lu-DOTATATE was given as a single injection. When combined, RAD001 was started either 3 days prior to or 3 days post administration of ^{177}Lu-DOTATATE. SPECT/CT was performed to quantify ^{177}Lu-DOTATATE tumor uptake. Where indicated, primary tumors were surgically removed when tumor size > 6000 mm^{3} to enable monitoring for possible metastasis. If metastases were suspected an ^{111}In-DTPA-octreotide SPECT/CT scan was performed. Seven rats with metastases were treated with 400 MBq ^{177}Lu-DOTATATE.

Results

^{177}Lu-DOTATATE tumor uptake was not significantly affected by RAD001 pre-treatment. The occurrence of metastases after RAD001 treatment was not dose dependent in the dose range tested, nor was it related to the duration of RAD001 treatment. In the experiment in which the LEW/SsNsd substrain was used only 12.5% of RAD001 treated rats showed complete response (CR), compared to 50% tumor regression in the control group. Re-treatment with a high dose of ^{177}Lu-DOTATATE resulted in CR in only two out of seven animals.

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Conclusion

Less effective anti-tumor effects after the combination of RAD001 + ^{177}Lu-DOTATATE could not be explained by reduced ^{177}Lu-DOTATATE tumor uptake after RAD001. Our current data support RAD001 induced immune suppression as the reason for this observation. No evidence was found that cessation of RAD001 treatment caused development of metastases. Metastases appeared to be less sensitive to ^{177}Lu-DOTATATE treatment than primary tumors.

Introduction:

Neuroendocrine tumors (NETs) consist of a heterogeneous group of neoplasms originating from cells characterised by the synthesis and release of amines/peptides[2]. Since 1973 the incidence of NETs has been increasing, in which genetic factors might play a role[3] and in addition improved diagnostics contributed to a higher registered incidence of NETs[2]. Because NETs are slowly proliferating tumors the prevalence of NETs is much higher than the incidence, resulting in a relatively high percentage of NET patients in the population of cancer patients[4]. In > 50% of the patients, NETs are diagnosed at a relatively late stage, often with metastatic spread[3], which leaves little chance for curative surgery. As a consequence of the slow proliferation rate most NETs are relatively resistant to chemotherapeutics.

Most NETs are characterized by overexpression of somatostatin receptors, mainly subtype 2 (sst_2). Targeting these receptors by administration of somatostatin analogs radiolabeled with e.g. beta particle emitting radionuclides, such as ^{177}Lu or ^{90}Y, allows peptide receptor radionuclide therapy (PRRT) of NET patients. This therapeutic approach is being performed since more than 10 years and has proven to be an effective treatment option in patients with inoperable disease. Therapeutic responses result in a significantly longer overall survival time compared to other treatments such as chemotherapy or external beam radiation therapy[5-7]. PRRT also improves patient's self-assessed quality of life[8]. Although PRRT is a successful therapy, complete remissions (CR) in patients with metastasized disease are still rare, so there is an urgent need for improvement.

The combination of PRRT with the mammalian target of rapamycin (mTOR) inhibitor Everolimus or RAD001 could be promising in this respect. Everolimus (RAD001) has recently received FDA approval for the treatment of pancreatic NETs. RAD001 has been reported to show anti-tumor and anti-angiogenic activity both in vitro as well

Table 1 Overview of the research questions and setup of the subsequent studies

Research question (rat strain)	Study	Groups	N	^{177}Lu-TATE (MBq)	RAD001 (dose: mg/kg, period)
(A) Potential synergistic effect of RAD001 in combination with 177Lu-TATE (LEW/HanHsd)	1	Control	7	-	Placebo
		RAD only	6	-	5.0, 4.5 w
		^{177}Lu-TATE low dose	7	125	Placebo
		^{177}Lu-TATE high dose	7	275	Placebo
		RAD + ^{177}Lu-TATE low dose	7	125	5.0, 4.5 w
		RAD + ^{177}Lu-TATE high dose	7	275	5.0, 4.5 w
(B) Prolonged follow-up of potential development of distant metastasis (LEW/HanHsd)	2	Control	8	-	Placebo
		Low-dose RAD	8	-	2.5, 4.5 w
		High-dose RAD	8	-	5.0, 4.5 w
		^{177}Lu-TATE	7	125	Placebo
		^{177}Lu-TATE + low-dose RAD	8	125	2.5, 4.5 w
		^{177}Lu-TATE + high-dose RAD	7	125	5.0, 4.5 w
(C) Influence of RAD001 on tumor uptake of ^{177}Lu-DOTATATE (LEW/HanHsd)	1 +2	^{177}Lu-TATE	21	125 or 275	Placebo
		RAD + ^{177}Lu-TATE	29	125 or 275	2.5 or 5.0, 4.5 w
(D) Effects of prolonged RAD001 treatment (LEW/SsNHsd)	3	Control	8	-	Placebo
		RAD	8	-	5.0, 4.5 w
		^{177}Lu-TATE + RAD	8	125	5.0, 4.5 w
		RAD prolonged treatment	8	-	5.0, 12 w
		^{177}Lu-TATE + RAD prolonged treatment	8	125	5.0, 12 w
(E) Effects of PRRT on growth of metastases (LEW/SsNHsd)	3	High-dose ^{177}Lu-TATE after diagnosis of metastases	7	400	-

^{177}Lu-TATE, ^{177}Lu-DOTA0,Tyr3-octreotate; RAD, RAD001; PRRT, peptide receptor radionuclide therapy; w, weeks.

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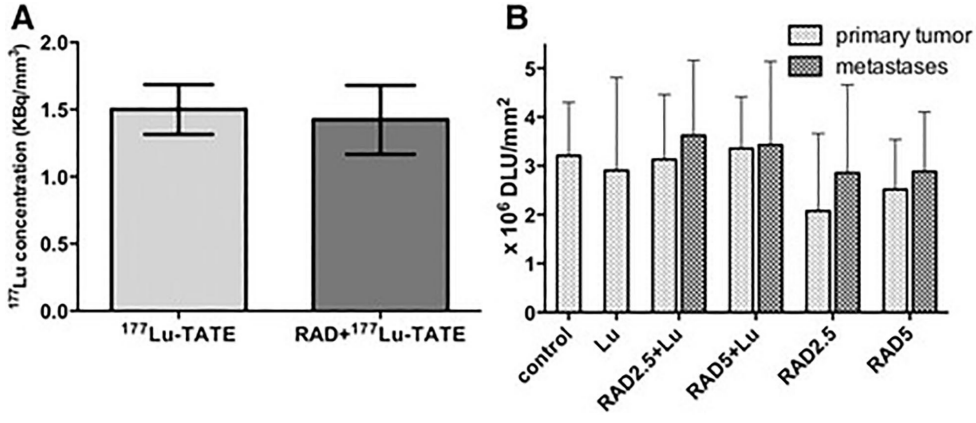

Figure 1 A) Tumor concentration of ^{177}Lu-DOTATATE in rats receiving ^{177}Lu-DOTATATE-only (light grey bar) and groups receiving ^{177}Lu-DOTATATE 4 days after RAD001 therapy was started (dark grey bar). B) Quantification of sst_2 expression in primary tumors and metastases based on in vitro autoradiography. (^{177}Lu-TATE: ^{177}Lu-DOTA^0,Tyr^3-octreotate, RAD: RAD001 2.5 or 5 mg/kg, DLU: digital light unit)

as in vivo, since both tumor proliferation and tumor angiogenesis are regulated by mTOR[9]. The clinical RADIANT III trial, a randomized, double-blind, placebo-controlled, multicenter Phase III trial in pancreatic neuroendocrine tumor (PNET) patients, showed a median progression-free survival of 11 months after daily administration of 10 mg RAD001 plus best supportive care versus 4.6 months in the placebo group[3]. Since it was shown that RAD001 may act as radiosensitizer in various tumor models[10], RAD001 and PRRT could have a synergistic effect. Antitumor efficacies of RAD001 treatment schedules in the CA20948 tumor model have been reported before by Boulay et al.[11]. In this study comparable anti tumor effects were shown for twice weekly and daily RAD001 administration.

We have performed a combination study of the two treatments in the sst_2-expressing CA20948 tumor-bearing rat model on which we recently reported the first data[1]. In this study we compared either ^{177}Lu-DOTATATE, RAD001, or their combination for treatment of tumor-bearing rats. RAD001 was administered orally twice a week for 4.5 weeks, a suboptimal dose of ^{177}Lu-DOTATATE (leaving room for additional effect of RAD001) was given once. Unexpectedly we observed that the majority (77%) of rats treated with RAD001 (single treatment or combined with PRRT) developed metastases. We have used this subcutaneous CA20948 tumor model in many PRRT studies for more than 10 years and metastases never occurred before. We have hypothesized that a rebound effect after stopping the RAD001 treatment after 4.5weeks initiated a metastasizing process. Furthermore, we observed that rats treated with the combination of RAD001 and ^{177}Lu-

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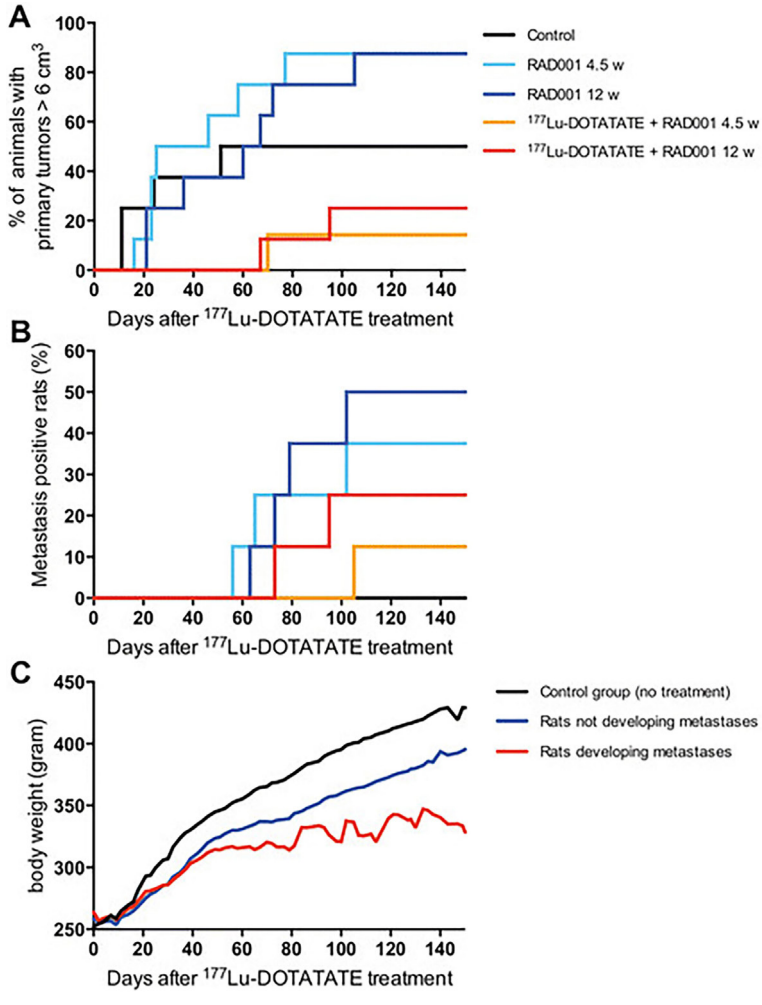

Figure 2 A) Percentage of (LEW/SsNHsd Lewis) rats with primary tumors that reached the max. size > 6 cm^3 and underwent surgery afterwards to remove the tumor. The control group received saline. RAD001 therapy started at day 4 (5 mg/kg administered twice weekly). ^{177}Lu-DOTATATE (125 MBq) was administered at day 1. B) Percentage of (LEW/SsNHsd Lewis) rats developing metastases in each group. C) Mean body weight of animals in the control group (black line) and of the rats treated with either RAD001 or a combination of RAD001 and ^{177}Lu-DOTATATE that did not develop metastases (blue line) versus the body weight of rats treated with RAD001 or a combination of RAD001 and ^{177}Lu-DOTATATE that developed metastases (red line).

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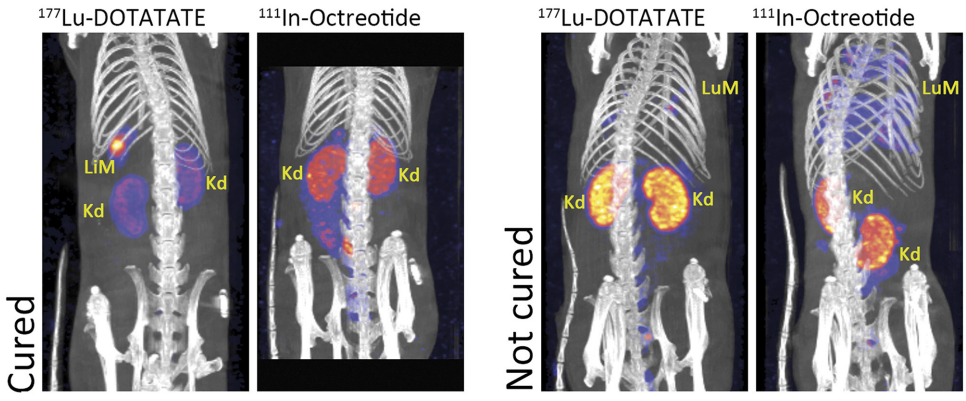

Figure 3 Two representative sets of SPECT/CT scans of rats before and after retreatment with ^{177}Lu-DOTATATE. The two images at the left represent a rat with liver metastasis; the lesion was not detectable anymore on the scan with ^{111}In-DTPA-octreotide made 8 days after ^{177}Lu-DOTATATE PRRT. The two images at the right represent a rat treated for lung metastases. On the right scan after injection of ^{1}In-DTPA-octreotide made before euthanasia of the rat because of on-going weight loss. (LuM = lung metastasis, LiM = liver metastases, Kd = kidney)

DOTATATE showed less impressive anti tumor effects compared to those treated with ^{177}Lu-DOTATATE-only.

In the current studies we performed additional in vivo experiments in the same rat model and obtained additional results from the previous studies. To find an explanation for the lower therapeutic effect of the combination vs. single ^{177}Lu-DOTATATE therapy, ^{177}Lu-DOTATATE tumor uptake was quantified in tumors with and without RAD001 pre-treatment. Moreover, in LEW/SsNsd rats we studied the effects of longer, i.e. 12 instead of 4.5 weeks RAD001 treatment on the potential induction of metastasis.

Materials and methods

Tumor Cell Lines

The rat sst_2-expressing pancreatic tumor CA20948 cell line[12] was cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Invitrogen Corp., Breda, the Netherlands) supplemented with 10% heat-inactivated fetal bovine serum.

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Animals and tumor model

The animal ethics committee of our institution has approved all experiments. Male Lewis rats (250–300 g) were obtained from Harlan (Heerlen, the Netherlands). We used LEW/HanHsd Lewis rats and where indicated we also included rats from the LEW/SsNHsd substrain. In the LEW/SsNHsd substrain the immune system shows an enhanced CD4+ and CD8+ T cell (auto)-immune response[13-17]. This autoimmunity is linked to an increased tumor-immunity[18,19] One week after arrival, rats were inoculated subcutaneously with 10^7 CA20948 cells in 0.5 ml HBSS. For all experiments, animals were randomized into matching treatment groups with regard to tumor size at the start of treatment. A person blinded for the treatment measured tumor size using a calliper and weighed the rats three times a week. Tumor volume was calculated using the formula: 0.4 x length x weight x height. Tumor response was defined as follows: partial response (PR): > 50% reduction of tumor volume, complete response (CR): 100% reduction of tumor volume. Tumors were allowed to develop until a maximum of 4–6 $cm^3$and were surgically removed where indicated. Rats were euthanized when > 10% loss of body weight (BW) was registered.

Anesthesia

2.5% Isoflurane/O_2 gas anaesthesia was used at 0.5 ml/min during tumor cell inoculation, administration of ^{177}Lu-DOTATATE, scanning or surgery.

Surgical procedure to remove the primary tumor

When primary tumors were > 4–6 cm^3, surgical tumor resection was performed where indicated. During surgery a heating pat was used to maintain body temperature. Routine shaving and disinfecting of the skin was performed. The tumor including the tumor capsule was carefully dissected from the surrounding tissue. After tumor resection the skin was closed using individual sutures (vicryl 3/0).

RAD001

RAD001 and its placebo, kindly provided by Novartis Pharmaceuticals; Basel; Switzerland was used for study 1 and was prepared according to the manufacturers protocol. For the next experiments RAD001 powder from LC laboratories, Woburn USA, was dissolved in 2 ml ethanol and diluted with 5% glucose solution in water to obtain 3

or 6 mg/ml. RAD001 was administered orally by gavage in a volume of 0.2 ml thus 0.8 or 1.6 mg/rat or 2.5 / 5 mg/kg BW was administered depending on treatment group. For each administration RAD001 was prepared freshly from powder.

Radionuclides and peptides

DOTA^{0},Tyr^{3}-octreotate was obtained from Mallinckrodt, St Louis, MO, US and $^{177}LuCl_2$ was obtained from IDB, Baarle-Nassau, the Netherlands. ^{177}Lu-DOTATATE was prepared as described before[20], with a specific activity of 100 MBq/2.75µg peptide, and injected iv via the tail vein under anesthesia. Labeling of ^{111}In-DTPA-octreotide (OctreoScan, Covidien, Petten, the Netherlands) in a specific activity of 30 MBq_2/0.5µg DTPA-octreotide was performed as described previously[21].

SPECT/CT scanning

Forty-eight hours after injection of ^{177}Lu-DOTATATE, helical SPECT/CT scanning of the tumor region was performed with the four-headed NanoSPECT/CT system (BioScan, Washington DC USA). Multi pinhole rat collimators with 9 pinholes (diameter 2.5 mm) per head were used: 24 projections, 90 sec per projection were applied. SPECT scans were reconstructed iteratively on a 256x256 matrix, using HiSPECT NG software (Scivis, GmbH Göttingen Germany) and ordered subset expectation maximization (OSEM). The total amount of radioactivity (MBq) in the tumor was quantified by drawing a sufficiently large volume of interest (VOI) around the tumor using InVivoScope software (IVS, Bioscan, Washington DC USA). To achieve accurate quantification, the camera was calibrated by scanning a 20 ml polypropylene tube rat phantom filled with a known amount of ^{177}Lu activity. The in vivo tumor volume was assessed by setting the lower threshold to 90% of the maximum voxel intensity of the tumor using the IRW program (Siemens). Before euthanasia, a whole body SPECT/CT scan of rats was acquired 24 h after intravenous injection of ^{111}In-DTPA-octreotide (50 MBq ^{111}In/ 0,5 µg DTPA-octreotide) to detect CA20948 metastases. During scanning, the rat body temperature was maintained using a heated bed.

In vitro autoradiography

Autoradiography was performed on primary tumors as well as metastases. Frozen

sections of 10 µm (Cryo-Star HM 560 M; Microm, Walldorf, Germany) were mounted on Superfrost plus slides (Menzel, Braunschweig, Germany) and incubated with 10^{-10}M ^{111}In-DTPA-octreotide with and without an excess (10^{-6}M) of unlabelled octreotide. Adjacent sections were used for Hematoxylin/Eosin staining. Tumor sections were exposed to SR phosphor imaging screens (Packard Instruments Co., Meriden, USA) in X-ray cassettes. After 48 h exposure, screens were read by a Cyclone phosphor imager and analysed using OptiQuant 03.00 (Perkin Elmer, Groningen, the Netherlands).

Statistics

Prism software version 5.0 (Graph Pad) was used to analyse tumor growth and determine statistical significance between groups. An unpaired T-test was used for statistical analysis of tumor uptake (Figure 1). Results are given as mean +/-SD. A log rank test was performed for curve comparison in Figures 2A and 2B. Body weight data in Figure 2C are expressed as mean values.

Experimental design

An overview of the different research questions (A-E) and treatment groups in all studies (1-3) is given in Table 1 and described below.

A) Potential synergistic effect of RAD001 in combination with ^{177}Lu-DOTATATE

To study whether RAD001 has an additional anti-tumoral effect on ^{177}Lu-DOTATATE, 6 different study groups were included with 6-7 rats per group. Besides the control group and the RAD001-only therapy groups (5 mg/kg twice a week for 4.5 weeks), 2 groups received ^{177}Lu-DOTATATE as a single therapy in different doses (125 or 275 MBq), and 2 groups received the same doses of ^{177}Lu-DOTATATE combined with RAD001 treatment (5 mg/kg twice a week for 4.5 weeks starting 3 days prior to PRRT)[1]. Rats were euthanized when tumor size exceeded 4 cm^3.

B) Prolonged follow up of potential development of distant metastasis in all groups after surgical resection of the primary tumor

Six additional groups of rats were included in the next study with 7-8 rats in each group. To enable prolonged follow up, in this experiment primary tumors were surgically removed when >4 cm^3. This enabled longer follow up to study the development of

metastases in relation to the combination of ^{177}Lu-DOTATATE and RAD001 or RAD001-only. 2.5 or 5.0 mg/kg RAD001 was administered twice weekly for 4.5 weeks, either alone or 3 days prior to 125 MBq ^{177}Lu-DOTATATE,

C) Influence of RAD001 on tumor uptake of ^{177}Lu-DOTATATE

^{177}Lu-DOTATATE-uptake in tumors was quantified based on SPECT/CT scans acquired 48 hours after administration of ^{177}Lu-DOTATATE in experiment 1 and 2 to examine if previous RAD001 treatment results in reduced ^{177}Lu-retention in CA20948 tumors.

D) Effects of prolonged RAD001 treatment

Five groups of 8 rats were included. 5 mg/kg RAD001 therapy was started 4 days after 125 MBq ^{177}Lu-DOTATATE. RAD001 was administered twice a week and continued for either 4.5 or 12 weeks. Surgical resection was performed if tumors reached a volume of 6 cm^3.

E) Effects of PRRT retreatment on metastases

If rats in experiment 3 showed lethargy or a >10% body weight loss, SPECT/CT was performed using ^{111}In-DTPA-octreotide. If metastases could be discriminated, 400MBq/10.8µg ^{177}Lu-DOTATATE PRRT was given. 24h after ^{177}Lu-DOTATATE injections, SPECT/CT was performed to image ^{177}Lu-DOTATATE uptake in metastases. Therapeutic effect was monitored by follow-up of body weight and ^{111}In-DTPA-octreotide SPECT/CT when ongoing decrease in body weight was registered.

Results

A) Potential synergistic effect of RAD001 in combination with ^{177}Lu-DOTATATE

In experiment 1 treatment with RAD001-only did not result in any complete or partial anti-tumor responses, defined as follows: partial response (PR): > 50% reduction of tumor volume, but no complete response (CR): 100% reduction of tumor volume (Table 2). Groups treated with ^{177}Lu-DOTATATE-only showed 57% CR after 125 MBq ^{177}Lu-DOTATATE and 71% after 275 MBq ^{177}Lu-DOTATATE. Combination of ^{177}Lu-DOTATATE and RAD001 however, resulted in only 29% CR after 125 MBq ^{177}Lu-DOTATATE + RAD001 and 14% after 275 MBq ^{177}Lu-DOTATATE + RAD001. So, in contrast to our hypothesis, no additive effect with regard to tumor response could be achieved by combining RAD001

and ^{177}Lu-DOTATATE. Moreover, unexpectedly, all rats treated with the combination of RAD001 and ^{177}Lu-DOTATATE and not showing CR eventually developed metastases as was reported earlier[1].

B) Prolonged follow up of potential development of distant metastasis in all groups after surgical resection of the primary tumor

Prolonged monitoring after primary tumor removal, the majority (77%) of rats treated with RAD001 (either 5.0 mg/kg or 2.5 mg/kg), developed metastases that resulted in mean body weight loss around 43 days after start of treatment. For these two doses no dose dependence of RAD001 was found (Table 2).

C) Influence of RAD001 on tumor uptake of ^{177}Lu-DOTATATE

The ^{177}Lu-tumor uptake in ^{177}Lu-DOTATATE-only treated rats was 1.51 ± 0,07 kBq/mm^3, while this was 1.42 ± 0,07 kBq/mm^{3}in rats pre-treated with RAD001; no significant different values were found between the groups (p=0.50, Figure 1A).

D) Effects of prolonged RAD001 treatment

In contrast to previous experiments 50% of the rats in the control group showed a CR. The rats from the combination groups (^{177}Lu-DOTATATE + RAD001 for 4.5 weeks resp. ^{177}Lu-DOTATATE + RAD001 for 12 weeks) showed a CR in 87.5% resp. 75% (not significantly different; p=0.63) of the animals, in comparison to only 12.5% of rats in both RAD001-only therapy groups. Within these 2 RAD001-only groups, there was no significant difference regarding both the number of animals that needed surgery as well as the time until surgery.

With regard to development of metastases, at day 150 p.t. no metastases were detected in untreated rats (Figure 2B). Also the time of appearance of the metastases in the combination group was later; 61 d p.t. vs. 91 p.t, respectively.

Monitoring the body weight of rats revealed the effects of treatment and the development of metastases. Rats in the control group showed a normal gain in body weight over time (Figure 2C). Rats treated with ^{177}Lu-DOTATATE + RAD001 or RAD001-only not developing metastases, also showed gain in body weight over time, although at a slower rate. However, the mean body weight of rats developing metastases stabilized as a result of their poor condition.

E) Effects of PRRT on growth of metastases

Although SPECT/CT confirmed significant uptake of ^{177}Lu-DOTATATE in metastases (Figure 3), in only two of the seven rats there was CR after retreatment with high dose

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^{177}Lu-DOTATATE. The average survival time of the non-responsive rats after detection and treatment of the metastases varied between 8 and 37 days, with an average of 27 days. One rat with CR recovered from a liver metastasis that was clearly visualized after administration of ^{177}Lu-DOTATATE. Eight days later there was no sign of this lesion as shown in the ^{111}In-octreotide scan, which was confirmed after dissection. On the other hand, a rat suffering from lung metastases did not respond to ^{177}Lu-DOTATATE. 8 days after re-treatment further loss in body weight was measured and the ^{111}In-DTPA-octreotide SPECT/CT still showed extended lung metastases, also found at autopsy. Determination of sst_2-density on CA20948 primary tumors and metastases using in vitro autoradiography revealed no significant differences (Figure 1B).

Discussion

We previously reported on the disappointing results of the combination of mTOR inhibitor RAD001 with ^{177}Lu-DOTATATE PRRT in the CA20948 rat tumor model. No hypothesised synergistic effect was found; the combination treatment appeared even less effective than ^{177}Lu-DOTATATE-only. This observation cannot be explained by reduced ^{177}Lu-DOTATATE uptake in the subcutaneous CA20948 tumors after RAD001 treatment as we demonstrated here. (Figure 1A).

Even more striking was the fact that the majority of RAD001 treated animals developed tumor metastasis to lymph nodes, liver and/or lung. RAD001 initially has been introduced as an immunosuppressive to protect patients from rejecting allografts after organ transplantation[22]. In 2005 Law et al.[23] reviewed the immunosuppressive effects of RAD001 in relation to its anti-tumor effects, and discussed immune suppression by RAD001 to be tumor growth accentuating. Therefore the application of RAD001 as an anti-tumor agent should be monitored carefully in the clinic, but to the best of our knowledge as yet no tumor accentuating effects in patients have been reported. Recently, after the publication of our Priority Report on our first findings, RAD001 has received FDA approval for the treatment of advanced NETs and is commonly used in clinical practice nowadays. Although we must consider the fact that RAD001 is used in patients with already advanced (metastasized) disease, so far no reports on accelerated metastasis in patients related to RAD001 treatment have been published. We earlier hypothesized multiple reasons for the occurrence of metastases: the twice-weekly dose regimen instead of daily dosing as is applied in clinical therapy, effects of RAD001 on

the immune system and/or tumor microenvironment, or the discontinuation of RAD001 treatment at 4.5-weeks after start of treatment. In the current studies we compared 4.5-weeks of RAD001 treatment with 12-weeks twice-weekly RAD001 treatment. This prolonged RAD001 treatment (with or without ^{177}Lu-DOTATATE therapy) did not reduce the number of rats developing metastases and in addition no delay in the occurrence of metastasis was seen. In fact, comparison of the 4.5-weeks RAD001 groups versus 12-weeks RAD001 groups showed a higher percentage of rats developing metastasis in the 12-weeks RAD001 groups, namely, 38% vs. 25% (p=0.45) in the 4.5-weeks RAD001 groups. The average time until detection of metastases was also not significantly different between groups receiving RAD001 for 4.5-weeks (82 days p.t.) versus 12-weeks (78 day p.t.). Moreover, in 67% of the rats developing metastases in the 12-weeks treatment groups, metastases were diagnosed while rats were still receiving RAD001. Therefore it is unlikely that the occurrence of metastases is due to cessation of RAD001 administration.

Experiment	Group	CR (%)	PR (%)	% rats with metastases	n
1	Control*	0	0	0	7
	RAD 5mg/kg*	0	0	0	6
	^{177}Lu-TATE 125 MBq	57	29	0	7
	^{177}Lu-TATE 278 MBq	71	29	0	7
	RAD 5mg/kg + ^{177}Lu-TATE 125 MBq	29	57	71	7
	RAD 5mg/kg+ ^{177}Lu-TATE 278 MBq	14	57	86	7
2	Control**	0	0	0	8
	RAD 5.0 mg/kg**	0	13	75	8
	RAD 2.5 mg/kg**	13	0	63	8
	^{177}Lu-TATE 125 MBq**	43	57	0	7
	^{177}Lu-TATE 125 MBq+ RAD 5.0 mg/kg**	0	63	88	8
	^{177}Lu-TATE 125 MBq + RAD 2.5 mg/kg**	14	43	86	7
3	Control **	50	0	0	8
	RAD 4½ weeks**	12.5	12.5	37.5	8
	RAD 12 weeks**	12.5	25	50	8
	^{177}Lu-TATE 125 MBq + RAD 4½ weeks**	87.5	12.5	12.5	8
	^{177}Lu-TATE 125 MBq + RAD 12 weeks**	75	25	25	8

Table 2: Overview of tumor responses. *rats did not survive until 42 - 146 days post start of treatment (p.t.), the time frame in which metastases became apparent in the other groups, because rats had to be euthanized when primary tumor size was > 4-6 cm^3. **Primary tumors were surgically removed when tumor size was >4-6cm^3. (CR: complete response: 100% reduction of tumor size, PR: partial response: > 50% reduction of tumor volume but no CR, n: number of animals/group, ^{177}Lu-TATE: ^{177}Lu-DOTA^0,Tyr^3-octreotate, RAD: RAD001)

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Potentially the twice-weekly administration could lead to a repetitive on-off effect on the mTOR pathway with repetitive up regulation/rebound effects of the mTOR pathway with varying plasma concentration levels of RAD001 in a twice weekly dose regimen[24]. In future experiments a daily RAD001 dose regimen will have to be compared to the twice-weekly dose regimen as was used in the current studies.

Compared to a treatment with RAD001-only, less animals receiving a combination of ^{177}Lu-DOTATATE and RAD001 developed metastases and mean time to diagnosis for those metastases was 30 days later compared to the rats receiving RAD001-only. Results from the combination therapy groups with ^{177}Lu-DOTATATE administered 4 days before RAD001 therapy suggested that ^{177}Lu-DOTATATE administered prior to RAD001 therapy reduced both incidence and time of onset of metastases in comparison to the reverse order combination. When ^{177}Lu-DOTATATE was administered 4 days after RAD001 therapy however, there was no reduction in the percentage of rats developing metastases in the combination therapy groups, indicating that rats with (some) tumor reduction already induced by PRRT were less likely to develop metastases than rats not treated with PRRT. This is in agreement with the fact that animals that showed CRs after PRRT or PRRT plus RAD001 did not develop metastases during follow up.

In studies 1 and 2 we used a syngeneic tumor model in rats with an uncompromised immune system. A significant role for T lymphocytes in the immune response to tumors after or during ionizing radiation therapy has been described, the latter resulting in upregulation of tumor-specific antigens[25-29]. As immune suppression by RAD001 has been proven to be mainly due to suppression of T-lymphocyte activation and proliferation[30,31], to our opinion immune suppression by RAD001 is a likely explanation for reduced tumor response to PRRT in combination with RAD001 as observed in our study. In studies 1 and 2 LEW/HanHsd rats were used, whereas in study 3 the LEW/SsNHsd substrain was used, providing the opportunity to test the hypothesis mentioned above. In this substrain the immune system is more active compared to the HanHsd strain and shows an enhanced (auto)-immune response[13-17]. In these rats 50% of tumors in the control group went into spontaneous regression after reaching an average tumor volume of ≈3cm^3, probably due to an immune response against the growing tumor. In such rats treated with RAD001 (4.5or 12 weeks) only 12.5% of the tumors went into regression. In a mouse model on rejection of an allogeneic subcutaneous tumor as created by Hammond-McKribben et al.[22], another mTOR inhibitor, the rapamycine derivate SDZ RAD, was used to prevent rejection of allogeneic tumors. So, immune suppression by RAD001 might have caused the reduced tumor response in our study with ^{177}Lu-DOTATATE administered after RAD001 as well.

Contrary to our results, a combination of ionizing radiation (IR) with rapamycin has been proven to be more effective than IR-only in preclinical studies [10,32,33]. These studies however have been performed in xenograft models using immunodeficient mice lacking T-cells.

As discussed in our priority report, reduced cell proliferation rate caused by a G1 arrest could also

be an explanation for the reduced tumor response to ^{177}Lu-DOTATATE in rats treated with the combination of RAD001 and ^{177}Lu-DOTATATE. RAD001 treatment has been shown to cause a G1 arrest, as mTOR is being linked to phosphatidylinositol 3-kinase (PI3K) pathways[34]. Within 24 h after RAD001 administration a significant increase of cells in G1 phase has been described[35,36]. As cells with a long cycling time, including NET cells, have a peak of radioresistance during early G1 phase[37], tumor cells may have been less sensitive to ^{177}Lu-DOTATATE when administered 4 d after the start of RAD001 treatment. Since clinical trials combining RAD001 and PRRT are being performed[38], to our opinion the decreased antitumor response in our study when RAD001 was administered prior to ^{177}Lu-TATE is rather relevant. Also in a clinical situation the combination of both therapies might be less effective compared to just PRRT.

For re-treatment of the rats with metastases we used a dose of 400MBq ^{177}Lu-DOTATATE, which is remarkably higher compared to the initial 125 or 275 MBq treatment doses. Still only 2 out of 7 rats with metastases were cured, suggesting metastases in our model to be more resistant to PRRT compared to the primary tumor. As it is quite complex to study responses of metastases in a preclinical model, there have not been many preclinical studies on therapies in metastatic models. However, to be able to get more solid information on sensitivity of metastases to PRRT in a preclinical model, certainly more studies in different models are necessary. The current CA20948 metastatic tumor model after RAD001 treatment could be a more realistic metastasis model for future experiments compared to often-applied metastasis-tumor models in which tumor cell suspensions are injected intravenously.

Concluding remarks: Results described here confirmed and provided more information on development of metastasis after RAD001 treatment in our in vivo rat tumor model. The impaired tumor response to the combination of RAD001 and ^{177}Lu-DOTATATE in comparison with that after ^{177}Lu-DOTATATE-only could not be attributed to a reduced tumor uptake of ^{177}Lu-DOTATATE in rats after RAD001 treatment. Moreover, the occurrence of metastases could not be attributed to the sudden cessation of RAD001 treatment, as we observed treatment for 12-weeks did not result in a lower metastasis rate compared to treatment for 4.5-weeks. Immune suppression by RAD001 could be a good explanation for reduced tumor response after RAD001 as well as for development of metastasis. More studies in different tumor models are needed now to provide proof and give detailed information on the translational value of these findings to the clinic.

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CHAPTER 5

6 MULTIMODALITY IMAGING OF SOMATOSTATIN RECEPTOR-POSITIVE TUMORS WITH NUCLEAR AND BIOLUMINESCENCE IMAGING

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CHAPTER 6

Molecular Imaging

Multimodal bioluminescence (BLI) and single-photon emission computed tomography/computed tomography (SPECT/CT) imaging were investigated as means to monitor somatostatin receptor subtype 2 (SST_2)-positive neuroendocrine tumors as both a subcutaneously implanted and a liver metastasis animal model in mice and rats. Ultimately, such a model will be of use for studying SST_2-targeted peptide receptor radionuclide therapy (PRRT). CA20948 cells were transfected with a green fluorescent protein/luciferase plasmid construct. Cells were inoculated subcutaneously in the shoulder of nude mice: nontransfected cells in the left shoulder and transfected cells in the right shoulder. BLI, SPECT/CT imaging, biodistribution analysis, and ex vivo autoradiography of the tumors were performed. BLI and SPECT/CT imaging were also performed on an intrahepatic tumor model in the rat. Caliper volume measurement of transfected tumors could be correlated with BLI measurements (R^2= .76). SPECT/CT imaging showed high levels of accumulation of ^{111}In-DTPA-octreotide in control and transfected tumors, which was confirmed by biodistribution analysis and autoradiography. Subcapsular inoculation of transfected cells in rat liver resulted in an intrahepatic tumor, which could be visualized by both SPECT/CT and BLI. Transfection of CA20948 tumor cells did not alter the growth properties of the cell line or the expression of SST_2. Transfected tumors could be clearly visualized by BLI and SPECT/CT imaging. The transfected SST_2-positive tumor cell line could represent a novel preclinical model for tumor monitoring in studies that aim at further optimizing PRRT for neuroendocrine tumors.

Introduction

Visualisation of somatostatin receptor-overexpressing neuroendocrine tumors by radiolabeled somatostatin analogues is being applied clinically for tumor detection, staging, and selection of therapy.[1] Equipping these peptides with therapeutic radionuclides for peptide receptor radionuclide therapy (PRRT) rendered promising results in preclinical[2] and clinical studies.[3] Kwekkeboom and colleagues demonstrated an increase in overall survival ranging from 40 to 72 months from diagnosis, with only minor side effects in comparison with historical controls in a clinical trial using ^{177}Lu-DOTAtate for treatment of 310 patients with gastroenteropancreatic neuroendocrine tumor (GEPNET).[4] Patients with liver metastases of GEPNETs have a median overall survival of 2 to 4 years and hardly benefit from classic therapeutic options such as surgery and chemotherapy,

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making PRRT an interesting new treatment option.

To further improve PRRT, new approaches, such as locoregional administration of PRRT and PRRT in combination with chemotherapy, are being tested in preclinical tumor models and in the clinic.[5-7] For this, we developed a somatostatin receptor subtype 2 (SST_2)-positive rat pancreatic tumor of acinar origin, CA20948.[8] For the various preclinical evaluations, these tumors can be grown either subcutaneously or as a liver metastasis model on implantation of subcapsulary tumor in the liver. To monitor tumor size and receptor expression in these models in a sensitive and quantitative way, we aimed to apply nuclear imaging in combination with bioluminescence imaging (BLI). For nuclear imaging, single-photon emission computed tomography (SPECT) is used for molecular imaging of receptor status and computed tomography (CT) for three-dimensional anatomy imaging. To allow for BLI, CA20948 pancreatic tumor cells were stably transfected with a green fluorescent protein (GFP)/luciferase plasmid construct. Tumor models based on these tumor cells in mice and rats were characterized by multimodal BLI and SPECT/CT imaging using ^{111}In-DTPA-octreotide (OctreoScan, Covidien, Petten, the Netherlands).

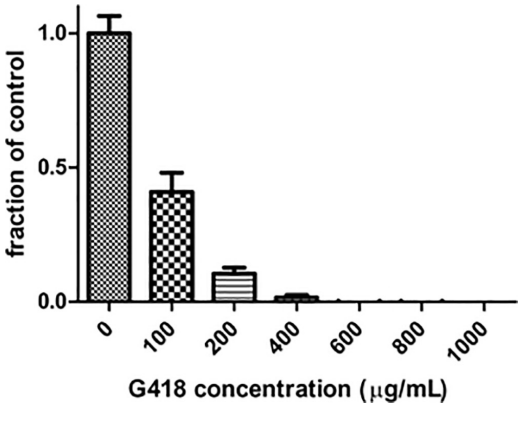

Figure 1. CA2094-wt (wild type) viability in vitro cultured with G418 in different concentrations. The concentration of 400 μg/mL was chosen as the optimal concentration for selecting CA20948-luc cells.

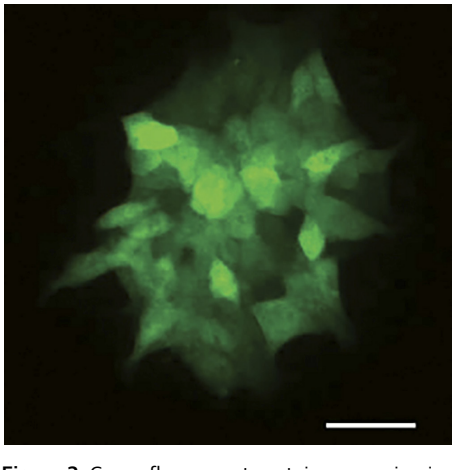

Figure 2. Green fluorescent protein expression in a CA20948-luc clone in vitro (scale bar 50 μm).

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Methods

Plasmid and Tranfection

The firefly luciferase gene luc+ was cloned as a < 1.7 kb Nhe1-Xba1 fragment in the Nhe1 site of a pIRES2-EGFP vector, enabling the expression of a single bicistronic transcript that encodes both luciferase and GFP. CA20948 cells were transfected with the construct using lipofectamin (Invitrogen) and cultured under geneticin (G418) selection. The G418 concentration for selecting transfected tumor cells was based on the G418 concentration killing almost all CA20948-wt (wild type) cells in an in vitro cytotoxicity test (SRB [Sulphorodamine B], Sigma Aldrich) of CA20948-wt cells treated for 24 hours at different concentrations of G418. GFP-positive clones were isolated, and a clone with high GFP/luciferase expression was selected and characterized in vitro for expression of SST_2.

Internalization Experiments

Twenty-four hours before the experiment, 10^6 CA20948 cells were plated per well in six-well plates. The cells were then washed with 2 mL phosphate-buffered saline (PBS, 37°C) and incubated in 1 mL incubation medium (RPMI-1640 medium [Gibco BRL] supplemented with 1% bovine serum and 20 mM Hepes) containing ^{111}In-DOTA-octreotate at a concentration of 10^{-9}M. To determine nonspecific internalization, cells were incubated with an excess of unlabeled peptide (10^{-6}M octreotate). Cellular uptake was stopped by removing medium from the cells, followed by washing twice with 2 mL PBS. To discriminate between internalized and non-internalized (surface bound) fraction, intact cells were incubated with 1 mL 20 mM sodium acetate (pH 5) as previously described.[9]

Fluorescence Microscopy

Cells grown on fibronectin-coated glass coverslips were analyzed by fluorescence microscopy using a Zeiss Axiovert 100M microscope and an ORCA-II-ER camera (Hamamatsu).

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Animals

NMRI Nu/Nu mice (body weight < 27 g) were used. Lewis rats (body weight around 275 g) were used. Animals were kept under standard laboratory conditions (12 hours light/12 hours dark) and given a standard laboratory diet and water ad libitum. During SPECT/CT imaging and BLI, animals were placed on a heated bed (38°C) and kept under isoflurane anesthesia (2.5% at 0.6 L/min). The animal studies conformed with the Animal Welfare Committee requirements of our institution and were conducted following generally accepted guidelines.

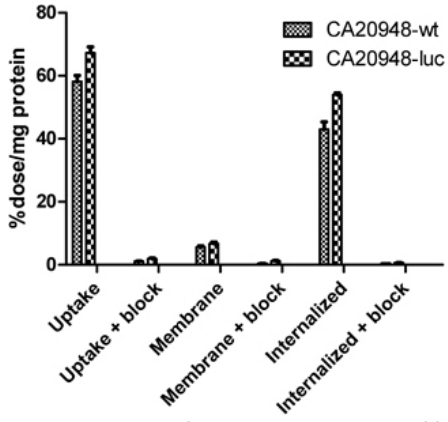

Figure 3. Internalization assay: comparable membrane binding (Membrane) and internalization (Internalized) of ^{111}In-DOTA-octreotate in CA20948-luc and -wt cells with and without blockade with 1,000 times excess octreotate. Data are presented as mean 6 SEM.

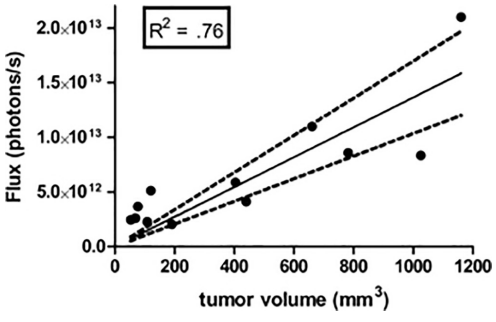

Figure 4. Correlation of caliper and bio-luminescence imagingmeasurements (R^2=.76).

For tumor induction, 2 × 10^6 living CA20948 cells in 0.2 mL were injected subcutaneously in the shoulder of nude mice: nontransfected cells in the left shoulder and transfected cells (CA20948-luc) in the right shoulder. Tumor growth was measured by caliper (tumor volume = length × diameter^2× 0.4) and BLI.

For the liver metastasis model, two male Lewis rats (Harlan, Horst, the Netherlands) were used. All surgical and injection procedures used isoflurane/O_2 anesthesia and a microsurgery microscope. During surgery, animals were kept warm with a heating pad. After laparotomy of the rats upper abdomen by a 1.5 cm incision, the main liver lobe was fixated between two swabs and around 100 mL CA20948 tumor cells (1.5 × 10^6cells) suspended in Matrigel basement membrane matrix (BD Biosciences, Breda,

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the Netherlands) were injected subcapsularly through a 27-gauge needle mounted on a 1 mL syringe. The abdomen was then closed by absorbable sutures.

Bioluminescence Imaging

BLI (IVIS 200, Xenogen, Hopkinton, MA) of tumor-bearing animals was performed after intraperitoneal injection of luciferin (0.15 mg/g body weight). During the procedure, animals were anesthetized with isoflurane and kept at 37°C.

SPECT/CT Imaging

SPECT/CT imaging was performed using the NanoSPECT/CT (Bioscan Inc., Washington, DC) 4 hours after intravenous injection of ^{111}In-DTPA-octreotide (30 MBq/rat or mouse). The exact injected activity was determined by measuring the syringe in a dose calibrator before and after injection. Multi-pinhole mouse and rat collimators with nine pinholes (1.4 mm diameter for mice and 1.5 mm diameter for rats) per head were used with a matrix of 256 x 256 and 24 projections (1 minute per projection).

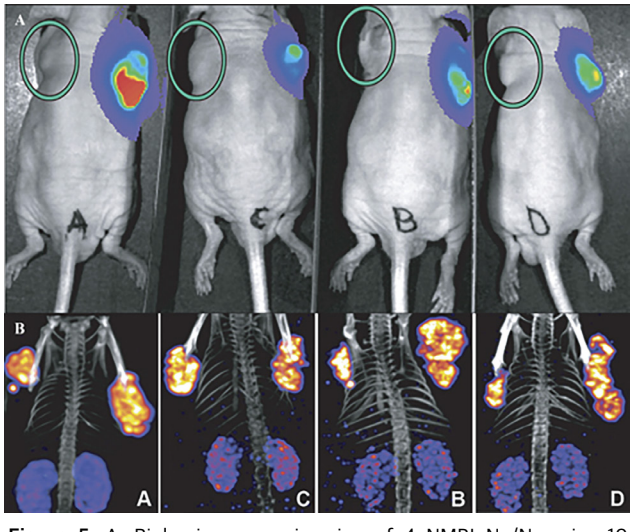

Figure 5. A, Bioluminescence imaging of 4 NMRI Nu/Nu mice 10 minutes after injection of luciferin showing luciferase activity in CA20948-luc tumor (right shoulder). On the left shoulder, the normal CA20948 tumor is visible, but without luciferase activity. The green circles indicate the CA20948- wt tumor. B, ^{111}In-DTPA-octreotide uptake in the CA20948 (left shoulder) and the CA20948-luc (right shoulder) tumors 4 hours after injection

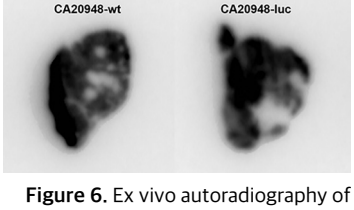

Figure 6. Ex vivo autoradiography of a CA20948-wt and a CA20948-luc tumor.

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CT was performed at 45 kV. SPECT scans were reconstructed iteratively using InVivoScope software version 1.32 (Bioscan) with medium noise reduction, a voxel size of 0.3 mm^3, and standard reconstruction settings.

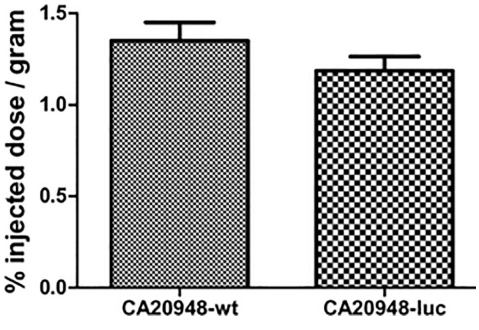

Figure 7. Tumor uptake of ^{111}In-DTPA-octreotide in CA20948-wt and CA20948-luc tumors as quantified in a gamma counter 24 hours after injection.

Tumor Uptake Quantification

Tumor uptake of ^{111}In-DTPA-octreotide was performed by gamma-photon counting of the tumor using a LKB-1282-Compugammasystem (Perkin Elmer, Waltham, MA) 24 hours after injection (counting time was 60 s/tumor). Before counting the tumor was weighed.

Ex Vivo Autoradiography

Intratumoral distribution of ^{111}In-DTPA-octreotide was investigated by ex vivo autoradiography. Tumor tissue isolated 24 hours after injection, was embedded in Tissue Tek (Sakura, Zoeterwoude, Netherlands) and quickly processed for cryosectioning. Sections (10 μm) were mounted on glass slides, which were placed on phosphor imaging screens (Packard Instruments Co., Meriden, CT) for 1 day. The screens were analyzed using a Cyclone phosphor imager and a computer-assisted OptiQuant 3.00 image processing system (Packard Instruments Co.).

Results

Transfected cells were selected by G418 resistance (400 μg/mL; Figure 1) and cloned. A clone with the highest GFP activity (Figure 2) was used for further experiments. Comparable SST_2 receptor binding and internalization of ^{111}In-DOTA-octreotate in the CA20948-wt and CA20948-luc cells were shown in an internalization experiment

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(Figure 3). Tumor growth of subcutaneous CA20948-luc and CA20948-wt tumor was monitored by caliper measurements and BLI and showed good correlation (R^2 .76) (Figure 4). At day 10 after inoculation, the CA20948-luc tumors on the right shoulder were clearly visible by BLI (Figure 5A), whereas the CA20948 tumors inoculated in the left shoulder were not. Both tumors were visualized by SPECT/CT imaging on intravenously injected ^{111}In-DTPA-octreotide, showing comparable levels of uptake and thus SST_2 expression by both the transfected and nontransfected tumors (Figure 5B).

Further comparison by ex vivo autoradiography of tumor sections (Figure 6) and by radioactivity counting of tumors 24 hours after injection (Figure 7) confirmed that levels of uptake of ^{111}In-DTPA-octreotide were comparable in transfected and nontransfected tumors in vivo.

An extra benefit of monitoring tumors by BLI is the possibility of monitoring orthotopically implanted tumors or metastastatic models. In a CA20948-luc liver metastasis rat model, intrahepatic tumor could be clearly visualized by both SPECT/CT (Figure 8A) and BLI (Figure 8B).

Discussion and Conclusion

In this study, we were able to demonstrate that luciferase transfected CA20948 pancreatic tumor cells show SST_2 expression and ^{111}In-DTPA-octreotide uptake in vivo comparable to that of nontransfected CA20948. Transfected tumors could be clearly visualized by both BLI and SPECT/CT imaging in mice and rats. BLI measurements of subcutaneous tumors in nude mice showed good correlation with caliper measurements. The use of BLI for measuring the therapeutic effects of PRRT in our animal models, especially in the liver metastasis model, is promising. This approach allows for daily monitoring of tumor growth in the same animal for several weeks. Tissue absorption of photons by overlying tissue could be a problem when the tumor is situated deep within the body. In the liver metastasis model used in this study, the tumor is covered by skin, abdominal wall, and liver capsule. The thickness of these structures is not expected to change in time. Emitted photons could be absorbed more and more by the tumor itself during growth of the tumor, making quantitative imaging uncertain. In our experiments, none of this was noticed. Other imaging modalities, such as CT and magnetic resonance imaging, could also be used for tumor size monitoring but are more time consuming. Using BLI, eight mice or four rats can be imaged simultaneously within 10 minutes. Taking these considerations into account, we conclude that BLI can be used

in the future in addition to nuclear imaging techniques and measurements of receptor expression levels to follow tumor response to PRRT in a sensitive and quantitative way. This will help optimize PRRT experiments in the liver metastasis model and will prevent unwanted discomfort to the animals and the use of large numbers of animals when using alternative invasive methods. Also, caliper measurements of liver tumor size on surgery could vary considerably between measurements and researchers. BLI is not expected to have this variation when imaging is performed using a standardized imaging protocol. In addition, in PRRT experiments, we often experience central necrotic areas within a tumor that will be taken into account when using caliper measurements but not when using BLI.

The transfected tumor cell line could be a promising new preclinical tool for tumor monitoring in studies that aim at further optimizing PRRT for neuroendocrine tumors, especially when the tumor is not located directly under the skin, as in the case of orthotopically inoculated tumors or in a liver metastasis model.

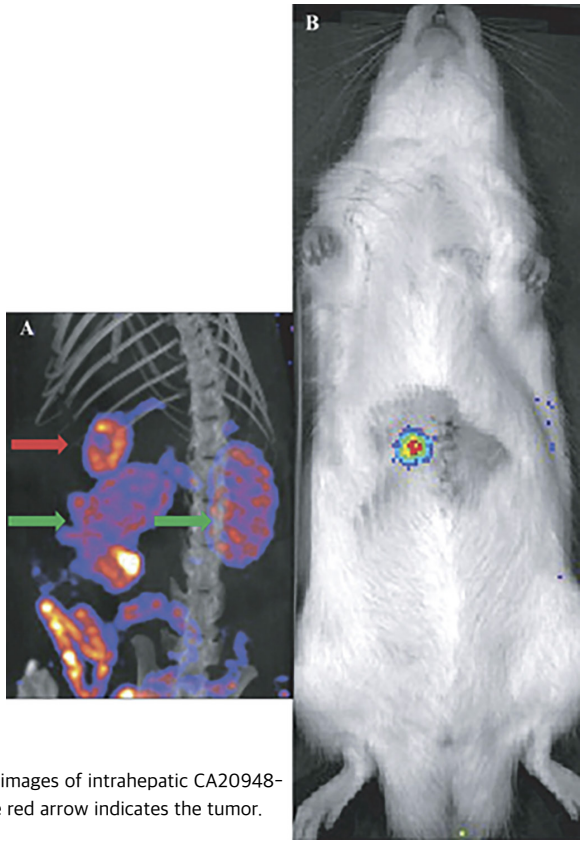

Figure 7. SPECT/CT (A) and bioluminescence (B) images of intrahepatic CA20948-luc tumor. Green arrows indicate the kidneys; the red arrow indicates the tumor.

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CHAPTER 6

SUMMARY

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SAMENVATTING

TOEKOMSTVISIE

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Summary, Future perspectives

Neuroendocrine tumors are rare neoplasms with an incidence of 2-5 per 100,000 inhabitants. When a NET is diagnosed, in the majority of cases it has already metastasized and the only curative treatment option available nowadays, i.e. surgery, is not an option any more. Standard treatment in most of these patients comprise long-acting somatostatin analogs, possibly in combination with (palliative) surgery, chemotherapy, radiotherapy, hepatic artery/transarterial (chemo-) embolization (H/TA(C)E), radiofrequency ablation (RFA), cryoablation and laser-induced thermotherapy (LITT) and intra-arterial radio embolization with yttrium-90/holmium-166 microspheres.

The widely used long-acting somatostatin analogs bind to somatostatin receptors (mainly sst_2), which are over-expressed on both functioning and non-functioning NETs, resulting in significant lengthening of time to tumor progression compared to placebo[1,2]. Also treatment with somatostatin analogs can reduce hormonal overproduction of functioning tumors and may result in symptomatic relief in most patients with metastasized disease[3-5]. The sst over-expression on NETs is also widely used for diagnosis/visualization of these tumors using radiolabeled somatostatin analogs, such as [^{111}Indium-DTPA]-octreotide (Octreoscan®, Covidien, Petten, the Netherlands) and the more recently developed PET tracers ^{68}Ga-DOTA-Tyr^3-octreotide[6] or ^{68}Ga-DOTA-Tyr^3-octreotate (^{68}Ga-DOTATATE)[7]. Based on the same principle, somatostatin analogs labeled with radionuclides with high-energy beta particle emission, including ^{177}Lu-DOTATATE and ^{90}Y-DOTATOC are commonly being applied for treatment of the metastasized NETs with most promising results. New is the promising application of alpha emitters like bismuth-213 (^{213}Bi), which is very likely to be a very effective strategy because of the high linear energy transfer (LET) of alpha radiation[8-10]. Some promising results with ^{213}Bi-DOTATOC PRRT have already been published in seven NET patients refractory ^{90}Y/^{177}Lu-DOTATOC PRRT[10].

In this thesis, several approaches to optimize the treatment of NETs have been described, as well as their application in different studies and evaluation of the results thereof. **Chapter 1** is a general introduction on NETs and the different treatment modalities currently being applied. Also, a short overview is given on the animal model and molecular imaging modalities described and applied in this thesis work.

Chapter 2 is a review of the literature on preclinical and clinical PRRT. The therapeutic

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effects of somatostatin analogs labeled with different radionuclides have been discussed. Also potential new peptides for PRRT, like glucagon-like peptide 1, gastrin-releasing peptide, CCK2, epidermal growth factor receptor and αvβ3 integrin targeting peptide analogues, have been included. Strategies to improve PRRT, including tumor size-specific use of ^{177}Lu for smaller tumors and ^{90}Y for larger tumors, locoregional administration and the combination of PRRT with chemotherapy, are described and discussed as well.

Chapter 3 describes the effect of intra-arterial (IA) administration compared to intravenous (systemic) administration on the [^{111}Indium-DTPA]-octreotide uptake in liver metastases in an animal model and in 3 patients with NET liver metastases ,varying in sst_2 density and tumor load. In rats with intrahepatic sst_2-positive tumors, IA administration resulted in a twice-higher uptake of ^{111}In in the tumor compared to that after systemic administration. The patient study showed that IA administration can result in significantly higher ^{111}In uptake in NET liver metastases, although the effect can differ between patients. The results obtained in one patient further showed accurate localization of the catheters tip to be essential for optimal IA administration to the whole liver. In this patient one of the NET liver metastases, situated in a part of the liver that was not perfused during locoregional administration, showed to have the same uptake as that in the setting of IV administration. Locoregional IA administration did not implicate reduction of uptake in tumor lesions elsewhere in the body compared to that after systemic administration. Estimation of ^{177}Lu dosimetry and pharmacokinetic modeling was performed based on the results obtained in one patient and showed a 30% reduction of the estimated radiation dose on the kidneys after intra-arterial administration.

In patients with diffuse or large, somatostatin receptor positive metastasic liver disease , IA PRRT administration could be preferable over IV administration. Future and more extended clinical studies will have to reveal if the high uptake after IA administration results in better tumor response and overall survival as well.

In **Chapter 4** we describe the combination study of ^{177}Lu-DOTATATE PRRT with the mTOR inhibitor RAD001 (Affinitor®, Novartis, Basel, Switzerland) in a NET model in rats. The aim of the study was to investigate a potential synergistic therapeutic effect of mTOR inhibition and PRRT, based on earlier reports showing radiosensitizing effects by mTOR inhibition[11,12]. Surprisingly, the combination treatment appeared to be less effective compared to ^{177}Lu-DOTATATE treatment alone, but even more importantly, we observed rats that received RAD001 treatment twice weekly for 4.5 weeks (with or

without ^{177}Lu-DOTATATE treatment) to develop distant metastasis, whereas control and ^{177}Lu-DOTATATE treated animals did not and never did before in our earlier studies during more than 10 years. We hypothesized that the discontinuation of RAD001 treatment at 4.5 weeks after start of treatment could have caused the occurrence of metastasis, probably by a rebound effect of the mTOR pathway. Inhibition of the immune system by RAD001 could also be an explanation for the occurrence of distant metastases.

Chapter 5 describes additional analyses derived from the in Chapter 4 described combination studies and from an additional animal study in which RAD001 treatment twice weekly for 4.5-weeks was compared to RAD001 treatment given for 12-weeks. Aim of the study was to investigate if the cessation of RAD001 after 4.5 weeks of treatment was causing the occurrence of metastasis. The 12-weeks RAD001 treatment also resulted in the development of metastasis. Also ^{177}Lu tumor uptake after ^{177}Lu-DOTATATE treatment with and without RAD001 co-treatment was quantified, to see if this could serve as an explanation for the fact that the combination treatment of RAD001 and ^{177}Lu-DOTATATE was less effective compared to that with ^{177}Lu-DOTATATE alone. ^{177}Lu-DOTATATE tumor uptake was not affected by RAD001 treatment. This experiment was performed in a different substrain of Lewis rats, often used for auto-immune studies, as these animals have a more active immune system showing an enhanced CD4+ and CD8+ T cell (auto)-immune response[13–17]. This autoimmunity is linked to an increased anti-tumor immunity[18,19]. In these rats 50 % of subcutaneous tumors disappeared spontaneously in the control group, whereas in the RAD001 treatment groups only 12.5 % of the tumors regressed, probably caused by RAD001 induced inhibition of the immune system and in line with our hypothesis.

A recent report by Yin et al.[20] showed comparable effects of mTOR inhibition in BALB/c mice bearing subcutaneous 4T1 murine breast cancer tumors. The mTOR inhibitor rapamycine accelerated lung metastasis. Rapamycin induced production of immunosuppressive molecules and cytokines, like TGF-β, arginase-1, indoleamine 2,3-dioxygenase, IL-6 and IL-10, in the lungs. The authors postulate treatment with rapamycin to result in an immunosuppressive microenvironment in the metastatic sites.

A definite answer on the occurrence of metastasis in the CA20948 tumor model after RAD001 treatment is not yet fully clear and more studies are needed to solve the still open questions. The use of mTOR inhibition treatment in the clinical setting should be closely watched for the development of unexpected, new metastasized disease.

Chapter 6 describes the transfection of CA20948 cells with a plasmid coding for green fluorescent protein and luciferase, for use of non-invasive tumor follow up by bioluminescence imaging (BLI) in future PRRT experiments in a liver metastasis rat model. We successfully applied

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these transfected CA20948-luc cells in a subcutaneous nude mouse as well in a rat liver-metastasis model as described in this chapter.

Future perspectives

Currently all clinical data published on the efficacy of ^{177}Lu-DOTATATE treatment for NETs were based on non-randomized studies, with data compared to those obtained in historical controls. In 2012 however, a multicenter, prospective, randomized, clinical trial in patients with inoperable, progressive midgut NETs, has started. This study randomizes between ^{177}Lu-DOTATATE (four administrations of 7.4 GBq (200 mCi) at 8±1-week intervals) combined with Octreotide LAR 30 mg (discontinued during PRRT) versus treatment with high dose Octreotide LAR 60 mg. Primary efficacy endpoint is progression-free survival as measured by objective tumour response, centrally assessed according to the response evaluation criteria in solid tumors (RECIST)[21], every 12±1 weeks from the first treatment date in 280 patients. Concomitant amino acids will be given with each administration for kidney protection. Estimated Primary Completion Date is December 2014. (www.clinicaltrials.gov; Identifier: NCT01578239)

Combination of PRRT with several new biologicals and chemotherapeutics could result in a higher therapeutic effect. For example, a prospective, randomized, clinical trial comparing RAD001 + Octreotide LAR versus ^{177}Lu-DOTATATE + Octreotide LAR in patients with advanced GEPNETs would be of very high interest now. Despite our observations in the studies described in Chapters 4 and 5, RAD001-induced metastasis has not been reported in patients.

Currently a randomized clinical trial, comparing ^{177}Lu-DOTATATE treatment with ^{177}Lu-DOTATATE treatment in combination with the oral chemotherapeutic drug capecitabine (Xeloda ; Roche, Basel, Switzerland) is ongoing in our center. Earlier studies already showed that combining these therapies is safe and at least as effective as ^{177}Lu-DOTATATE PRRT alone[22,23]. The aim of this study is to show that chemosensitization with capecitabine improves the percentage of patients with objective tumor responses who are also treated with ^{177}Lu-DOTATATE. In the near future the results of this study will be published.

Despite the disappointing and surprising results of Everolimus (RAD001) with ^{177}Lu-DOTATATE treatment combinations as described in Chapters 4 and 5 in this thesis, this combination therapy has now in Australia been given to patients with metastasized NET with tolerable side effects. In the future, a phase II randomized controlled trial of ^{177}Lu-DOTATATE with capecitabine/temozolomide versus ^{177}Lu-DOTATATE with RAD001 is planned to be performed in Australia[24]. With our preclinical results in mind, a close watch is needed for possible increase in metastases after/during RAD001 treatment in patients.

Another very promising targeted therapy, Sunitinib (Sutent®, Pfizer), a VEGF-receptor

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inhibitor[25], is an interesting option for comparison to PRRT in future randomized clinical trials. Combination therapy of Sunitinib with PRRT would be the next step. Transient normalization of the tumor vasculature by VEGF-receptor inhibition results in decreased intratumoral pressure and better oxygenation with possible facilitation of higher intratumoral radiopeptide penetration and increased radiation effects by higher oxygenation[26].

Intra-arterial PRRT is of high value in selected NET patients, as described in Chapter 3 of this thesis and elsewhere[27-29]. In the near future selected NET patients with a predominant tumor load in the liver will be offered intra arterial ^{177}Lu-DOTATATE PRRT in our center.

In selected NET patients with the tumor-mass mainly located in the liver, a combined approach of intra-arterial ^{177}Lu-DOTATATE PRRT with ^{166}Ho-loaded poly(L-lactic acid) microsphere radio-embolisation[30] of NET liver metastases compared to intra-arterial ^{177}Lu-DOTATATE PRRT alone is a strategy worth investigating. ^{166}Ho-microspheres are preferable over ^{90}Y-microspheres, because ^{166}Ho emits low energy gamma photons for gamma camera imaging and is paramagnetic, allowing magnetic resonance imaging (MRI).

In the near future, ^{177}Lu-DOTATATE PRRT dosimetry will hopefully be performed at our institution, resulting in patient tailored dose calculation, resulting in optimal tumor radiation with minimal radiation damage to the healthy tissues. Possibly many patients treated with the standard dose of 4 times 7.4 GBq ^{177}Lu-DOTATATE are under-treated and could be treated with higher dosages without significant side effects and better tumor responses are to be expected. Patients with, e.g., a suboptimal kidney function could receive a lower dose preventing end stage renal damage.

Varying the specific activity of ^{177}Lu (amount of radioactivity/gram), the peptide mass of DOTATATE (now around 200 µg) and infusion rate are very interesting methods of increasing tumor ^{177}Lu uptake and resulting therapeutic effects. Further (pre)clinical studies will have to be performed to explore the effects of these strategies.

The use of ^{177}Lu-DOTATATE PRRT as a neoadjuvant treatment in patients with initially irresectable pancreatic NETs has been performed at our center and is promising. We therefore propose a prospective study on neo-adjuvant use of ^{177}Lu-DOTATATE PRRT to be initiated.

The recently obtained and promising results on the use of the alpha emitter ^{213}Bi for α-PRRT in preclinical animal experiments[8,9] and in patients with NETs refractory to ^{90}Y- or ^{177}Lu-DOTATOC β-PRRT[10] will certainly result in clinical trials using ^{213}Bi α-PRRT as single treatment for NETs or in combination with e.g. ^{90}Y or ^{177}Lu β-PRRT. Acute and mid-term toxicity seems to be low, but

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long-term follow up is still needed.

Preclinical and clinical studies showed higher higher uptake of radiolabeled sst antagonists than of sst agonists[31,32] in neuroendocrine tumors and promising tumor responses. Future studies will have to show the potential benefit of these antagonists over agonists.

In conclusion, the field of PRRT is rapidly evolving and will hopefully result in increasing progression-free survival and higher quality of life for NET bearing patients. Hopefully, these rapid developments will ultimately result in curing a higher percentage of these patients.

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Samenvatting, toekomstvisie

Neuro endocriene tumoren zijn relatief zeldzaam met een incidentie van 2-5 per 100.000 inwoners. Bij initiële diagnose is de tumor in de meerderheid van de gevallen al gemetastaseerd en is de enige behandeling met kans op volledige curatie, chirurgie, geen optie meer. De standaard behandeling bestaat meestal uit het gebruik van een langwerkend somatostatine analoog mogelijk gecombineerd met (palliatieve) chirurgie, chemotherapie, radiotherapie, transarteriële (chemo) embolisatie, radiofrequente ablatie (RFA), cryoablatie en laser geïnduceerde thermotherapie (LITT) en intra-arteriële radio embolisatie met yttrium-90 of holmium-166 microsferen.

De veelgebruikte langwerkende somatostatine analogen binden aan somatostatine receptoren (voornamelijk de somatostatine-2-receptor) die tot overexpressie worden gebracht op zowel functionele als niet functionele neuro endocriene tumoren wat resulteert in een significante verlenging van progressie vrije overleving in vergelijking met placebo[1,2]. De behandeling met somatostatine analogen kan ook de overproductie van hormonen door functionele neuro endocriene tumoren remmen en hiermee een verlichting van de symptomen bij patiënten met gemetastaseerde ziekte [3-5]. De over expressie van somatostatine receptoren op neuro-endocrine tumoren wordt ook gebruikt voor diagnose/visualisatie van deze tumoren door middel van radioactief gelabelde somatostatine analogen zoals [^{111}Indium-DTPA]-octreotide (Octreoscan®, Covidien, Petten, Nederland) en de meer recent ontwikkelde positron emissie tomografie (PET) tracers ^{68}Ga-DOTA-Tyr^{3}-octreotide[6] of ^{68}Ga-DOTA-Tyr^{3}-octreotate (^{68}Ga-DOTATATE)[7]. Gebaseerd op hetzelfde principe wordt voor therapie gebruik gemaakt van somatostatine analogen gelabeld met hoog energetische bèta deeltjes uitzendende radionucliden zoals bijvoorbeeld ^{177}Lu-DOTATATE en ^{90}Y-DOTATOC. De resultaten bij deze vooralsnog niet geregistreerde middelen zijn veelbelovend. Relatief nieuw is de toepassing van radionucliden die alfa deeltjes uitzenden. Het gebruik van deze radionucliden zal waarschijnlijk een erg succesvolle strategie zijn door de hoge lineaire energie transmissie (LET) van alfa straling[8-10]. Recent zijn veelbelovende resultaten gepubliceerd met ^{213}Bi-DOTATOC peptide receptor radionucliden therapie (PRRT) bij zeven patiënten met neuro endocriene tumoren die weer progressief waren na ^{90}Y/^{177}Lu-DOTATOC PRRT[10].

In dit proefschrift zijn verschillende manieren beschreven om de therapie voor neuro endocriene tumoren te optimaliseren. Zo ook de toepassing en resultaten hiervan in

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verschillende studies. **Hoofdstuk 1** is een algemene introductie in het veld van de neuro endocriene tumoren en in de verschillende behandelingsmogelijkheden. Ook wordt er een korte uitleg gegeven over het gebruikte diermodel in de beschreven studies in dit proefschrift.

Hoofdstuk 2 is een review van de literatuur over preklinische en klinische PRRT. De therapie effecten van somatostatine analogen met verschillende radionucliden worden er beschreven. Ook mogelijke nieuwe peptiden voor PRRT zoals glucagon-like peptide 1, gastrin-releasing peptide, CCK2 en peptiden die binden aan de epidermal growth factor receptor en $\alpha_2\beta_2$ integrine. Mogelijke strategieën zoals bijvoorbeeld het gebruik van ^{177}Lu voor kleinere tumoren en ^{90}Y voor grotere tumoren, locoregionale toediening en de combinatie PRRT met chemotherapie, worden besproken.

Hoofdstuk 3 beschrijft de effecten van intra-arteriële (IA) toediening in vergelijking met intra veneuze (systemische) toediening op de opname van [^{111}Indium-DTPA]-octreotide in levermetastasen (CA20948 tumor) in een diermodel en in 3 patiënten met neuro endocriene levermetastasen met een variërende somatostatine 2 receptor expressie en een verschillende tumormassa. In ratten met een somatostatine 2 receptor positieve tumor in de lever resulteerde IA toediening in een tweemaal zo hoge opname van ^{111}In in de tumor vergeleken met systemische toediening. In de patiëntenstudie bleek dat IA toediening ook kan resulteren in een significant hogere ^{111}In opname in NET levermetastasen maar dat dit effect kan variëren per patiënt. De resultaten bij één patiënt lieten zien dat lokalisatie van de tip van de katheter essentieel is voor optimale IA toediening in de gehele lever. Bij deze patiënt bleek dat een in de linker kant van de lever gelegen levermetastase niet werd geperfundeerd met het radiofarmacon tijdens de IA toediening. Deze levermetastase bleek uiteindelijk dezelfde hoeveelheid ^{111}In te bevatten als na systemische toediening. IA toediening via de leverslagader hoeft dus niet te resulteren in een lagere opname van het radiofarmacon in tumoren elders in het lichaam in vergelijking met systemische toediening. Farmacokinetische modelering en theoretische schatting van ^{177}Lu dosimetry werd uitgevoerd op de verkregen metingen bij één patiënt en liet een 30% reductie van de stralingsdosis op de nieren zien na IA toediening.

In patiënten met diffuse of grote somatostatine receptor positieve levermetastasen kan locoregionale PRRT de voorkeur hebben boven systemische toediening. Toekomstige meer uitgebreide klinische studies zullen moeten bewijzen dat hogere opname van het

radiofarmacon na IA toediening resulteert in een betere tumor respons en een betere overleving.

In **Hoofdstuk 4** beschrijven wij een studie waarin ^{177}Lu-DOTATATE PRRT wordt gecombineerd met de mTOR inhibitor RAD001 (Affinitor®, Novartis, Basel, Switzerland) bij ratten met een onder de huid geplaatste neuro endocriene (CA20948) tumor. Het doel van deze studie was om te onderzoeken of deze twee behandelingen elkaar zouden versterken. In eerdere studies is namelijk aangetoond dat RAD001 tumoren gevoeliger kan maken voor bestraling[11,12]. Verrassend genoeg bleek de combinatietherapie minder effectief te zijn dan ^{177}Lu-DOTATATE behandeling alleen. Maar belangrijker was dat dieren die voor 4½ week twee maal per week RAD001 toegediend hadden gekregen (in combinatie met / zonder ^{177}Lu-DOTATATE) uiteindelijk uitzaaiingen bleken te ontwikkelen. Controledieren en dieren die alleen ^{177}Lu-DOTATATE hadden gekregen bleken geen uitzaaiingen te krijgen. Dit tumormodel wordt op onze afdeling al meer dan 10 jaar gebruikt en niet eerder hebben wij uitzaaiingen bij deze dieren gevonden. Onze hypothese was dat het stoppen met de RAD001 therapie na 4½ week mogelijk het ontstaan van de uitzaaiingen had veroorzaakt. Mogelijk door een versterkte reactivatie van de intracellulaire mTOR pathway. Ook remming van het immuunsysteem door RAD001 zou een oorzaak van de metastasering kunnen zijn.

Hoofdstuk 5 beschrijft verdere analyses van de resultaten gevonden bij de in hoofdstuk 4 beschreven combinatiestudies. Ook wordt een bijkomende studie beschreven waarin RAD001 behandeling gedurende 4½ week wordt vergeleken met 12 weken durende RAD001 therapie. Het doel van deze studie was om te onderzoeken of het stoppen van de therapie na 4½ week de oorzaak van het ontwikkelen van uitzaaiingen was. De dieren die 12 weken RAD001 kregen bleken echter ook uitzaaiingen te ontwikkelen.

Ook werd de mate van ^{177}Lu-DOTATATE opname in de tumor gemeten met en zonder de combinatie met RAD001. Een lagere opname in de tumor zou namelijk een verklaring kunnen zijn voor het feit dat de combinatie therapie minder effectief was dan ^{177}Lu-DOTATATE alleen. De mate van ^{177}Lu-DOTATATE opname in de tumor was echter niet afhankelijk van de behandeling met RAD001.

In dit experiment werden ratten gebruikt van een andere sub-stam van Lewis ratten die vaak gebruikt wordt voor auto-immuun studies omdat deze dieren een immuunsysteem hebben met een hogere CD4+ en CD8+ T cel (auto)-immuun respons[13-17]. Deze auto-immuniteit wordt gelinkt aan een hogere anti tumor immuniteit[18,19]. In deze dieren

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verdwenen 50% van de tumoren spontaan in de controle groep terwijl in de RAD001 groep maar 12½% van de tumoren verdwenen, waarschijnlijk veroorzaakt door remming van het immuunsysteem en in lijn met onze hypothese.

Een recente publicatie door Yin et al.[20] laat soortgelijke effecten zien na behandeling met een mTOR inhibitor in BALB/c muizen met onderhuidse 4T1 muizen borstkankers. De mTOR remmer Rapamycine bleek het ontstaan van uitzaaiingen naar de longen te versterken. Rapamycine bleek de productie van immuun remmende moleculen en cytokines zoals TGF-β, arginase-1, indoleamine 2,3-dioxygenase, IL-6 en IL-10 in de longen te induceren. De auteurs postuleren dat behandeling met Rapamycine resulteert in een immuun remmend microklimaat ter plaatse van de uitzaaiingen.

Een definitief antwoord op het ontstaan van uitzaaiingen bij RAD001 behandeling in het CA20948 tumormodel in de rat is er helaas nog niet en meer studies zijn nodig om de resterende vragen te beantwoorden. Het gebruik van mTOR remmers in de klinische setting zal echter goed moeten worden gemonitord voor het ontstaan van onverwachte (nieuwe) uitzaaiingen.

Hoofdstuk 6 beschrijft de transfectie (genetische modificatie) van CA20948 tumor cellen met een plasmide dat het gen voor een groen fluorescerend eiwit en voor het eiwit luciferase bevat. Dit met het doel om in toekomstige PRRT experimenten de tumoren op een niet invasieve manier te kunnen vervolgen middels bioluminescentie imaging (BLI). We hebben, zoals beschreven in dit hoofdstuk, deze getransfecteerde CA20948-luc cellen succesvol onderhuids in een naakte muis model en in een levermetastase model in de rat kunnen gebruiken.

Toekomstvisie

De huidige gepubliceerde klinische data over de effectiviteit van ^{177}Lu-DOTATATE PRRT voor neuro endocriene tumoren zijn gebaseerd op niet gerandomiseerde studies waarbij de uitkomsten vergeleken worden met historische controles. In 2012 is er echter een prospectieve gerandomiseerde multicenter klinische studie gestart waarin patiënten gerandomiseerd ^{177}Lu-DOTATATE (vier toedieningen van 7.4 GBq (200 mCi) met 8±1-week interval) gecombineerd met Octreotide LAR 30 mg (gediscontinueerd tijdens PRRT) of alleen behandeling met een hoge dosis Octreotide LAR (60 mg) krijgen. Het primaire eindpunt van deze studie is progressie vrije overleving gebaseerd op objectieve centrale meting van tumor respons elke 12±1 weken na de eerste behandeling bij 280 patiënten middels de RECIST criteria (response evaluation criteria in solid tumors)[21].

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Voor bescherming van de nieren worden er bij de toediening van ^{177}Lu-DOTATATE aminozuren gegeven. De geschatte primaire einddatum van deze studie is December 2014. (www.clinicaltrials.gov; Identifier: NCT01578239)

Het combineren van PRRT met nieuwe biologicals en/of chemotherapeutica zou kunnen resulteren in een beter therapeutisch effect. Een prospectieve gerandomiseerde klinische studie bij patiënten met gastro entero pancreatische neuro endocriene tumoren (GEPNET) waarin RAD001 met Octreotide LAR wordt vergeleken met ^{177}Lu-DOTATATE PRRT + Octreotide LAR zou zeer interessant zijn. Gelukkig zijn de effecten van RAD001 behandeling zoals beschreven in hoofdstuk 4 en 5 niet bij patiënten gerapporteerd.

Op dit moment is er een gerandomiseerde klinische studie gaande in ons ziekenhuis waarin ^{177}Lu-DOTATATE PRRT wordt vergeleken met ^{177}Lu-DOTATATE PRRT in combinatie met het orale chemotherapeuticum capecitabine (Xeloda ; Roche, Basel, Switzerland). Eerdere studies hebben al laten zien dat de combinatie van deze twee therapieën veilig en tenminste even effectief is als ^{177}Lu-DOTATATE PRRT alleen[22,23]. Het doel van de studie in ons ziekenhuis is om aan te tonen dat chemosensitisatie met capecitabine resulteert in een hoger percentage van patiënten met een objectieve tumor respons na ^{177}Lu-DOTATATE PRRT. De resultaten van deze studie worden waarschijnlijk in de nabije toekomst gepubliceerd.

Ondanks de teleurstellende en verrassende resultaten van de combinatie van Everolimus (RAD001) met ^{177}Lu-DOTATATE PRRT zoals beschreven in hoofdstuk 4 en 5 is deze combinatietherapie in Australië al gegeven met minimale bijwerkingen aan patiënten met uitgezaaide neuro endocriene tumoren. In Australië zijn er plannen voor een fase II gerandomiseerde gecontroleerde trial waarin ^{177}Lu-DOTATATE PRRT met capecitabine/temozolomide zal worden vergeleken met ^{177}Lu-DOTATATE met RAD001[24]. Met onze preklinische resultaten in het achterhoofd zullen deze patiënten extra goed gemonitord moeten worden voor het ontstaan van nieuwe uitzaaiingen tijdens/na behandeling met RAD001.

Een andere veelbelovende therapie, Sunitinib (Sutent, Pfizer), een VEGF-receptor remmer[25], is ook een zeer interessante optie om te vergelijken met PRRT in een gerandomiseerde klinische studie. Combinatie van Sunitinib met PRRT zou dan een logische volgende stap zijn. Tijdelijke normalisatie van de vaatvoorziening van de tumor door middel van VEGF-receptor remming resulteert in een verlaging van de intratumorale druk en een betere zuurstof toevoer met hierdoor facilitatie van een betere penetratie van het radiopeptide in de tumor en een verhoogd effect van de bestraling door de

betere oxygenatie[26].

Intra-arteriële PRRT is van grote waarde in bepaalde patiënten met neuro endocriene tumoren zoals beschreven in Hoofdstuk 3 van dit proefschrift en elders[27-29]. In de nabije toekomst zullen patiënten met een tumormassa die voornamelijk in de lever is gelegen IA ^{177}Lu-DOTATATE PRRT worden aangeboden.

Bij deze patiënten zou het ook interessant zijn om een gecombineerde aanpak van IA ^{177}Lu-DOTATATE PRRT met ^{166}Ho-bevattende poly(L-lactic acid) microsphere radio-embolisatie[30] te vergelijken met IA ^{177}Lu-DOTATATE PRRT alleen. ^{166}Ho-microspheren hebben de voorkeur ten opzicht van ^{90}Y-microspheren, vanwege het feit dat ^{166}Ho laag energetische gamma fotonen uitzendt die gebruikt kunnen worden voor gamma camera imaging. Tevens is ^{166}Ho paramagnetisch, waardoor het goed zichtbaar is met magnetic resonance imaging (MRI).

Hopelijk zal er op korte termijn ^{177}Lu-DOTATATE PRRT dosimetry worden uitgevoerd. Dit zal dan resulteren in een patiënt specifieke dosis berekening wat uiteindelijk zal resulteren in een optimale bestraling van de tumor met een minimale bestralingsdosis op de gezonde organen. Mogelijk worden veel patiënten die nu de standaard dosis van 4 keer 7,4 GBq ^{177}Lu-DOTATATE krijgen onder behandeld en zouden zij een hogere dosis kunnen krijgen zonder significante bijwerkingen en waarschijnlijk een hogere tumor respons. Patiënten met bijvoorbeeld een gestoorde nierfunctie zouden juist een lagere dosis krijgen waarbij nierfalen voorkomen zou worden.

Het variëren van de specifieke activiteit van ^{177}Lu (hoeveelheid radioactiviteit/gram), van de peptide massa van DOTATATE (nu ongeveer 200 µg/7,4 GBq) en van de infusiesnelheid zijn interessante opties om de opname van ^{177}Lu in de tumor te verhogen en zo mogelijk ook het therapeutisch effect. Meer (pre)klinische studies zullen gedaan moeten worden om deze strategieën te onderzoeken.

^{177}Lu-DOTATATE PRRT als neoadjuvante therapie bij patiënten met initieel niet-resecteerbare pancreatische neuro endocriene tumoren is reeds gebruikt in ons centrum en is veelbelovend. Een prospectieve studie waarin ^{177}Lu-DOTATATE PRRT als neoadjuvante therapie wordt gegeven zou daarom gestart moeten worden.

De onlangs gepubliceerde en veelbelovende resultaten over het gebruik van de alfa emitter ^{213}Bi voor α-PRRT in preklinische dierexperimenten[8,9] en in patiënten met ^{90}Y- of ^{177}Lu-DOTATOC β-PRRT refractaire neuro endocriene tumoren[10] zullen zeker resulteren in klinische studies waarin ^{213}Bi α-PRRT alleen als behandeling voor neuro-endocriene tumoren wordt gegeven of in combinatie met bijvoorbeeld ^{90}Y or ^{177}Lu β-PRRT. De acute en middellange bijwerkingen lijken minimaal te zijn maar opvolging op

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de lange termijn is nog nodig.

Preklinische en klinische studies hebben een hogere opname van somatostatine receptor antagonisten in vergelijking met somatostatine receptor agonisten in neuro-endocriene tumoren laten zien met een veelbelovende tumor respons.[31,32]. Toekomstige studies zullen deze antagonisten moeten vergelijken met de huidige agonisten met betrekking tot therapeutisch effect en mate van bijwerkingen.

We kunnen in ieder geval concluderen dat het veld van PRRT snel evolueert en dat dit hopelijk zal resulteren in een steeds hogere progressie vrije overleving van patiënten met neuro endocriene tumoren met hierbij een hogere kwaliteit van leven. Hopelijk zullen al de hierboven beschreven ontwikkelingen uiteindelijk resulteren in een hoger percentage echte curatie van deze patiënten met een neuro endocriene tumor.

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Curriculum Vitae

Stefan Pool was born on 6th of March 1978 in Hilversum, the Netherlands. He grew up with his sister in Capelle aan den IJssel. He attended secondary school at 'het Emmaus College' in Rotterdam from which he graduated the gymnasium in 1997. Due to numerus fixus he started Medical school in Antwerp, Belgium. He continued medical school at the ErasmusMC, Rotterdam, the Netherlands. Beside Medical school he started a Master in Molecular Medicine at the MolMed Postgraduate school at the ErasmusMC. In 2005 he obtained his medical degree and his bachelor's degree in Molecular Medicine.

After finishing Medical school he started in a PhD project in a collaboration of the Department of Nuclear Medicine and the Department of Surgery, ErasmusMC. In 2011 he started his residency in Nuclear Medicine under supervision of Dr. D.J. Kwekkeboom. This will be completed in 2016.

He lives together with Dorien Rijkaart and their child Sofie (2011) and in joyful expectation of a second child.

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List of publications

Pool SE, Bison SM, Koelewijn SJ, van der Graaf LM, Melis M, Groen HC, de Jong M. Peptide receptor radionuclide therapy (PRRT) with [177Lu-DOTA0,Tyr3]octreotate in combination with RAD001 treatment; further investigations on tumor metastasis and response in a rat pancreatic cancer model. European Journal of Nuclear Medicine and Molecular Imaging Research. 2014 June;4:21

Pool SE, Kam B, Koning GA, Konijnenberg M, ten Hagen TLM, Breeman WAP, Krenning EP, de Jong M, van Eijck CHJ. [^{111}In-DTPA]octreotide tumor uptake in GEPNET liver metastasis after intra-arterial administration - an overview of preclinical and clinical observations and implications for tumor radiation dose after peptide radionuclide therapy. Cancer Biotherapy and Radiopharmaceuticals. 2014 May;29(4):179-87

Pool SE, Bison S, Koelewijn SJ, van der Graaf LM, Melis M, Krenning EP, de Jong M. mTOR Inhibitor RAD001 Promotes Metastasis in a Rat Model of Pancreatic Neuroendocrine Cancer. Cancer Research 2013 Jan;73(1): 12-8

Pool SE, ten Hagen TL, Koelewijn SJ, de Jong M, Koning GA. Multimodality imaging of somatostatin receptor-positive tumors with nuclear and bioluminescence imaging. Mol Imaging. 2012 Feb;11(1):27-32

Pool SE, Krenning EP, Koning GA, van Eijck CH, Teunissen JJ, Kam B, Valkema R, Kwekkeboom DJ, de Jong M. Preclinical and clinical studies of peptide receptor radionuclide therapy. Semin Nucl Med. 2010 May;40(3):209-18

Bison SM, Konijnenberg MW, Melis M, **Pool SE**, Bernsen MR, Teunissen JJM, Kwekkeboom DJ, de Jong M. Peptide receptor radionuclide therapy using radiolabeled somatostatin analogs: focus on future developments. Clinical Translational Imaging. 2014 Feb; (2):55-66

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PHD PORTFOLIO

Summary of PhD training and teaching

Erasmus MC Departments:	Nuclear Medicine and Surgical Oncology
Research School:	Molecular Medicine
PhD period:	2005 - 2013
Promotor:	Prof.dr. M. de Jong, Prof.dr. C.H.J. van Eijck
Supervisor:	Dr. G.A. Koning

1. PhD training

General academic skills	Year	ECTS
Photoshop and Illustrator CS-4 Workshop (molmed)	2010	0,3
Biomedical English Writing and Communication (Molmed)	2009	2
Research management for PhD-students	2009	1
Get out of your lab days (Molmed)	2006	0,6
JNI weekly meetings	2005-2010	1
JNI oncology lectures	2005-2010	0,3
Journal club meetings LECO and Nuclear medicine	2005-2010	1
Research skills		
Course 'Radiation safety, level 3'	2013	12
AMIE Imaging workshop & symposium	2009	1,4
BROK (Basiscursus Regelgeving Klinisch Onderzoek) course	2006	0,6
In Vivo Imaging 'From Molecule to Organism' (molmed)	2006	1,8
Course 'Radiation safety, level 5B'	2005	1,5
Course 'proefdierkunde ex art. 9'	2005	3
In-depth courses		
Course 'Geavanceerde beeldvormende technieken voor dokters'	2009	0,2
Analysis of Microarray Gene Expression Data (Molmed)	2005	2
Course Basic and Translational Oncology (molmed)	2005	1,8
Research meetings, Department of Nuclear Medicine	2005-2010	1

Research meetings, Laboratory of Experimental Surgical Oncology (LECO)	2005–2010	1

Presentations / posters at conferences

European Congress of Radiology (ECR), Vienna, Austria 2014	2014	1
Annual meeting of the Radiological Society of North America (RSNA) 2012, Chicaco, USA	2012	1
Annual congress of the Society of Nuclear Medicine (SNM) 2011, San Antonio, USA (poster)	2011	1
Annual meeting of the European Association of Nuclear Medicine (EANM) 2011, Birmingham, England (oral)	2011	1
EANM 2010, Wien, Austria (2 posters)	2010	1
EANM 2009, Barcelona, Spain (2 posters)	2009	1
EANM 2007, Copenhagen, Denmark (oral)	2007	1
NKRV workshop, UMC St Radboud, Nijmegen, the Netherlands (oral)	2010	1
COST meeting 'Targeted radionuclide therapy', Freiburg 2010, Germany (oral)	2010	1
COST meeting 'Advances in targeted radionuclide therapy', Nantes 2009, France (oral)	2009	1
Symposium 'The Netherlands platform for targeted nanomedicine', Groningen, the Netherlands (oral)	2008	1
SEOHS (Symposium Experimenteel Onderzoek Heelkundige Specialismen), Leiden, the Netherlands (poster)	2008	1
15th Molmed day, Rotterdam, the Netherlands (poster)	2011	1
14th Molmed day, Rotterdam, the Netherlands (oral)	2010	1
13th Molmed day, Rotterdam, the Netherlands (oral)	2009	1
12th Molmed day, Rotterdam, the Netherlands (poster)	2008	1
Chirurgendagen, Ede, the Netherlands (oral)	2007	1
Chirurgendagen, Veldhoven, the Netherlands (poster)		
Imagination meeting, Rotterdam, the Netherlands (poster)	2007	1

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Seminars, workshops

Interactieve nascholingscursus Pancreas NET Rotterdam	2012	0,2
Imagination meetings, Rotterdam/Nijmegen	2006-2008	1
NKRV workshop, UMC St Radboud, Nijmegen	2006	0,3
3th symposium of the Netherlands Platform for Targeted Nanomedicine, Leiden	2010	0,3

2. Teaching activities

Supervising HLO trainees (Satish, Petra, Stuart)	2006-2010	6
Supervising medical student (Peter)	2007	2
Amie imaging course, practical part (micro-PET + nano-SPECT)	2009-2010	1
Supervising practicals medical students 2th Year	2010-2011	0,3
Education nuclear medicine at start internships	2012-2013	0,5
	Total ECTS	62

Optimisation of Neuroendocrine Tumor Treatment

Stefan Pool