Therapy of neuroendocrine tumors with ^{177}Lu-octreotate

Human tumor cell types and models and optimization of treatment

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Front cover: Illustration of ^{177}Lu-[DOTA^{0}-Tyr^{3}]-octreotate based on 1YL8.pdb and 1NC2.pdb.

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“Ever tried. Ever failed. No matter. Try again. Fail again. Fail Better."
Samuel Beckett

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Abstract

Neuroendocrine (NE) tumors (NET) have often metastasized at the time of diagnose, which makes it hard to cure patients with NET. Radiolabeled hormone analogues (especially somatostatin analogues, SS) can be used for diagnostics (e.g. ^{111}In-octreotide) and therapy (e.g. ^{177}Lu-octreotate). For development of the treatment methods, realistic tumor cell lines and models are valuable. Human NET cell lines and models are few, and there is a need to find suitable models for different types of NET, with e.g. relevant expression of hormone receptors, e.g. somatostatin receptors (SSTR), cholecystokinin-2/gastrin receptors, and catecholamine transporters.

In this work, several types of human NET models (paraganglioma, gastrointestinal stromal tumor (GIST), human medullary thyroid cancer (GOT2), and midgut carcinoid (GOT1)) were studied, with the aim to evaluate the binding and/or uptake of radiolabeled hormone analogues (^{177}Lu-octreotate, ^{111}In-octreotide, ^{111}In-MG0, and ^{131}I-MIBG). Activity concentration in tumor and non-tumor tissues was measured *in vitro* or *in vivo* in different NETs. The activity concentration after ^{111}In-octreotide injection indicated a large variation in somatostatin receptor expression in different NETs. A specific uptake and internalization of radiolabeled ^{111}In-octreotide or ^{177}Lu-octreotate was found *in vitro* in paraganglioma and in GIST, respectively, as well as a specific uptake of ^{131}I-MIBG in paraganglioma. The tumor uptake of ^{111}In-octreotide and ^{131}I-MIBG in the patient with paraganglioma, and of ^{111}In-octreotide in several individuals with GIST showed that some of these patients might benefit from radionuclide therapy. All studied human NETs in this work will serve as good models in the development of increased therapeutic effect of different NETs.

^{177}Lu-octreotate is today routinely used for treatment of carcinoids and endocrine pancreatic tumors, but needs to be optimized. A novel treatment schedule was tested, giving a priming administration of ^{177}Lu-octreotate before administering the therapeutic amount. This procedure resulted in higher mean absorbed dose to tumor tissue and increased therapeutic effect compared with those for a single administration.

To improve the individual following-up after fractionated treatment with ^{177}Lu-octreotate, the possibility to use urinary retinol binding protein (RBP) and valine hydantoin (VH) in blood as biomarkers for radiation induced nephrotoxicity was studied. RBP4 was shown to be a potential biomarker for nephrotoxicity, before kidney injury was demonstrated by morphology.

Keywords: somatostatin, radionuclide therapy, receptor up-regulation, RBP, nephrotoxicity
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List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Dalmo J, Rudqvist N, Spetz J, Laverman P, Nilsson O, Ahlman H, Forssell-Aronsson E. *Biodistribution of ^{177}Lu-octreotate and ^{111}In-minigastrin in female nude mice transplanted with human medullary thyroid carcinoma GOT2.* Oncology reports 27: 174-181, 2012

II. Spetz J, Dalmo J, Nilsson O, Wängberg B, Ahlman H, Forssell-Aronsson E. *Specific binding and uptake of ^{131}I-MIBG and ^{111}In-octreotide in metastatic paraganglioma –tools for choice of radionuclide therapy*. Hormone and Metabolic Research, 44(5): 400-404, 2012

III. Arne, G., Nilsson B., Dalmo J, Kristiansson E, Arvidsson Y, Forssell-Aronsson E, Nilsson O and Ahlman H. *Gastrointestinal stromal tumors (GISTs) express somatostatin receptors and bind radiolabeled somatostatin analogs.* Acta Oncol 52(4): 783-792, 2013

IV. Dalmo J, Spetz J, Montelius M, Langen B, Arvidsson Y, Johansson H, Parris T, Helou K, Wängberg B, Nilsson O, Ljungberg M, Forssell-Aronsson E. *Increased therapeutic effect using priming administration before the main administration of ^{177}Lu-octreotate in nude mice bearing human carcinoid tumor GOT1.* Manuscript

V. Dalmo J, Westberg E, Barregård L, Svedbom L, Johansson M, Törnqvist M, Forssell-Aronsson E. *Evaluation of retinol binding protein 4 and carbamoylated haemoglobin as potential renal toxicity biomarkers in adult mice treated with ^{177}Lu-octreotate.* Manuscript

Preliminary results have been presented as follows

Dalmo J, Svedbom L, Westberg E, Barregård L, Törnqvist M, Forssell-Aronsson E.
Potential renal toxicity biomarkers indicating radiation injury after ^{177}Lu-octreotate treatment.
Posters walk presentation at the European Association of Nuclear Medicine Congress, Lyon, France, Oct 2013

Dalmo J, Spetz J, Nilsson O, Wängberg B, Forssell-Aronsson E.
Increased therapeutic effect of fractionated ^{177}Lu-octreotate administration on nude mice bearing human carcinoid tumor GOT1.
Poster at the annual meeting with SWE-RAYS, Swedish radiation research association for young scientists, Uppsala, Aug 2013

Dalmo J, Rudqvist N, Spetz J, Laverman P, Nilsson O, Ahlman H, and Forssell-Aronsson E.
Biodistribution of ^{177}Lu-octreotate and ^{111}In-minigastrin in GOT2 (human MTC) animal model.
Poster at the annual meeting with SWE-RAYS Swedish radiation research association for young scientists, Stockholm, Aug 2012

Dalmo J, Rudqvist N, Ahlman H, Nilsson O, Forssell-Aronsson E.
Radiation induces higher somatostatin receptor expression in carcinoid tumours in mice.
Poster at the European Radiation Research Society annual meeting, Stockholm, Sept 2010

Spetz J, Dalmo J, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E.
Increased therapeutic response from fractionated ^{177}Lu-octreotate therapy of GOT1 tumors in nude mice. Cancerfondens planeringsgrupp för radionuklidterapi. Nov 2013

Spetz J, Dalmo, J, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E.
Fractionated ^{177}Lu-octreotate therapy of GOT1 tumors in nude mice increases treatment efficacy, possibly via SSTR up-regulation. Poster at the annual meeting with Radiation Research Society 2013. New Orleans, Sep 2013

Spetz J, Langen B, Dalmo J, Parris TZ, Wängberg B, Helou K, Forssell-Aronsson E. *Combination therapy of GOT1 tumours in nude mice using ^{177}Lu-octreotate and the hedgehog inhibitor LDE225.*
Poster at the annual meeting with SWE-RAYS, Swedish radiation research association for young scientists, Uppsala, Aug 2013

Spetz J, Dalmo J, Nilsson O, Wängberg B, Ahlman H, Forssell-Aronsson E. *Uptake of radiolabeled meta-iodobenzylguanidine and octreotate - tumor/blood values and in vitro studies as tools for choice of radionuclide therapy.* International Symposium on Pheochromocytoma and Paraganglioma. Paris, Sep 2011

Content

1 Abbreviations

^{111}In	indium-111
^{131}I	iodine-131
^{177}Lu	lutetium-177
CA	catecholamine
CCK	cholecystokinin
DOTA	dodecanetetraacetic acid
DOTA-MG0	DOTA-DGlu-$(Glu)_5$-minigastrin
DTPA	diethylenetriaminepentaacetic acid
GFR	glomerulus filtration rate
GIST	gastrointestinal stromal tumor
GOT1	human midgut carcinoid cell line
GOT2	human MTC cell
Hb	hemoglobin
MG	minigastrin
MIBG	meta-iodobenzylguanidine
MTC	medullary thyroid carcinoma
NE	neuroendocrine
NET	neuroendocrine tumor
PET	positron emitting tomography
PRRT	peptide receptor radionuclide therapy
qPCR	quantitative real-time polymerase chain reaction
RBP	retinol binding protein
SS	somatostatin
SSTR	somatostatin receptor
T/B	tumor-to-blood activity concentration ratio
T/N	mean tumor-to-normal-tissue activity concentration ratio
TKI	tyrosine kinase inhibitor
VH	valine hydantoin

2 Introduction

The first radiolabeled somatostatin analogue used in patients was ^{123}I-Tyr^{3}-octreotide followed by ^{111}In-DTPA-octreotide (OctreoScan®, Mallinckrodt, Inc., St. Louis, MO, USA). ^{111}In-DTPA-octreotide was introduced for scintigraphy of somatostatin (SS) receptor (SSTR) positive tumors (e.g. neuroendocrine tumors) in the late 1980ies (Krenning et al., 1989, Krenning et al., 1992), and soon became a routine method for diagnosis of such tumors. The first clinical trials of radiolabelled somatostatin analogues used for therapy were soon thereafter started (Fjalling et al., 1996, Krenning et al., 1996), and numerous radiopharmaceuticals targeting somatostatin positive neuroendocrine tumors, both for diagnostics and therapy, have since then been developed. However, clinical results obtained so far are modest, and optimization of the existing radiopharmaceutical and further developments of new tracers are required. For that purpose, reliable models are needed which should mimic the human situation as much as possible. One type of such a model is human tumor tissues transplanted on mice (Forssell-Aronsson et al., 2013).

Xenografted animal models with human neuroendocrine tumors have been developed in order to investigate new radiopharmaceuticals both for diagnostics and in therapy, and to optimize use of already existing ones. A few different neuroendocrine tumor types have successfully been transplanted to mice, with different characteristics such as receptor expression, growth rate and the origin of the neuroendocrine tumor. These models are of great value in order to evaluate and optimize new radiopharmaceuticals or treatment schedules.

2.1 Neuroendocrine system and neuroendocrine tumors

The neuroendocrine system is the link between the nervous system and endocrine system; the neuroendocrine cells receive signals from the nervous system to regulate, store and release hormones in the endocrine system. The regulation is done by the endocrine glands and tissues, which are found throughout the body, e.g., adrenal glands, pancreas, pineal gland, pituitary gland, ovaries, testes, thyroid gland, parathyroid gland, hypothalamus, and the gastrointestinal tract. Somatostatin, serotonin, histamine, cholecystokinin and gastrin are examples of neuroendocrine hormones in the gastrointestinal tract (Rehfeld, 1998). The secretions of these hormones are regulated by G-protein-coupled receptors, ion-gated receptors, and receptors with tyrosine-kinase activity (Modlin et al., 2008).

Neuroendocrine tumors (NET) are slow growing neoplasms of the disseminated neuroendocrine cell system. NETs are characterized by its overexpression of hormone receptors and ability to regulate the secretion of different peptides and neuroamines (Vinik et al., 2010). NETs are predominantly found in the gastrointestinal tract and the bronchopulmonary system that are the largest systems of neuroendocrine cells (Ahlman et al., 2001, Hauso et al., 2008). Gastrointestinal NETs often metastasize to abdominal lymph nodes and the liver.

After surgery, the most common treatment of metastatic NETs is hormone analogues that can regulate the hormone secretion, e.g. long-acting octreotide and lanreotide autogel, used for palliation and symptom relief. Symptoms are caused by disturbance in the hormone regulation of the NET (storage and secretion of various peptides and neuroamines), and are dependent of the origin of the NET. The most common symptoms are diarrhea, wet or dry flushing, vasomotor phenomenon that causes redness and warmth in the face and upper torso (Modlin et al., 2008, Strosberg et al., 2011, Vinik et al., 2010).

The disturbances in the hormone regulation give rise to an overexpression of somatostatin receptors (SSTR) on different types of neuroendocrine tumor cells. Taking advantages of that

gives the possibility to use peptide receptor radionuclide therapy (PRRT). The recent advances in diagnostic imaging and radionuclide therapy of NETs using radiolabeled SS analogues have been prominent (Kwekkeboom et al., 2010). However, depending on origin of the tumor, the receptor expression may vary considerably (Hofland et al., 2003). Minor short- and long-term side effects of the endocrine function in organs that normally express SSTR and release hormones have been seen after PRRT with somatostatin analogues (Teunissen et al., 2009).

Tumors overexpressing SSTR typically include pituitary adenoma, gastrointestinal and pancreatic endocrine carcinoma (the so-called gastroenteropancreatic tumors), paraganglioma, pheochromocytoma, small cell lung cancer, medullary thyroid carcinoma (MTC), breast cancer, and malignant lymphoma (Ahlman et al., 2000).

2.1.1 NE hormones and its receptors

Hormones are endocrine signals that are produced in the endocrine glands or in endocrine cells throughout the body but most frequently in hypothalamus, central and peripheral nervous system and in peripheral tissues (spleen and gastrointestinal tract). To all hormones, there are specific receptors that interact with the hormone. The receptor can be situated on the cell membrane, in the cytoplasm or in the nucleus (Erlansson-Albertsson, 2007).

Somatostatin and its analogues and receptors, SSTR1-5

Somatostatin (SS) is a polypeptide hormone produced throughout the body by neuroendocrine cells, as well as by inflammatory and immune cells (Patel, 1999). There are two native forms of somatostatin, with 14 (SS-14) or 28 (SS-28) amino acids, where SS-14 dominates in the central nervous system and in most peripheral organs, while SS-28 are mostly produced along the gastrointestinal tract (Van Op den Bosch et al., 2009, Wangberg et al., 1997). The somatostatin hormone is double acting with direct actions on organs via regulation of different pathways, predominantly in the gastrointestinal tract. In the indirect actions somatostatin have inhibitory effects on synthesis and secretion of growth factors, e.g. growth hormone and insulin-like growth factor 1 (Oberg et al., 2010, Van Op den Bosch et al., 2009).

Somatostatin acts with high affinity via five different subtypes of G-protein-coupled plasma membrane receptors, SSTR1-5, where SSTR2 is spliced in two, SSTR2A and SSTR2B. NET often overexpresses all SSTR subtypes.

Hormones and their receptors can be used in treatment of NETs as carrier and receivers of radionuclide-bound ligand, respectively. Due to the short biological half-life of native somatostatin (approx. 3 min), several somatostatin analogues have been developed (Grozinsky-Glasberg et al., 2008). The first commercially available somatostatin analogue for diagnostic use was octreotide, which is a synthetic and metabolic stable analogue (Krenning et al., 1989, Krenning et al., 1993, Lamberts et al., 1990).

Modified versions of octreotide are long-acting octreotide, which is used for symptom relief, and octreotate that is more suitable for PRRT of NETs (e.g. ^{177}Lu-octreotate) (Froidevaux et al., 2002, Reubi et al., 2000). Other radiolabelled analogues are for example pan-somatostatin analogues, which bind to all SSTR subtypes with high affinity. However, the level of internalization and the uptake by tumors seem to be low, and are thus not considered for therapeutic purposes (Ginj et al., 2008, Oberg et al., 2010).

Other hormones and their receptors

Somatostatin analogues are the most used analogue in PRRT today. Much effort has been spent on finding other overexpressed receptors on NETs since not all NETs overexpress SSTRs, but also because of heterogeneity according to low or high SSTR expression between and within

tumors in a patient. Alternative to somatostatin and its receptors are e.g. cholecystokinin-2/gastrin, with CCK_2/gastrin receptors expressed on MTC and catecholamines that are secreted by pheochromocytomas/paragangliomas.

Cholecystokinin-2/gastrin and CCK_2/gastrin receptor

Cholecystokinin (CCK) and gastrin are peptide hormones and act as neurotransmitters in the central nervous system and in the gastrointestinal tract. The receptors, CCK_1 and CCK_2 that CCK and gastrin acts through, belongs to the G-protein-coupled receptor family (Dupre et al., 2013). CCK and gastrin regulates, among others, the secretion of gastrointestinal acids and regulates absorption of nutrients. Minigastrin (MG) is an example of an analogue to CCK_2/gastrin.

Catecholamines

Catecholamines (CA) are for example the neurotransmitters epinephrine, norepinephrine, and dopamine (Purves, 2001), and act by activating G-protein-coupled receptors. Catecholamines are produced in chromaffin cells, the molecules contains a catechol ring and an amino group. An analogue to norepinephrine is meta-iodobenzylguanidine (MIBG).

2.2 Human NET models

Different NET models have been established. Those used today include BON, TT, HEK293, A431, KRJ-I, H69, GIST cells, GOT1 and GOT2 (Forssell-Aronsson et al., 2013). These cell studies are used as *in vitro* models or are transplanted on rat or mice as *in vivo* models. In this work four different models have been used, paraganglioma and GIST *in vitro* models, and GOT1 and GOT2 *in vivo* models.

2.2.1 Cell culture from human tumor tissue

Paraganglioma

Paragangliomas can be derived from chromaffin cells of the adrenal medulla and from sympathetic (thoracoadominal region) and parasympathetic (head-neck region) paraganglia cells. Malignant paragangliomas have often distant metastasis at the time of diagnosis and are hard to cure (Forssell-Aronsson et al., 2006, Forssell-Aronsson, 2011).

Paragangliomas secrete catecholamines (CA). The CA status in these tumors are of great importance in the treatment since the norepinephrine analogue MIBG labelled with radio-iodine can be used in the visualization and therapy of paragangliomas (Forssell-Aronsson et al., 2006, Forssell-Aronsson, 2011, Grogan et al., 2011, Kolby et al., 2003). Furthermore, some patients with paraganglioma are presented with high expression of SSTR, qualifying them for consideration for ^{177}Lu-octreotate treatment (Forssell-Aronsson, 2011, Grogan et al., 2011). To validate if PRRT treatment can be applied, individual uptake profiles for each patient needs to be validated.

Gastrointestinal stromal tumors, GIST

GIST is the most common mesenchymal tumor of the gastrointestinal tract but more often it arises from the stomach and the small intestine. The symptoms are non-specific and include, among others, gastrointestinal bleeding and fatigue from anemia. GIST that carries mutation in KIT and/or PDGFRA can successfully be treated with the tyrosine kinase inhibitor, imatinib. Unfortunately, both primary and secondary resistance to imatinib is seen in patients, 10-15 % and 50-70 %, respectively. Sunitinib follows imatinib in a second line therapy but a new resistance will be developed after some time and new treatment modalities are needed for these patients (Arne, 2012).

Some GISTs have neuroendocrine features with high expression of SSTR, which opens new treatment possibilities for imatinib resistant GIST patients (Palmieri et al., 2007). GIST with high expression of SSTR can be visualized by ^{111}In-octreotide scintigraphy, and if a higher uptake in GIST is verified than in normal tissues, treatment with ^{177}Lu-octreotate might be possible.

2.2.2 Transplantable tumor tissues

Tumor tissue can be transplanted to immune deficient mice, which gives the opportunity to study different aspects on tumor and non-tumor tissue *in vivo*. Tumor characteristics regarding biokinetics and biodistribution and radiosensitivity to radiopharmaceuticals, tumor growth, and therapeutic optimization strategies can be studied as well as toxicity profiles and tolerance doses to non-tumor tissue.

GOT2 model (medullary thyroid carcinoma)

The origin of medullary thyroid carcinoma (MTC) is the calcitonin producing parafollicular C-cells in the thyroid, which are neuroendocrine cells. MTC metastasizes early, both in the paratracheal and lateral cervical lymph nodes, but metastases can occur outside the neck, in the liver, bones, lungs, brain, and skin (Pacini et al., 2010). The response rate to treatment of MTC is dependent on the nature of the tumors, and if it is sporadic or hereditary. In general, the response rate to systemic chemotherapy is low, and pain relief can be achieved by external radiation therapy (Pacini et al., 2010).

The receptor expression of CCK/gastrin are often high in human MTC, these receptors (gastrin-like peptides) can therefore be used as targets in PRRT (Amiri-Mosavi et al., 1999, Behe et al., 2005, Behr et al., 1999, Behr et al., 1998, Laverman et al., 2011, Reubi et al., 1997, Reubi et al., 1996). In general, the SSTR expression is moderate in MTC, but with large individual differences depending on the nature of the MTC (Forssell-Aronsson et al., 2000). Higher expression of SSTR has been found in aggressive MTCs, which makes it possible to visualize MTC by ^{111}In-octreotide scintigraphy and also treat with ^{177}Lu-octreotate (Johanson et al., 2007).

GOT2 is a cell line that has its origin in MTC. The GOT2 tumor cells have been xenografted on nude mice and have well-preserved phenotypic properties, and a slow growth rate. RET mutation was found, and a low expression of somatostatin receptors (Johanson et al., 2007).

GOT1 model (midgut carcinoid)

Midgut carcinoid cells are derived from the enterochromaffin cells in the small intestine with the basis in the distal duodenum, right colon, jejunum and ileum (Vinik et al., 2010). The symptoms that classify the midgut carcinoid are that these tumors and its metastasis (primarily in the liver) produce serotonin, tachykinins, and other vasoactive substances (Strosberg et al., 2011), and that it has an overexpression of SSTR. The overexpression of SSTR makes it possible to treat these tumors with the radiolabeled SS analogue ^{177}Lu-octreotate.

GOT1 cells were obtained at surgery of a liver metastasis with the origin of a human midgut carcinoid and were developed into a cell line. GOT1-tumor cells can be transplantable on nude mice and have well-preserved NE differentiation, express SSTR1-5 (mainly SSTR2 and SSTR 5), and have a slow growth rate (doubling time of 2-3 weeks) (Kolby et al., 2001, Kolby et al., 2005, Nilsson et al., 2004). Several biodistribution and therapeutic studies using radiolabeled SS analogues, such as ^{177}Lu-octreotate has been performed on GOT-bearing nude mice (Bernhardt et al., 2007, Kolby et al., 2005, Oddstig et al., 2012).

2.3 Radiopharmaceuticals

^{111}In-octreotide is routinely used for scintigraphy of tumors overexpressing SSTR (Reubi et al., 2008). The first radiolabeled somatostatin analogue used for PRRT was ^{111}In-[DTPA^{0}]-octreotide due to the lack of SS analogues labeled with a more optimal radionuclide for therapy (Table 2.1) (Fjalling et al., 1996, Krenning et al., 1996). To improve the therapeutic effect, other β-emitting radionuclides were coupled to SS analogues, and to create a stable complex with other radionuclides DTPA was replaced by dodecanetetraacetic acid (DOTA). DOTA can form a more thermodynamic and kinetically stable metal complex with several β-emitting radionuclides, such as ^{111}In, ^{67}Ga, ^{68}Ga, ^{86}Y and ^{64}Cu for imaging and ^{90}Y and ^{177}Lu for PRRT (de Jong et al., 2002, Reubi et al., 2008). DOTA is the chelator routinely used today in the labeling process of forming stable complexes with ^{177}Lu and octreotate, se Figure 2.1.

Octreotate is a somatostatin analogue that differs from octreotide in C-terminal threoninol which is replaced with threonine, this gives a higher affinity to SSTR2 than [DTPA^{0}]-octreotide.

^{177}Lu is a radiolanthanide, which is produced in a reactor by fast neutron activation or thermal neutron activation (Lund nuclear data, (Breeman et al., 2003)) of ^{176}Yb (indirect) or ^{176}Lu (direct). ^{177}Lu emits β^{-}-particles, but also γ, and decays to the stable daughter of hafnium, ^{177}Hf (Eckerman et al., 2008). The range of the β^{-}-particles in tissues is between 0.5-2 mm, with a mean range of 0.67 mm (Breeman et al., 2001, de Jong et al., 2001, Forssell-Aronsson et al., 2013). ^{177}Lu suits well for radionuclide therapy since the β^{-}-particles have a relatively short range in tissue and are therefore a good option in the treatment of small metastases. The γ energy is in favor for gamma camera imaging, which make it possible to do biodistribution and dose assessments after treatment.

It is important to have as high specific activity as possible of ^{177}Lu-octreotate after labeling, to avoid possible SSTR saturation after administration of high amounts of ^{177}Lu-octreotate (Bernhardt et al., 2007).

Table 2.1 The physical properties of the radionuclides ^{177}Lu, ^{111}In and ^{131}I, including the physical half-life and daughter nuclide, decay mode, and energy emitted per nuclear transformation (nt). Data were retrieved from ICRP publication 107 (ICRP, 2008).

Radionuclide	Half-life Daughter nuclide	Decay mode	Emitted energy per nt (MeV)		
			Electron	Photon	Total
^{177}Lu	6.6 d ^{177}Hf	β^{-} 1.00	0.15	0.035	0.18
^{111}In	2.8 d ^{111m}Cd ^{111}Cd	EC 5.0E-5 1.0E-1	0.035	0.41	0.44
^{131}I	8.0 d ^{131m}Xe ^{131}Xe	β^{-} 1.2E-2 9.9E-1	0.19	0.38	0.57

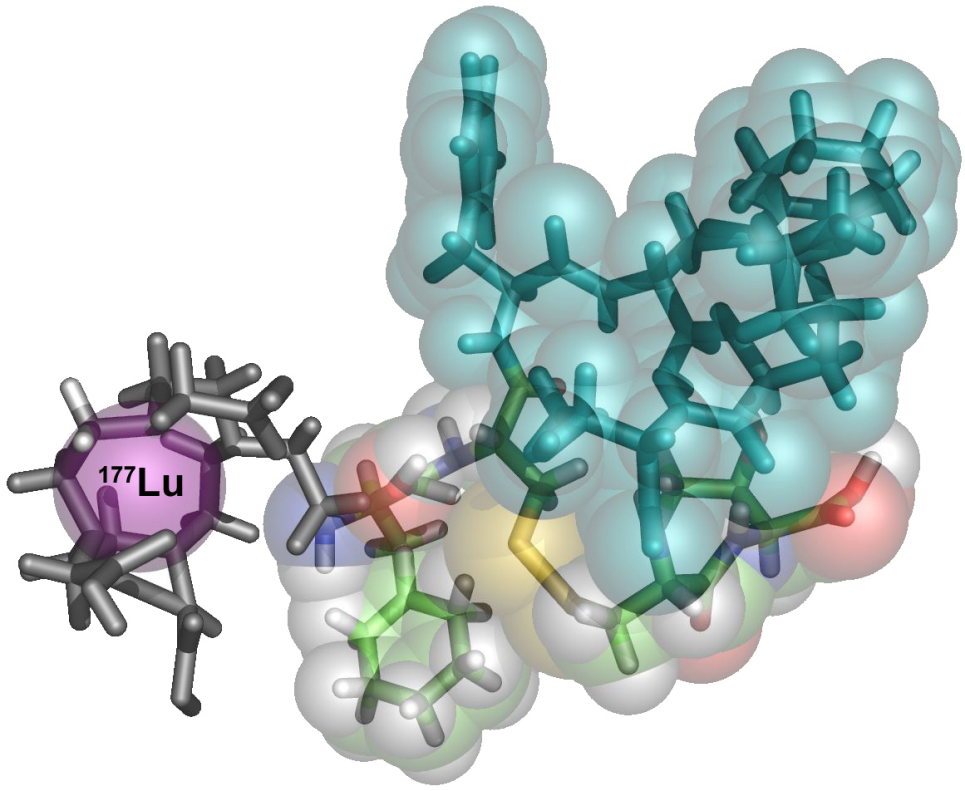

Figure 2.1 Illustration of the somatostatin analogue ^{177}Lu-[DOTA^{0}-Tyr^{3}]-octreotate showing a potential conformation built with 1YL8.pdb (Tyr^{3}-octreotate) and 1NC2.pdb (adapted DOTA) using PyMOL. The DOTA is colored with black and encloses ^{177}Lu (in lilac). In turquoise color is the part of octreotate that binds to the receptor.

DOTA-DGlu-$(Glu)_5$-minigastrin (DOTA-MG0), is an analogue that acts via the CCK_2/gastrin receptor, and has been investigated for NETs and specifically for MTC but also in GIST. DOTA-MG0 can be used in scintigraphy when labeled with ^{111}In (Froberg et al., 2009, Laverman et al., 2011, Laverman et al., 2008).

Catecholamine/guanidine analogue meta-iodobenzylguanidine (MIBG) has similar features as norepinephrine and can thereby be used for localizing tumors that has its origin in chromaffin cells (paragangliomas and pheochromocytomas), and that overexpress these receptors. (Blanchet et al., 2012, Jakobsen et al., 2001, Kolby et al., 2006, Kolby et al., 2003). Different iodine isotopes can be labelled to MIBG, most common is ^{123}I used in diagnostics and ^{131}I are used in therapy (and diagnostic) (Table 2.1). MIBG is primarily transported into the cells by the norepinephrine transporter on the cell membrane and into intracellular vesicles by vesicular monoamine transporters (VMATs) and organic cation transporters (OCTs) (Kolby et al., 2003).

2.3.1 Internalization of ^{177}Lu

^{177}Lu-octreotate acts as an agonist when it reaches SSTR, in general SSTR2 and 5, at the cell membrane, the agonist is phosphorylated by G-protein-coupled-receptor- kinases, Figure 2.2**.** β-arrestin forms a stable complex to the receptor and are internalized together through a clathrin-dependent mediated pathway into the endocytotic vesicles. The receptors are recycled by recycling endosomes (Jacobs et al., 2008). ^{177}Lu-octreotate are probably degraded in lysosomes and ^{177}Lu might be accumulated in the cytoplasm or in the nucleus, which was shown to be the case for ^{111}In studied in midgut carcinoid cell culture incubated with ^{111}In-octreotide (Andersson et al., 1996).

The uptake of the cell (both healthy and tumor tissue) will be dependent of all three parts of the molecule; the radionuclide, the chelator and the analogue since the receptors change and act as agonists or antagonists depending on the molecule that they receive. The internalization process can be stopped or changed depending on these three parts.

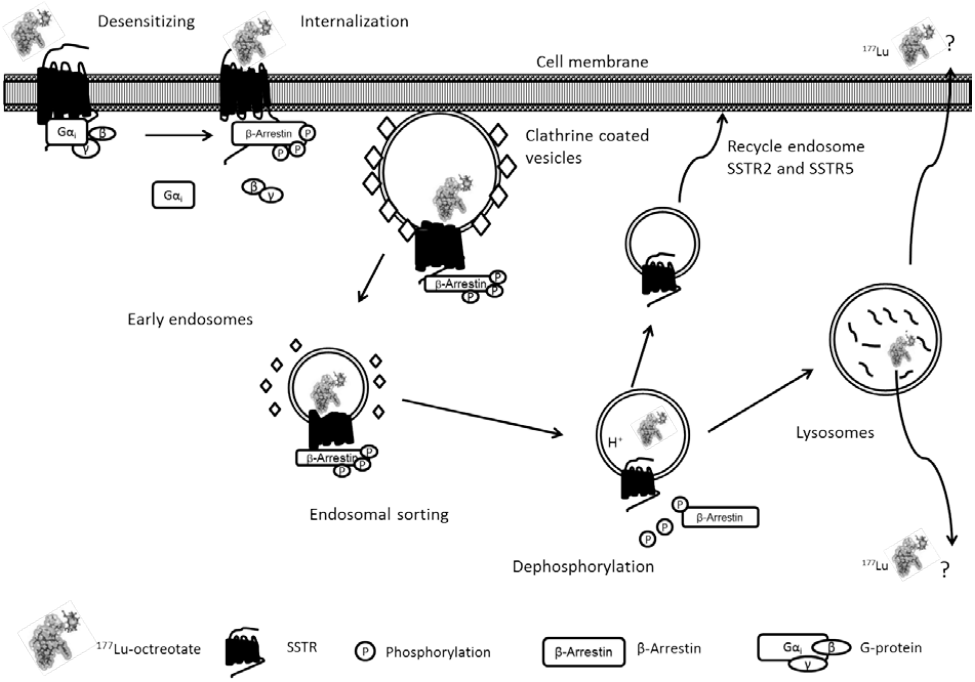

Figure 2.2 A basic model of the recycling of somatostatin receptors and internalization of ^{177}Lu-octreotate. After binding of ^{177}Lu-octreotate the G-proteins are desensitized. Induction of phosphorylation and recruitment of β–arrestin start short before internalization via endocytosis of clathrine coated vesicles which results in intracellular vesicles, so called endosomes. The ligand will dissociate from the receptor which are recycled back to the plasma membrane (degradation without recycling of receptors can also occur, especially for SSTR3). The ^{177}Lu-octreotate are transported to the lysosomes for processing, and thereafter probably transported to the cytoplasm, the nucleus or out through the cell membrane (Breeman et al., 2001, Jacobs et al., 2008, Wangberg et al., 1997). Redrawn after inspiration from Jacobs et al 2008 (Jacobs et al., 2008).

2.4 Optimization of ^{177}Lu-octreotate treatment

The main goal with peptide receptor radionuclide therapy, PRRT, is to deliver as high absorbed dose to tumor as possible while avoiding side effects on non-tumor tissues (de Visser et al., 2008, Forssell-Aronsson et al., 2013). In the treatment protocols that are routinely used in the clinics, the kidneys, which are one of the dose limiting organs, are blocked with amino acids (often lysine and arginine). These amino acids reduce the relative retention of ^{177}Lu-octreotate in the kidneys, with the purpose to be able to administer higher activity amounts (de Jong et al., 2002, Rolleman et al., 2003). The administration is also fractionated so that normal tissues have time for recovery. These arrangements seem not to be enough since only a few percent of the patient are presented with complete remission (Bodei et al., 2011, Kwekkeboom et al., 2008), while the tumors in animal models can go to complete remission in almost all animals without obvious side effects (Forssell-Aronsson et al., 2013, Kolby et al., 2005, Schmitt et al., 2004).

Optimization strategies of therapy with radiolabeled SS analogues have been purposed (Forssell-Aronsson et al., 2013). Individualized treatment for each patient, method to increase the treatment effect on tumor tissues, and methods to reduce non-tumor tissue toxicity (especially renal toxicity) is issues that need to be considered.

2.4.1 Up-regulation of SSTR

Radiation can induce up-regulation of SSTR on the cell membrane (Bernhardt et al., 2007, Oddstig et al., 2006, 2011). The therapeutic effect might thereby be increased if a small amount of ^{177}Lu-octreotate (priming) is administered before the main therapeutic administration of ^{177}Lu-octreotate, with the task to up-regulate the receptors and thereby increase the possibility to a higher uptake of ^{177}Lu-octreotate of the main administration, without increasing the total amount of activity.

2.4.2 Kidney toxicity

The kidneys are together with the bone marrow the dose limiting organs in PRRT using radiolabeled SS analogues (Forrer et al., 2009). The tolerance doses for the kidneys after ^{177}Lu-octreotate therapy is not known, and the tolerance doses derived for uniform irradiation by external-beam radiotherapy are used, with $TD_{5/5}$ and $TD_{50/5,}$ of 23 Gy and 28 Gy, respectively (Emami et al., 1991, Wessels et al., 2008). However, irradiation properties of these two therapy modalities are very different. In PRRT, for example, there is a much lower and varying dose rate with time, continuous irradiation, and usually inhomogeneous dose distribution. Therefore, tolerance doses for PRRT should be defined (Lambert et al., 2004).

^{177}Lu-octreotate is mainly excreted via the urine. After injection with ^{177}Lu-octreotate, the highest activity concentration in the kidneys is found in the cortex region (Melis et al., 2007). Small radiolabelled peptides are filtered through the glomerular and are thereafter reabsorbed or retained in the proximal tubular cells, most probably via megalin/cubulin receptor-mediated endocytosis and ligand-specific receptors (e.g. SSTR), pinocytosis, amino acid transporters, and passive diffusion (Akizawa et al., 2008, de Jong et al., 2005, Trejtnar et al., 2008, Vegt et al., 2010).

The biodistribution of ^{177}Lu-octreotate is very different in different patients, and the absorbed dose to kidneys per administered activity can differ by a factor of up to 8 between patients (Larsson et al., 2012).

In general, radiation injury on the kidneys includes both short-term and long-term effects on kidney function. Impairment of the kidney function is routinely estimated by measurement of

glomerulus filtration rate (GFR), e.g. using ^{99m}Tc-DTPA scintigraphy (Hauser et al., 1970), but can also be estimated by changes in serum creatinine (eGFR) (National Kidney Foundation, 2002). However, the correlation between radiation induced kidney injury after ^{177}Lu-octreotate treatment and serum creatinine levels seems not to be high (Bodei et al., 2011). Reason for that might be that both the glomerulus and the proximal tubule are injured, which than cause a more severe injury than demonstrated by reduction in GFR alone (Bodei et al., 2011, Gupta et al., 2012, Svensson et al., 2012).

Reduced GFR is an effect that appear late after irradiation, and serum creatinine levels depend also on other factors than kidney function, and cannot be used as biomarkers for late kidney toxicity during ongoing therapy. There is thus a need for methods/biomarkers to detect renal impairment early, especially for tubular injury. Biomarkers that indicate for acute or late/chronic radiation induced renal injury at an earlier time-point than the methods used today can enable a more individualized treatment with ^{177}Lu-octreotate. If a radiation induced renal impairment could be detected by a biomarker, then patients with less radiation sensitive kidneys could receive higher absorbed dose to kidneys than those with higher sensitive ones, which would give higher absorbed dose to tumor and thus higher therapeutic effect.

Retinol binding protein (RBP) is a plasma protein that passes freely through the glomerulus and is nearly completely reabsorbed in the proximal tubule cells via the megalin/cubulin receptor complex where it is catabolized (Frey et al., 2008, Vaidya et al., 2008). When the functions in the proximal tubule are impaired, the protein will be excreted in the urine. The possibility to use urinary RBP (RBP4 is used when analyzing mouse urine) as a sensitive biomarker of impairment of the reabsorption of proximal tubular cells seems therefore promising (Bernard et al., 1982, Trof et al., 2006). Another potential indicator of GFR reduction is valine hydantoin (VH) level in blood (erythrocytes). VH has been proposed as a good indicator of the uremic status (serum urea level) in patients with acute and chronic renal failure, since VH is a stable product and an adduct bound to hemoglobin (Hb) (Davenport et al., 1996, Smith et al., 1988, Wynckel et al., 2000), and might also be a biomarker for radiation induced renal injury.

3 Aims

When studying the use of radiolabeled hormones and hormone analogues for diagnosis and therapy of NE tumors, realistic tumor models are valuable. Human NE cell lines and models are few and there is a need to find suitable models for different types of NE tumors, with, e.g., relevant expression of hormone receptors. There is also a need to study the possibility to use radiolabeled hormone analogues for treatment of other types of NE tumors beside those treated today (mainly carcinoids and endocrine pancreatic tumors).

There is a need to optimize radionuclide therapy. One way is to increase the uptake and absorbed dose to the tumor tissue, e.g. by increasing the SSTR expression before administration of therapeutic amount of ^{177}Lu-octreotate. Another way is to give as high amount of ^{177}Lu-octreotate as possible without exceeding risk for side effects, primarily on kidneys. This requires individual follow-up using appropriate biomarkers of nephrotoxicity during fractionated treatment with ^{177}Lu-octreotate.

The specific aims of this work were to study

- the biodistribution of ^{177}Lu-octreotate and ^{111}In-DOTA-MG0 in a GOT2 (human MTC) animal model (paper I)
- the tumor uptake of ^{111}In-octreotide and ^{131}I-MIBG in a patient with paraganglioma, and to study binding and internalization characteristics of ^{111}In-octreotide and ^{131}I-MIBG in primary culture of collected paraganglioma cells (paper II)
- the tumor uptake of ^{111}In-octreotide in patients with GIST, and to study binding and internalization characteristics of ^{177}Lu-octreotate in primary culture of GIST cells (paper III)
- the therapeutic effects on GOT1 (human midgut carcinoid) animal model after a priming administration 24 h before the main administration of ^{177}Lu-octreotate, compared with a single administration of ^{177}Lu-octreotate (paper IV)
- the potential of using urinary retinol binding protein 4 (RBP4) and carbamoylated hemoglobin (Hb) measured as valine hydantoin (VH) in blood as biomarkers of nephrotoxic effects on adult mice after ^{177}Lu-octreotate treatment (paper V)

4 Material and Methods

4.1 Radiopharmaceuticals and chemicals

$^{177}LuCl_3$ and [$DOTA^0$, Tyr^3]-octreotate was purchased from the Nuclear Research & consultancy Group (IDB Holland, the Netherlands or Tyco Healthcare, Mallinckrodt, St Louis, MO, USA).

^{111}In, $^{111}InCl_3$, and [DTPA-D-Phe^1]–octreotide was purchased from the Covidien, Mallinckrodt Medical B.V. (Covidien, Mallinckrodt Medical B.V., Petten, The Netherlands).

DOTA-MG0 was obtained from the Department of Nuclear Medicine at Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands. Human minigastrin (MG) was purchased from Sigma-Aldrich Sweden AB (Sigma-Aldrich Sweden AB, Sweden).

^{177}Lu-octreotate and ^{111}In-octreotide (OctreoScan®) were produced according to the instructions of the manufacturers. ^{111}In-DOTA-MG0 was produced according to paper I.

The quality control of ^{177}Lu-octreotate (26 MBq/µg), ^{111}In-octreotide, and ^{111}In-DOTA-MG0 was performed by instant thin layer chromatography (ITLC™ SG, Pall Life Sciences, PALL Corporation, USA or Gelman Instrument Company, Ann Arbor, MI, USA). The mobile phase was 0.1 M sodium citrate (VWR International AB, Sweden). The fraction of peptide-bound ^{177}Lu and ^{111}In, was 98-99 %, 99 %, and 95 %, respectively.

^{131}I-labeled CA/guanidine analogue meta-iodobenzylguanidine (MIBG) was purchased from GE Healthcare Buchler GmbH & Co. KG, Braunschweig, Germany.

Octreotide (Sandostatin®) was obtained from Novartis (Basel, Switzerland). Reserpine and clomipramine were purchased from Sigma-Aldrich (St. Louis, USA).

4.2 Detectors and radioactivity measurements

4.2.1 Ionization chamber

A direct reading well-type ionization chamber was used for activity measurements (CRC-15R, Capintec, USA) during the labelling process and activity measurements of syringe. The ionization chamber filling gas is argon (high pressure) and the chamber wall is made of aluminum, the measuring rage is up to 200 GBq (of ^{99m}Tc) (Capintec, 2004).

4.2.2 Gamma counter

Wallac 1480 gamma counter (WIZARD™ 3", Wallac Oy, Finland), equipped with a single crystal of thallium-activated sodium iodine, NaI(Tl), detector, with 80 mm x 75 mm crystal size (Aalto, 1997). A 20 % broad energy window over the photon peak for respective radionuclide (over the 208 keV ^{177}Lu photon peak, 250 keV ^{111}In photon peak and 360 keV ^{131}I photon peak) was used to measure the activity in the samples. The efficiency in the gamma counter was calibrated against the efficiency of a well-type ionization chamber. Correction was determined and used for detector background, dead time loss of less than 3 %, spill over (both from the ^{131}I photon peak into the ^{111}In energy window, and from the ^{111}In coincidence peak, at 420 keV, into the ^{131}I energy window) and for radioactive decay.

4.2.3 Gamma camera

Scintigraphy was performed to visualize the uptake of ^{111}In-octreotide in patients with GIST (paper III) on a gamma camera (General Electric 400 AC/T, General Electric, London, UK) with a mean energy parallel hole collimator. Static anterior and posterior images from the base of the scull to the pelvis were acquired for six GIST patients (Paper III) using a 20 % broad energy window.

4.3 Studies on patients with NET

4.3.1 Paraganglioma (paper II)

Tumor samples were collected from a 58-year-old woman with metastatic paragangliomas (liver and bone metastases). Prior to surgery the patient was i.v. injected with 37 MBq ^{131}I-MIBG and 120 MBq ^{111}In-octreotide, 3 h and 27 h before surgical removal of the primary tumor, respectively. Tumor, muscle and blood samples were collected at surgery and the ^{131}I and ^{111}In activities were determined by the gamma counter.

Informed consent was given by the patient and approval was obtained from the Regional Ethical Review Board in Gothenburg, Sweden.

4.3.2 GIST (paper III)

Tumor biopsies were collected from 34 patients with GIST during surgery 1997–2008. Seven of these patients were i.v. injected with 170-240 MBq ^{111}In-octreotide prior to surgery. Six of them underwent scintigraphy, and in five of them tumor and blood samples were collected at surgery, and the ^{111}In activity was determined in each tissue sample using the gamma counter.

Informed consent was given by the patients and approval was obtained from the Regional Ethical Review Board in Gothenburg, Sweden.

4.3.3 GOT2 (paper I) and GOT1 (paper IV)

The tumor types used, GOT2 and GOT1, are xenografted medullary thyroid carcinoma and human midgut carcinoid, respectively (see section 2.2.2 for more information about the models). The tumor tissue was established as previously described, cf. Johanson et al and Kolby et al (Johanson et al., 2007, Kolby et al., 2001).

4.4 Studies in cell cultures (paper II and III)

Paraganglioma cells were grown as described in paper II. Paraganglioma cells were incubated with 10 nM and 1.5 kBq per well ^{131}I-MIBG, or with 10 nM and 20 kBq or 2 nM and 5 kBq, a high or low concentration, of ^{111}In-octreotide, respectively, for 4, 24, or 46 h. The control groups vs. ^{111}In-octreotide were simultaneously incubated with an excess of unlabeled octreotide. Blocking of ^{131}I-MIBG binding/uptake at the granule membrane or at the plasma membrane on the paraganglioma cells, were studied by adding 10 µM reserpine or 10 µM clomipramine, respectively, to the cells 30 min before incubation with ^{131}I-MIBG.

The GIST cells were grown on culture plates as described in paper III. GIST cells were incubated with approximately 8-9 kBq ^{177}Lu-octreotate (corresponding to 10 nM octreotate) for 4, 24, or 48 h. Control groups were simultaneously incubated with an excess of 5 µM octreotide (Sandostatin; Novartis, Basel, Switzerland).

The amount of membrane bound, internalized, and unbound radionuclide was determined with the gamma counter.

4.5 Animal studies

BALB/c female nude mice (Charles River, Japan and Germany) were used in paper I, IV and V.

GOT2 and GOT1 tumor tissue (paper I and IV) was subcutaneously xenotransplanted in the neck of 4 weeks old female nude mice. The transplantation was performed as described in paper I and IV. A wide range of tumor sizes were used (30-1600 mm^3). However, the tumor size distribution within the groups was similar.

Adult (6 month old) non-tumor bearing mice were used in paper V.

Water and autoclaved food were available *ad libitum*.

The animal studies were approved by the Ethics committee for Animal Research at University of Gothenburg, Göteborg, Sweden.

4.5.1 Biodistribution of ^{111}In-MG0 or ^{177}Lu-octreotate in GOT2-bearing mice (paper I)

GOT2 tumor-bearing mice were i.v. injected with 5, 10, or 30 MBq ^{177}Lu-octreotate, or with 22 kBq ^{111}In-DOTA-MG0 (^{111}In-MG0). Control animals were simultaneously injected with 22 kBq ^{111}In-MG0 and a 100-fold molar excess of unlabeled human minigastrin, MG.

Animals injected with 5 and 10 MBq ^{177}Lu-octreotate were killed at 24, 72, or 168 h after injection. Animals injected with 30 MBq were only sacrificed 24 h after injection. The animals receiving ^{111}In-MG0 were killed at 1 h after injection by cardiac puncture under anesthesia (2.5 % Avertin, Sigma-Aldrich, Sweden AB). Samples of blood, adrenals, heart, liver, pancreas, spleen, kidneys, tumor, and muscle from femur were excised. All tissue samples were weighed and the ^{177}Lu or ^{111}In activity in each sample was measured by a gamma counter.

4.5.2 Biodistribution of ^{177}Lu-octreotate in GOT1-bearing mice (paper IV)

GOT1 tumor-bearing mice were administered with a priming activity of 5 MBq followed by a main administration of 10 MBq ^{177}Lu-octreotate 24 h later, or with a single injection of 15 MBq ^{177}Lu-octreotate.

Animals were killed 24, 72, or 168 h after the last injection by cardiac puncture under anesthesia with 2.5 % Avertin (Sigma-Aldrich Sweden AB). Samples of blood, and adrenals, liver, kidney, spleen, pancreas, brain and lung, were excised. All tissue samples were weighed and the ^{177}Lu activity in each sample was measured by a gamma counter.

The tumor tissue was excised and divided into two pieces, one was instantly frozen in liquid nitrogen for qPCR and global gene analysis, one part was weighted and fixed in 4 % paraformaldehyde for activity measurements and histological evaluation.

4.5.3 Therapeutic effect of GOT1-bearing animals (paper IV)

The therapeutic effect was studied (in two settings) on GOT1 tumor-bearing nude mice using priming activities of ^{177}Lu-octreotate. A priming activity of 5 or 10 MBq ^{177}Lu-octreotate was i.v. injected in study 1, followed by the main administration, 24 h later. All mice received a total

amount of 15 MBq ^{177}Lu-octreotate, while in study 2 the priming activities were 0.5, 2.5, 5, or 10 MBq ^{177}Lu-octreotate. Control groups received 15 MBq or 30 MBq as a single administration. The tumor size in the treatment groups was measured twice a week with a digital slide caliper until sacrifice 6 weeks after the first injection. A non-treated group of tumor bearing mice served as control, and tumor sizes was measured once a week during 7 weeks.

At sacrifice, tumor tissue was excised and divided into two pieces, one was instantly frozen in liquid nitrogen for global gene expression analysis and one part was weighted and fixed in 4 % paraformaldehyde for activity measurements and histological evaluation.

4.5.4 Study on renal toxicity from ^{177}Lu-octreotate in mice (paper V)

Adult nude mice were injected with saline solution (the control group), or with 60 MBq, or 120 MBq of ^{177}Lu-octreotate (n=6/group). Urine were collected at different time-points during 90 days (before injection, used as baseline value, and subsequently 14, 30, 60 and 90 days after injection), and frozen in -20°C until analysis of RBP4 and creatinine. Blood samples were taken after 90 days analyzing valine hydantoin in erythrocytes. Kidneys were fixed in 4 % paraformaldehyde for histological evaluation.

4.6 Dosimetry

The fraction of the injected activity in the sample per unit mass (tissue or cells or medium) was calculated according to

$$C_{tissue} = \frac{A_{tissue}/M_{tissue}}{IA} \cdot 100\ [\%\mathrm{IA}/g],$$

where A_{tissue} is the activity in the sample, corrected for radioactivity decay to the time of injection, M_{tissue} is the mass of the sample and IA is the injected activity.

In paper IV, the C_{tissue} could not be determined alone for the 5 MBq and the 10 MBq, respectively, and was therefore calculated as described in paper IV.

The mean tumor-to-normal-tissue activity concentration ratio (T/N), at the time of injection was calculated as

$$T/N = \frac{C_{tumor}}{C_{tissue}}.$$

The mean absorbed dose to the tissue was calculated according to Medical Internal Radiation Dose Committee (MIRD) pamphlet 21 formalism (Bolch et al., 2009).

$$D(r_T, T_D) = \frac{\tilde{A}(r_S, T_D)\sum_i E_i Y_i \phi(r_T \leftarrow r_S, E_i, T_D)}{M(r_T, T_D)}\ [Gy],$$

where Ã is the area under the curve from C_{tissue} calculations at different time-points using $\tilde{A} = \int_0^{T_D} C_{tissue}(r_s, t)\ dt\ [Bq/s]$ or the trumpets method. At t=0, C_{tissue} was assumed to be the same as after t=24 h. The mean energy emitted per nuclear transformations, $\sum_i E_i Y_i$, was approximated for ^{177}Lu to 147.9 keV/decay (Eckerman et al., 2008), including β^- particles, Auger and conversion electrons. The absorbed fraction, $\phi(r_T \leftarrow r_S, E_i, T_D)$ was set to 1 for all tumors and organs, and, $M(r_T, T_D)$, is the mass of the tissue. The mean absorbed dose after

administration of a priming activity followed by the main activity was calculated as described in paper IV.

4.7 Methods of analyses

4.7.1 Histological and morphological evaluation

Histological examinations on tumor tissues and kidney tissues were performed in paper IV and V, respectively. Tissues were put into 4 % formaldehyde in PBS after sacrifice, and then embedded in paraffin wax. Parallel sections of 2-4 μm thick slices were stained with hematoxylin-eosin of both tumor (paper IV) and kidney (paper V) tissues for histological evaluation. Experienced pathologists made the evaluation and grading.

4.7.2 SSTR expression analyses

Expression levels of SSTR1-5 in GIST tumors from patients and GOT1 tumors in mice were analyzed by quantitative real-time polymerase chain reaction (qPCR) (paper III and IV).

TaqMan® assays (Applied Biosystems, CA, USA) with specific probes for SSTR1-5 described in paper III and IV, were used in the qPCR analysis. Triplicate analyses of each sample were performed according to the manufacturer's instructions. The mRNA expression values of SSTR1-5 were determined relative to the housekeeping gene (ACTB was used as a housekeeping gene in paper III and TUBB and GAPDH were used in paper IV).

4.7.3 Analysis of renal toxicity (paper V)

RBP4 and creatinine

RBP4 and creatinine was analyzed from urine samples at different time-points (baseline, start, and at 14-90 days) with mouse RBP4 ELISA kit (R&D Systems Europe Ltd., Abingdon, UK) and Creatinine kit (R&D Systems Europe Ltd., Abingdon, UK), respectively, according to the manufacturer's instructions. Repeated analysis of the kit's control samples were performed for both RBP4 and creatinine, both analyses showed good reproducibility.

VH in erythrocytes

Erythrocytes were separated from whole blood samples collected 90 days after injection. Its globin was isolated through precipitation with ethyl acetate after the cell residues was removed. Valine hydantoin (VH), in the globin samples were analyzed with HPLC-MS/MS (Davies et al., 2010, Kwan et al., 1990), and phenylvaline hydantoin (PVH) was used as a volumetric standard. Triplicate analysis of the samples were performed and showed good reproducibility.

5 Results

5.1 Studies on human NE tumor types

5.1.1 Uptake, binding and internalization of ^{111}In-octreotide and ^{131}I-MIBG in paraganglioma (paper II)

Tumor-to-blood activity concentration ratio (T/B), for tumor tissue from the patient with paraganglioma was 590 at 27 h after injection of ^{111}In-octreotide, and 180 three hours after injection of ^{131}I-MIBG.

Figure 5.1 demonstrates the fraction of membrane bound and internalized ^{111}In and ^{131}I in paraganglioma cell cultures after 4, 24 and 46 h of incubation. The specific membrane bound and the internalized amount of ^{131}I increased with time in paraganglioma cells. Clomipramine and reserpine reduced membrane bound and the internalized amount of ^{131}I by 60-70 % at 46 h after incubation.

The fraction of membrane bound ^{111}In after incubation with high amount of ^{111}In-octreotide was higher than with low amount, but the internalization of ^{111}In was lower for high amount than for low amount. A 60 % reduction of membrane bound and internalized radiopharmaceutical were seen after 46 h in cells given a high amount of ^{111}In-octreotide with excess of unlabeled octreotide, while no difference was found with or without excess of octreotide after a low amount of ^{111}In-octreotide.

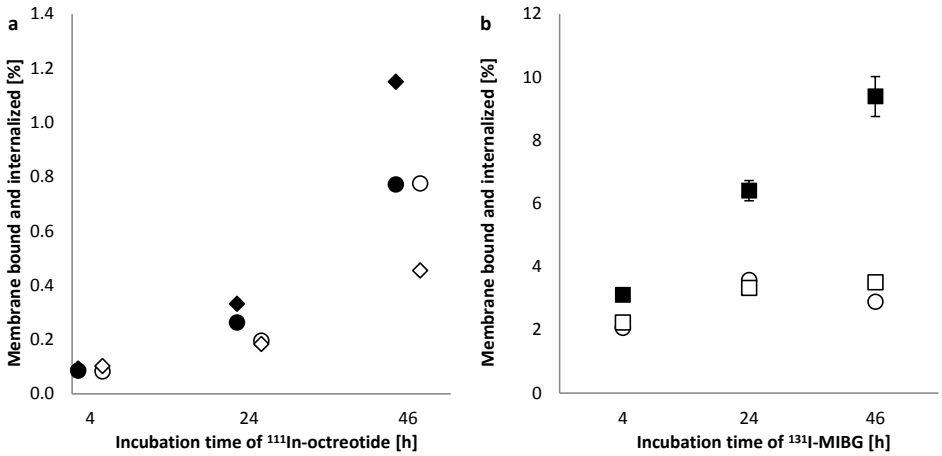

Figure 5.1 The fraction of membrane bound and internalized ^{111}In or ^{131}I in paraganglioma cells, measured after 4, 24, and 46 h of incubation with a) ^{111}In-octreotide, circles and rhombs indicate the uptake of the low and high concentration of ^{111}In-octreotide, respectively; filled and empty symbols represent experiments without or with excess of octreotide, respectively, and with b) ^{131}I-MIBG, visualized by filled squares, and empty squares and dots represent cells incubated with excess of clomipramine or reserpine, respectively. Error bars indicate SEM (can be smaller than symbols).

5.1.2 Visualization, uptake, binding and internalization in GIST (paper III)

Table 5.1 shows a summary of the results received from GIST patients that were injected with ^{111}In-octreotid for scintigraphy. Biopsies were taken from patients, that underwent surgery (2-22 days after scintigraphy), and the T/B ratios were 8.0-19 for 4 patients, and a relatively high value of 96 for one patient at a late time-point after scintigraphy (13 days).

The expression profile of SSTR1-5 was analyzed with qPCR, and demonstrated low expression of SSTR 2-5 and high expression of SSTR1 in GIST in some patients (Table 5.1).

GIST cells in primary culture from patient 25 with the T/B value of 96 were incubated with ^{177}Lu-octreotate, and the fraction of membrane bound and internalized ^{177}Lu increased with time of incubation, Figure 5.2. The specific membrane bound and the internalized amount of ^{177}Lu increased with time. The reduction of membrane bound and internalized ^{177}Lu with an excess of octreotide was 96 % after 48 h. Similar results were also obtained for GIST cells from patients 15.

Table 5.1 A summary of the results in GIST patients related to ^{111}In-octreotide: tumor visualization at scintigraphy with ^{111}In-octreotide, tumor-to-blood ^{111}In activity concentration ratios (T/B) for biopsies collected at surgery, and expression profiles of SSTR1-5 in GIST tissue analyzed by qPCR.

Patient	Scintigraphy ^{111}In-octreotide	T/B	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
8 P	no tumor visualized	14	110	0.66	-	-	-
15	n.a.	-	110	0.38	-	-	-
19 P	no tumor visualized	19	0.27	0.10	-	-	-
25 R	high tumor uptake	96	440	0.60	-	0.01	0.01
29 P	high tumor uptake	12	0.13	0.38	-	0.01	-
31 P	no tumor visualized	n.a.	240	0.18	-	-	-
32 P	n.a.	8.0	400	0.16	-	12	-
34 P	high tumor uptake	n.a.	0.04	0.08	-	0.03	-

P, primary tumor analyzed; R, recurrent tumor analyzed; -, not detectable; tumor samples from patient 15 and 25 were analyzed in cell culture.

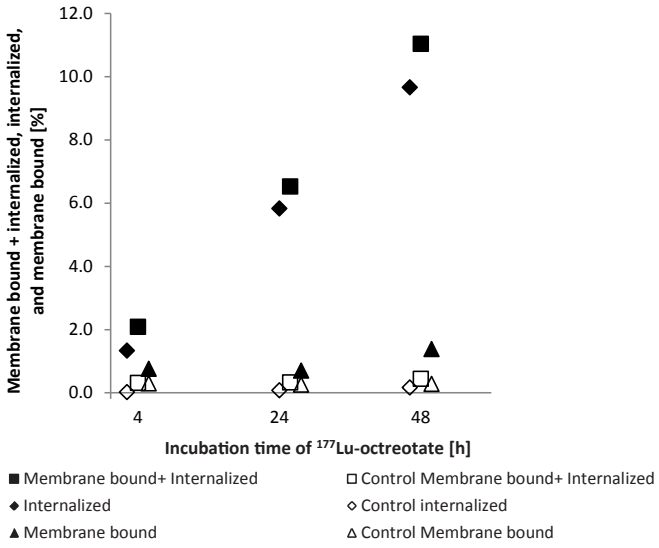

Figure 5.2 The fraction of membrane bound+internalized, internalized, and membrane bound ^{177}Lu in GIST cells from patient no. 25, incubated with ^{177}Lu-octreotate for 4 h (n=4), 24 h (n=4), and 48 h (n=8). Filled symbols represent data from cells only incubated with ^{177}Lu-octreotate and open symbols represents cells which also incubated with excess of octreotide. Error bars represent SEM, but are smaller than symbols.

5.2 Animal studies

5.2.1 Biodistribution of ^{111}In-MG0 and ^{177}Lu-octreotate in GOT2-bearing mice (paper I)

GOT2 tumor-bearing nude mice were i.v. injected with ^{177}Lu-octreotate or with ^{111}In-MG0. Table 5.2 shows the ^{111}In and ^{177}Lu activity concentration in tissues collected. Uptake of ^{177}Lu in SSTR expressing organs (tumor, adrenals and pancreas) were reduced with higher amounts of ^{177}Lu-octreotate administered.

T/B ratios increased from 25 to 50 from 1d to 7d after injection for both 5 and 10 MBq. T/B ratio at 1 h after ^{111}In-MG0 administration was ca 3.

Mean absorbed dose to GOT2 tumors per administered activity was 0.025 Gy/MBq and 0.013 Gy/MBq after administration 5 and 10 MBq of ^{177}Lu-octreotate, respectively. Higher mean absorbed doses per administered activity were also seen in almost all normal tissues after administration of 5 MBq compared to the 10 MBq ^{177}Lu-octreotate. SSTR expressing organs (adrenals and pancreas) and the kidneys received higher mean absorbed dose per administered activity than the GOT2 tumors, Figure 5.4.

Table 5.2 ^{177}Lu and ^{111}In activity concentration in tissues collected from GOT2-bearing nude mice previously injected with ^{177}Lu-octreotate, ^{111}In-MG0 or with ^{111}In-MG0+excess of MG. Data are corrected for physical decay and values are given as mean (SEM).

	Activity concentration (SEM) [%IA/g]				
Radiopharmaceutical	^{177}Lu-octreotate			^{111}In-MG0	
Time after administration	24 h			1 h	
Injected activity	5 MBq	10 MBq	30 MBq	22 kBq	22 kBq + excess MG
Tissue					
Adrenals	0.87 (0.17)	0.72 (0.10)	0.0030 (0.0107)	0.42 (0.13)	0.22 (0.02)
Blood	0.020 (0.006)	0.0099 (0.0023)	0.00011 (0.00187)	0.32 (0.05)	0.28 (0.03)
Heart	0.054 (0.004)	0.036 (0.001)	0.027 (0.003)	0.15 (0.03)	0.13 (0.01)
Kidneys	5.0 (0.1)	6.2 (0.3)	0.054 (0.480)	71 (11)	79 (4)
Liver	0.14 (0.01)	0.12 (0.00)	0.0013 (0.0141)	0.24 (0.03)	0.16 (0.01)
Muscle	0.012 (0.002)	0.023 (0.013)	0.01 (0.01)	2.3 (0.8)	0.88 (0.41)
Pancreas	2.0 (0.4)	0.77 (0.12)	0.0048 (0.0765)	-	-
Spleen	0.12 (0.01)	0.092 (0.006)	0.0011 (0.0183)	0.17 (0.03)	0.10 (0.01)
Tumor	0.37 (0.01)	0.23 (0.02)	0.0013 (0.0096)	0.79 (0.19)	0.32 (0.08)

5.2.2 Optimization of therapy with ^{177}Lu-octreotate

Therapeutic effect in GOT1-bearing animals (paper IV)

The therapeutic effect on tumors in GOT1 bearing nude mice after a priming activity 24 h before the main administration of ^{177}Lu-octreotate and single administration is shown in Figure 5.3. Best therapeutic effect was seen after administrations of 5+10 MBq (both studies) and 2.5+12.5 MBq, and statistically significance was obtained between the 15 MBq group and 5+10 MBq in study 1, and 2.5+12.5 MBq group.

High mean absorbed doses to the tumor was found in the 5+10 MBq group (0.36 Gy/MBq), compared with that in the 15 MBq group (0.20 Gy/MBq), values estimated from biodistribution data, Figure 5.4.

No differences were found in the SSTR1-5 gene expression (mRNA) between the administration schedules.

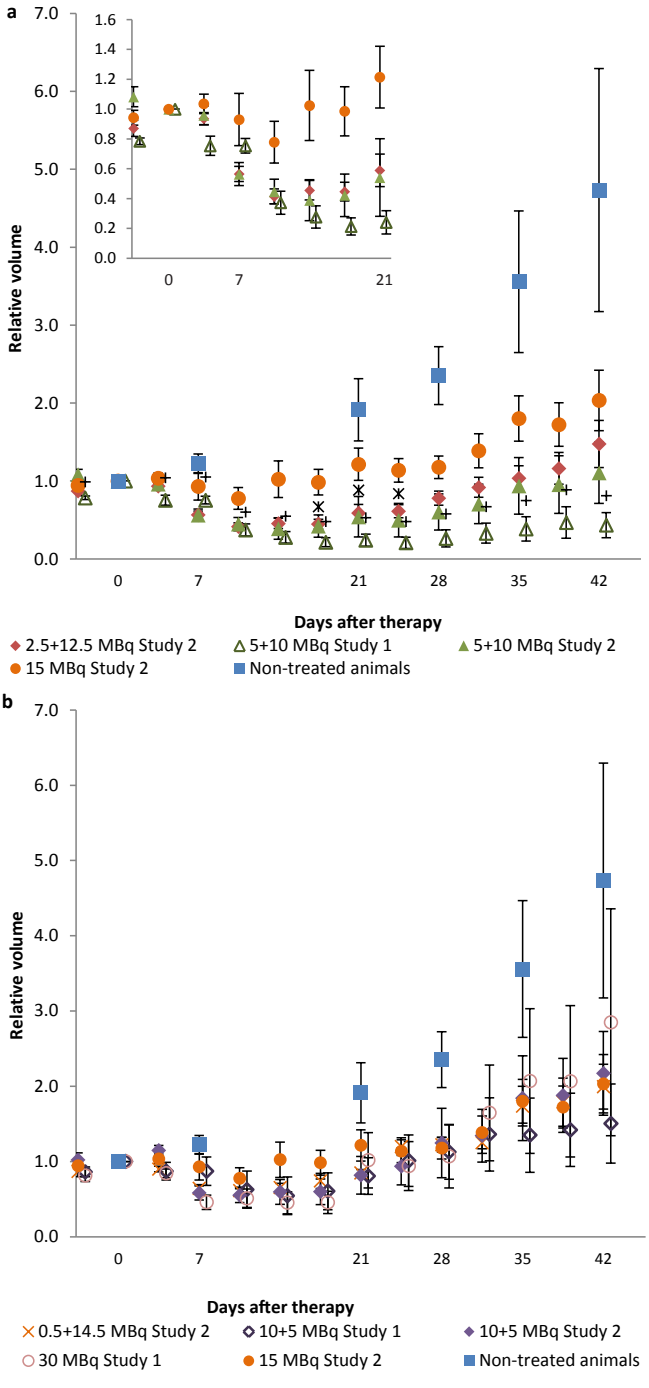

Figure 5.3 The therapeutic effect (paper IV) after administration of different priming activities given 24 h before main administration compared to single injection of ^{177}Lu-octreotate in GOT1-bearing mice.
a) Mean relative tumor volumes for the groups receiving the best therapeutic results i.e. 2.5+12.5 MBq, 5+10 MBq (study 1 and 2), and as a comparison the 15 MBq and non-treated animals. The insert shows the early phase. ⨯ and + indicate statistically significant lower relative volume in the 2.5+12.5 MBq group and 5+10 MBq group (study 1) compared to the 15 MBq group respectively.
b) Mean relative tumor volumes for 0.5+12.5 MBq, 10+5 MBq (study 1 and 2), 15 MBq, 30 MBq group and non-treated animals. Error bars indicate SEM.

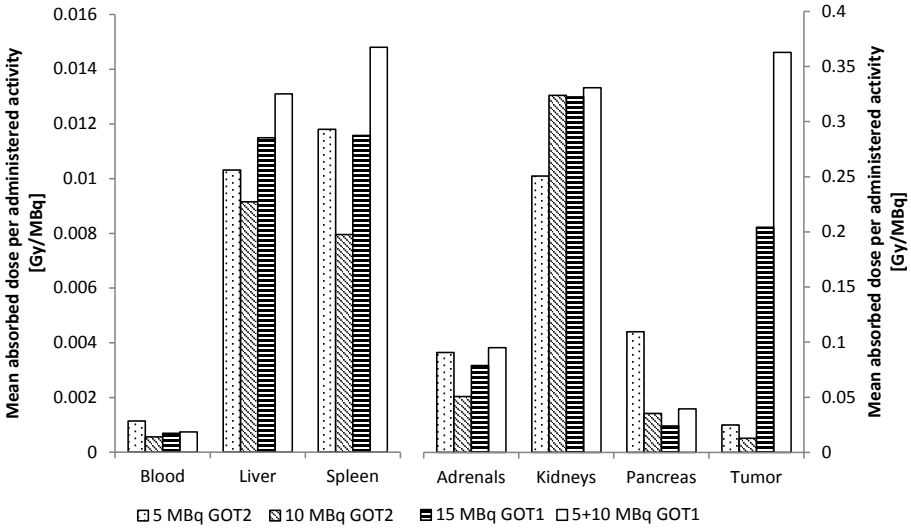

Figure 5.4 Mean absorbed dose per administered activity (Gy/MBq) to GOT1- or GOT2- tumor tissues and normal tissues in BALB/c nude mice transplanted with GOT1 or GOT2. GOT2-bearing animals were either injected with 5 or 10 MBq ^{177}Lu-octreotate and GOT1-bearing animals were injected with 15 MBq, or with a priming administration of 5 MBq followed by a main administration of 10 MBq 24 h later of ^{177}Lu-octreotate. Note different scales of the y-axes.

Study on renal toxicity from ^{177}Lu-octreotate in mice (paper V)

Urinary RBP4/creatinine ratio and carbamoylated Hb measured as VH as biomarkers of renal toxicity, are summarized in Figure 5.5. A time and dose dependence for urinary RBP4 was found. Changes in RBP4 values compared to the baseline value were obtained already after 14 days in the 120 MBq group, but after 30 days in the 60 MBq group, with statistically significant evaluations after 30 days and 60 days, respectively. No statistically significant differences in VH in treated animals and the control group was found.

No morphological changes in the form of sclerosis and atrophy were found on the proximal tubules or the glomeruli, except for a few focal signs of tubulointerstitial nephritis. The areas of these changes were however small compared to the whole kidney.

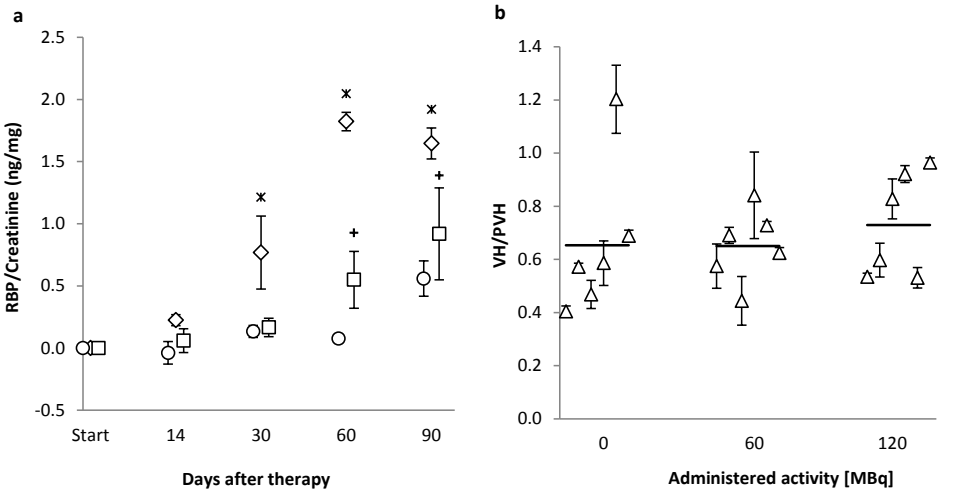

Figure 5.5 Levels of potential biomarkers for nephrotoxicity in nude mice treated with 60 MBq or 120 MBq ^{177}Lu-octreotate or untreated control mice (0 MBq). a) Urinary RBP4/creatinine. A baseline value was taken before administration of ^{177}Lu-octreotate (study start). Creatinine was used to correct for differences in urinary flow. ⁎ and + indicate statistically significance between values after therapy and baseline for the 120 MBq and the 60 MBq groups, respectively. b) VH/PVH ratio in blood samples taken at sacrifices (90 days after study start); mean values are indicated as horizontal lines for each group. Error bars indicate SEM.

6 Discussion

In this work, several types of human NET (GIST, paraganglioma, MTC, and midgut carcinoid) were investigated with the aim to find or optimize diagnostics and therapeutic methods using radiolabeled receptor binding hormone analogues (^{177}Lu-octreotate, ^{111}In-octreotide, ^{111}In-MG0, and ^{131}I-MIBG). These radiopharmaceuticals bind to SSTR, CCK2/gastrin receptor or norepinephrine transporter, all G-protein coupled receptors. Activity concentration in tumor tissue, and non-tumor organs was measured. The findings from these studies indicate large variation in receptor expression in different tumors and within the same tumor type, and a possibility to use other tracers than somatostatin analogues in pursuing a higher treatment effect on NETs.

Compared with GOT1, a relatively low SSTR2 expression in GOT2-tumor animal model was found in paper I, maybe because this specific MTC tumor cell line was well differentiated, since tumors with a higher degree of differentiation seem to have less SSTR (Forrer et al., 2007). The GOT2-animal model will serve as a good model for testing the binding specificity of new gastrin analogues (as was done with ^{111}In-MG0 in paper I). It will also be useful for investigation of other types of radiopharmaceuticals, combination therapies using ^{177}Lu-octreotate together with other radiopharmaceutical or with drugs which act on specific signaling pathways, for example the hedgehog pathway (Arne, 2012, Bohinc et al., 2013). With this animal model, new possible treatment options for medullar thyroid carcinoma based on SSTR can be more realistically studied, since the T/B ratio of radiolabeled SS analogues was similar to those found in patients (Forssell-Aronsson et al., 1995).

Both the paraganglioma patient and several GIST patients (paper II and III) were shown to be candidates for ^{177}Lu-octreotate treatment. The amount of ^{111}In-octreotide that was membrane bound and internalized in the paraganglioma cells were low, but the T/B ratios for ^{111}In (and ^{131}I-MIBG) was high, which can make the ^{177}Lu-octreotate treatment possible. The paraganglioma cells had a specific binding and internalization with rapid and continuous uptake of ^{131}I-MIBG. Thereby, the paraganglioma patient can have the opportunity to receive a combined treatment with both ^{131}I-MIBG and ^{177}Lu-octreotate. The advantage with such a combination is that the critical organs are different for these radiopharmaceuticals, which enables higher absorbed dose to the tumor tissue without increasing the risk of side effects. The tumor tissue from this patient has been xenografted on mice and can now most probably serve as a new animal model. The transplantable paraganglioma cells will after final validation most likely be named GOT3.

Tumor samples from two GIST patients were analyzed in paper III and a specific binding and internalization of ^{177}Lu was found. These results were in concordance with the activity uptake and T/B values after scintigraphy with ^{111}In-octreotide. However, some patients had low T/B values that indicated that not all patients are suitable for therapy with ^{177}Lu-octreotate, and individual SSTR expression needs to be determined before PRRT. A reason for why the reduction of internalization after adding an excess of octreotide were more prominent for GIST cells (98 %) than for paraganglioma cells (75 %) may be caused by the higher affinity of SSTR2 for ^{177}Lu-octreotate than for ^{111}In-octreotide that GIST cells and paraganglioma cells were incubated with, respectively.

The information collection with respect to the accumulation of different radiopharmaceuticals used in this work should be under consideration for GIST patients with primary and secondary resistance to tyrosine kinase inhibitors (TKI), for paraganglioma patients, and for patients with medullary thyroid carcinoma. For patients with GIST without resistance to TKI, PRRT with ^{177}Lu-octreotate is maybe not the best option since most GISTs have a higher expression of SSTR1, and

^{177}Lu-octreotate has low affinity to SSTR1. Then a ^{177}Lu-labeled SSTR1 specific analogue or a pan-analogue would be valuable.

When comparing uptake after ^{111}In-octreotide scintigraphy of GIST patients with tumor biopsies, the values did not match for all patients. In some cases, the T/B ratio was low but the visualization was good, and vice versa. This situation is also seen when comparing the internalization results between paraganglioma cells and GIST cells, and T/B ratio for the two tumor types. The internalization of ^{111}In-octreotide in paraganglioma cells was low but the T/B ratio was high, while the opposite was seen in GIST cells. The internalization in GIST cells was almost 10 times higher than in the paraganglioma cells but the T/B were moderate. This type of finding may indicate that the tumors in a patient are heterogeneous both concerning SSTR expression and to tumor cell heterogeneity within the tumor, which was shown with MRI in the *in vivo* study on GOT1-tumor bearing mice (paper IV). Another reason is the difference in visualization of different sized tumors in a patient, and also the influence of the location of the tumor in the body. To overcome this problem tumor biopsies should be collected at surgery, since T/B ratio is a good validation of uptake in the tumor compared to the normal tissues (Forssell-Aronsson et al., 2004).

The heterogeneity of tumor cell density in tumor tissue will also become a problem when evaluating the tumor growth or therapeutic effects based on tumor volume measurements. Histological evaluations on GOT1 tumor tissues after regrowth (41 days after injection of ^{177}Lu-octreotate), showed that some tumors had very heterogeneous composition, with a tumor cell count of less than 50 % in some tumors (paper IV). This indicates that only a part of the tumor volume is active which causes errors when estimating the therapeutic effect.

The therapeutic effect of a new treatment schedule including priming administration shortly before administration of the therapeutic amount of ^{177}Lu-octreotate was studied on GOT1-beraring animals (paper IV). The study demonstrated that the administration of 5 MBq ^{177}Lu-octreotate 24 h before administration of the actual therapeutic amount of ^{177}Lu-octreotate resulted in a higher therapeutic effect. The reason for this was expected to be due to up-regulation of SSTR from the priming administration, as was previously demonstrated in vitro and suggested from in vivo studies (Bernhardt et al., 2007, Melis et al., 2007, Oddstig et al., 2012, Oddstig et al., 2006, 2011). However, qPCR evaluation of mRNA expression 1-7 days after administration could not verify enhanced expression. Further *in vivo* studies should be performed to elucidate the mechanisms of priming, and the optimal priming activity and time-point after first injection need to be verified. Patient studies should also be initiated in order to study if priming would increase uptake and therapeutic effects.

The activity concentration (in %IA/g) varied between different tumor types and different activity amounts in the animal models (Table I in paper I and Table 1 in paper IV). The variation between the tumor types depends mainly on difference in SSTR expression on the cell surface, both also on the SSTR subtype expression. Octreotide and octreotate are known to mainly bind to SSTR2, 3 and 5 (Esser et al., 2006, Reubi et al., 2000).

The reason for different biokinetics of different amounts of radiolabeled SS analogues in tumor bearing animals is probably due to receptor saturation, since higher tumor uptake was found after injection of lower amount of radiolabeled SS analogues. Also variations in concentration in non-tumor tissue were found between animals and animal models. General reasons for differences in biokinetics of radiolabeled SS analogues between animals within a study and between studies can be differences in age of the animals, different generations of mice in the same study, difference in tumor size (30-1600 mm^3 in our studies), which might result in differences in e.g. hormone levels. Other factors might be differences in environment, such as humidity and temperature in animal room.

Biomarkers that reflect radiation induced renal injury can be a good tool in the assessment of the number of ^{177}Lu-octreotate treatments that can be given to a certain patient with NETs, and to verify the individual function of the kidneys after a certain mean absorbed dose to the kidney. Several different methods are used today to measure or estimate glomerular filtration rate e.g. ^{99m}Tc-DTPA scintigraphy (Hauser et al., 1970, Levey et al., 2003). Renal toxicity after irradiation from ^{177}Lu-octreotate is supposed to mainly occur in the proximal tubule of the nephron, since much of the ^{177}Lu is reabsorbed at that site. Proposed biomarkers should be evaluated based on suitable dose-response relationship and time for response, together with correlation with the risk or severity of the toxic effect or injury. Furthermore, long time studies are needed to verify if a radiation induced kidney detriment detected by a biomarker at an early time-point really indicates a more severe injury later in life, or if the radiation-induced injury can be repaired.

The current functional imaging techniques used today for NETs are ^{111}In-octreotide scintigraphy and for pheochromocytomas and paragangliomas ^{131}I-/^{123}I-MIBG scintigraphy. Nowadays more suitable methods have been developed using positron emitting tomography (PET) to visualize tumors and their metastases. The advantages with PET are not only that a more precise visualization can be obtained. PET can also give a broader understanding of how the individual tumors behave e.g. aggressiveness, receptor expression, and response to treatments. Radiotracers that are available today are for example ^{68}Ga-DOTA-octreotide for SSTR expression, ^{124}I-MIBG, ^{18}F-FDOPA, ^{18}F-FDA and ^{11}C-epinephrine for catecholamine metabolism, and ^{18}F-FDG for glucose metabolism (Blanchet et al., 2012, Bodei et al., 2011, Buchmann et al., 2007).

Animal models should mimic the human situation as much as possible because there are many factors that influence the therapeutic effect and uptake of the radiopharmaceutical. Before new radiopharmaceuticals can be used in the clinical settings, optimization strategies can be investigated, combination therapies can be tried, and toxic effects evaluated in realistic animal models. The animal model should be clinically relevant so that biodistribution data, tumor characteristics and dosimetric analysis can give a hint of the human situation (Forssell-Aronsson et al., 2013). Tumors in animal models are more predictable according to growth-rate, and the phenotype, the histology and the genetics are often known. Therefore, in the evaluation of an new drug or the therapeutic effect, the result from the specific cell line are probably clearer than in clinical trials, when patients with many different tumor characteristics are included (Ruggeri et al., 2014). One drawback of using cell culture and animal models is that the tumor cell line may change its characteristics with time.

It is hard to translate animal data to human, but animal data may give good indications on how the outcome after a change in treatment schedule will be, or how a new radiotracer will affect tumor and normal cells (Ruggeri et al., 2014). Reasons for difficulties in translation between mice and humans are many: biological differences between mice and humans; differentiation of the NET, the receptor expression on the NET, the metastatic spread, the tumor in mice is growing subcutaneously and in human the growth is orthotopic; the mice used for transplantation are immune deficient animals; and comparable studies between mice and human are lacking.

7 Conclusions

The main observations and conclusions in this work can be summarized as follows.

The biodistribution of ^{177}Lu-octreotate and ^{111}In-DOTA-MG0 was studied in the GOT2 animal model (paper I). The results showed overexpression of both SSTR and CCK2/gastrin receptors in tumor tissue, and that the uptake in tumor of ^{111}In-DOTA-MG0 was specific. Furthermore, the study showed that this model can be used in studies with radiolabeled somatostatin-, CCK2-, and gastrin- analogues for diagnostic use. For realistic therapy studies higher expression of SSTR would probably be needed. If efficient kidney-blocking agents are found, CCK2 and gastrin analogues can be therapeutic alternatives.

The tumor uptake of ^{111}In-octreotide and ^{131}I-MIBG in a patient with paraganglioma showed relatively high T/B values for both radiopharmaceuticals (paper II). The *in vitro* study showed specific binding and internalization of ^{131}I-MIBG and ^{111}In-octreotide in primary culture of collected paraganglioma cells. The results indicate that this patient would most probably benefit from radionuclide therapy using both ^{177}Lu-octreotate and ^{131}I-MIBG.

The tumor uptake of ^{111}In-octreotide in patients with GIST was in general moderate, probably due to a lower expression of SSTR2 than in some other NETs (paper III). Specific binding and internalization of ^{177}Lu-octreotate was found in primary culture of GIST cells. In conclusion, selected GIST patients with high T/B may benefit from ^{177}Lu-octreotate therapy. Development of new SS analogues with higher affinity to the receptors on the cell membrane, primarily SSTR1 and SSTR2, is needed to increase the possibilities for PRRT of all patients with GIST.

The GOT1 animal model was used in the optimization procedure when a priming activity was administered 24 h before the main administration of ^{177}Lu-octreotate. A higher mean absorbed dose to tumors treated with a priming activity was seen compared with that after a single injection. Although it was not verified that these findings are due to up-regulation of SSTR, we suggest that this type of treatment schedule should be considered when optimizing treatment protocols for patients with NETs.

Urinary RBP4 level increased with absorbed dose to the kidneys, and the increase started at an earlier time-point after a higher absorbed dose to kidneys in mice. RBP4 (RBP in humans) seems to be a biomarker that indicates renal effects after ^{177}Lu-octreotate treatment before any renal detriments can be visualized morphologically. RBP may thus be a potential biomarker used in ^{177}Lu-octreotate therapy to avoid late nephrotoxic effects. VH concentration in blood did not change with amount of administered ^{177}Lu-octreotate, and is presently not sensitive enough. If a change in urinary RBP level will reflect the degree of radiation induced acute or chronic renal injury remains to be investigated.

8 Future perspectives

In this work studies on human NET cells and models have been presented. Human NET cell lines and animal models are rare but most valuable for various types of studies, including evaluation and optimization of treatment methods. The results indicate that the usefulness of therapy using radiolabeled SS analogues such as ^{177}Lu-octreotate should be considered not only for patients with midgut carcinoid and endocrine pancreatic tumors. Individual patients with other NETs, such as MTC, GIST and pheochromocytoma and paraganglioma might also benefit from such treatment.

After the promising results from the optimization study made in paper IV the study design should be used on other animal models (for example GOT2 and NCI-H69 a transplantable human small cell lung cancer cell line) to investigate if these results can be repeated in other NETs or tumors with NE features. Furthermore, the mechanisms behind the increased uptake after priming should be determined. This also gives rise to the question if it is possible to change the administration schedule for humans, since neither the neuroendocrine organs nor the kidneys had increased doses.

A clinical study built on the findings from paper IV would be possible to perform since the total activity amount administered to the patient are not changed, only the administration schedule. Such a study can be motivated even though the most optimal priming activity is not known for humans. The estimated mean absorbed tumor dose 24 h after the priming administration was 0.05 Gy, and the most optimal absorbed dose after priming administration for patients needs to be defined in clinical trials.

The other way to enhance the treatment effect, studied in this work, is to by individually determined the radiosensitivity of the kidneys after treatment with ^{177}Lu-octreotate. The results in paper V demonstrate one potential biomarker, urinary RBP that early might signal late toxicity. However, further studies are needed both concerning this biomarker but also study other potential biomarkers. Moreover, tolerance doses for the kidneys must be determined for radionuclide therapy, not only for external radiotherapy. A long term study is ongoing on normal mice after receiving different amounts of ^{177}Lu-octreotate, where different protein biomarkers both from urine and from blood samples are analyzed. The aim is to investigate if the dose relationship found for RBP4 in paper V, also can be found for other proteins at different time-points after treatment. The correlation between a (late) radiation kidney detriment and the protein amount in blood or urine will be investigated. Methods of analyses suitable for small samples of both urine and blood will be used. When reliable kidney toxicity markers are defined, they should be applied to and evaluated in clinical trials, where urine and blood samples are collected and analyzed from patients that undergo ^{177}Lu-octreotate treatment.

Further studies on animal models and patients that would be necessary to enhance therapeutic effects on NETs are additional studies where ^{177}Lu-octreotate treatment is combined with another type of therapy. A higher treatment effect is needed to increase the fraction of patients with complete remission after treatment with ^{177}Lu-octreotate. Several types of combination therapy can be discussed. By using two different radionuclides with different range of emitted particles it may be easier to obtain high absorbed dose to different sized tumors. The use of various radionuclide bound ligands might avoid receptor saturation and enable different ways of internalization into the cells. Another way is to combine radiosensitizing agents with ^{177}Lu-octreotate. Other possible agents to combine with radiation are those acting on angiogenic molecules or on those leading to apoptosis by a different signaling pathway (e.g. sonic hedgehog or TRAIL) (Bohinc et al., 2013, Modlin et al., 2008, Strosberg et al., 2011).

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