Lutetium-177-octreotate treatment of small intestine neuroendocrine tumors

Radiation biology as basis for optimization

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Front cover: Word cloud in the shape of an ionizing radiation symbol, representing the words used in the thesis and papers. The size of a word in the visualization is proportional to the number of times the word appears in the text. Illustration created by Johan Spetz using http://www.wordclouds.com.

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Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.

Robert Frost, The Road Not Taken (1916)

Abstract

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Patients with neuroendocrine tumors (NETs) often have metastatic spread at the time of diagnosis. NETs frequently express somatostatin receptors (SSTR) that can be targeted by radiolabeled somatostatin analogs (*e.g.* ^{177}Lu-octreotate). Despite being highly effective in animal models (*e.g.* the human small intestine NET GOT1 transplanted to nude mice), ^{177}Lu-octreotate-based therapies have shown low cure rates in clinical studies. The cellular processes that underlie positive treatment response to ^{177}Lu-octreotate are largely unknown.

The aim of this work was to study the possibilities to optimize the therapeutic effects of ^{177}Lu-octreotate in the GOT1 model in nude mice.

A literature study of available data on radiolabeled somatostatin analogs on NETs in animal models was performed, to identify strategies for treatment optimization. To test these strategies, GOT1-bearing BALB/c nude mice were treated with non-curative amounts of ^{177}Lu-octreotate in different treatment schedules including single administrations, priming (fractionated) administrations and combination treatment with hedgehog inhibitor sonidegib. Biodistribution and dosimetry studies were performed and anti-tumor effects were monitored by measuring tumor volume. Global transcriptional and proteomic responses in tumor samples were evaluated using RNA microarray and liquid chromatography mass spectrometry, respectively.

^{177}Lu-octreotate therapy of GOT1 tumors xenotransplanted in nude mice resulted in tumor volume reduction. Priming administration resulted in increased anti-tumor effects and increased therapeutic window. Combination therapy using sonidegib and ^{177}Lu-octreotate resulted in prolonged time to progression. The global transcriptional and proteomic analyses of ^{177}Lu-octreotate treated tumor samples revealed time-specific responses in terms of affected biological functions.

In conclusion, time-dependent changes in p53-related cell cycle regulation and apoptosis, angiogenesis, endoplasmic reticulum stress, and oxidative stress-related processes suggest possible niches for combination therapy at different time-points after radionuclide therapy. Priming ^{177}Lu-octreotate therapy and combination therapy using sonidegib and ^{177}Lu-octreotate could be beneficial to patients with NE-tumors.

Keywords: Peptide receptor radionuclide therapy, PRRT, somatostatin receptors, SSTR, midgut carcinoid, radiogenomics

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Populärvetenskaplig sammanfattning

De vanligaste behandlingarna mot cancer är idag kirurgi, kemoterapi och extern strålbehandling. Även om dessa metoder förbättrats med åren så är det fortfarande en stor utmaning att behandla patienter där cancern spridit sig i kroppen. När det gäller neuroendokrina tumörer har de flesta oftast redan hunnit ge upphov till spridd sjukdom vid diagnosen.

Vid radionuklidterapi använder man radioaktiva ämnen, ofta kopplade till tumörsökande ämnen – så kallade radioaktiva läkemedel. Denna behandlingsform går ut på att det radioaktiva läkemedlet ges till patienten, och kan via blodet nå alla tumörer i kroppen, även om cancern spridit sig. Det radioaktiva läkemedlet tas upp i tumörerna och bestrålar dem inifrån.

I detta arbete har det radioaktiva ämnet ^{177}Lu använts, kopplat till den tumörsökande substansen octreotate (kallat ^{177}Lu-octreotate). Många neuroendokrina tumörceller har en stor mängd somatostatinreceptorer på sin utsida som fångar upp ^{177}Lu-octreotate ur blodet, vilket gör att tumörerna tar upp mer ^{177}Lu-octreotate än friska organ och därför får en högre stråldos. Behandling med ^{177}Lu-octreotate har visat lovande resultat i patienter med neuroendokrina tumörer, med tumörvolymsminskning och flera års ökad överlevnad. Dock botas i dagens läge endast ett fåtal patienter, och mängden läkemedel som kan ges begränsas av bieffekter på framförallt njurarna.

Målet med denna avhandling var att undersöka möjliga sätt att förbättra terapieffekterna av ^{177}Lu-octreotate i neuroendokrina tunntarmstumörer. Detta genomfördes genom att först undersöka tidigare studier som gjorts i djurförsök, och definiera strategier för hur terapieffekterna skulle kunna optimeras. Sedan studerades de biologiska effekterna av en icke-botande mängd ^{177}Lu-octreotate på mänskliga tumörer (kallade GOT1) transplanterade till möss, och två olika metoder för att förbättra behandlingseffekterna testades.

Resultaten visar att förbehandling med en liten mängd ^{177}Lu-octreotate kan göra så att tumörerna tar upp en större andel av en andra behandling med ^{177}Lu-octreotate som utförs 24 timmar senare. ^{177}Lu-octreotate kan också kombineras med läkemedlet sonidegib som också ger effekter på tumören men har andra biverkningar än ^{177}Lu-octreotate. Båda dessa metoder gav en större behandlingseffekt på tumörerna trots att samma totala mängd ^{177}Lu-octreotate gavs till djuren. Studierna av ^{177}Lu-octreotate-behandlingens biologiska effekter gav även upphov till flera andra möjliga förbättringsmetoder. Dessa metoder skulle potentiellt kunna användas för öka behandlingseffekten även i patienter utan att mängden läkemedel (och på så sätt även biverkningarna) behöver ökas.

List of papers

This thesis is an introduction to and a summary of the work contained in the following six papers, referred to in the text by Roman numerals:

I. Eva Forssell-Aronsson, Johan Spetz, Håkan Ahlman: **Radionuclide therapy via SSTR: Future aspects from experimental animal studies**. *Neuroendocrinology*, **2013**; 97(1):86-98. Reprinted by permission of S. Karger AG, Basel.

II. Johan Spetz, Nils Rudqvist, Britta Langen, Toshima Z Parris, Johanna Dalmo, Emil Schüler, Bo Wängberg, Ola Nilsson, Khalil Helou, Eva Forssell-Aronsson: **Time-dependent transcriptional response of GOT1 human small intestine neuroendocrine tumor after ^{177}Lu-octreotate therapy**. *In revision.*

III. Johan Spetz, Mikael Montelius, Evelin Berger, Carina Sihlbom, Maria Ljungberg, Khalil Helou, Ola Nilsson, Eva Forssell-Aronsson: **Profiling proteomic responses in small intestinal neuroendocrine tumor GOT1 after ^{177}Lu-octreotate therapy.** *Submitted.*

IV. Johanna Dalmo, Johan Spetz, Mikael Montelius, Britta Langen, Yvonne Arvidsson, Henrik Johansson, Toshima Z Parris, Khalil Helou, Bo Wängberg, Ola Nilsson, Maria Ljungberg, Eva Forssell-Aronsson: **Priming increases the anti-tumor effect and therapeutic window of ^{177}Lu-octreotate in nude mice bearing human small intestine neuroendocrine tumor GOT1**. *EJNMMI Research,* **2016;** in press.

V. Johan Spetz, Britta Langen, Nils Rudqvist, Toshima Z Parris, Johanna Dalmo, Bo Wängberg, Ola Nilsson, Khalil Helou, Eva Forssell-Aronsson: **Transcriptional effects of ^{177}Lu-octreotate therapy using a priming treatment schedule on GOT1 tumor in nude mice**. *Manuscript.*

VI. Johan Spetz, Britta Langen, Nils Rudqvist, Toshima Z Parris, Khalil Helou, Ola Nilsson, Eva Forssell-Aronsson: **Hedgehog inhibitor sonidegib potentiates ^{177}Lu-octreotate therapy of GOT1 human small intestine neuroendocrine tumors in nude mice**. *Submitted.*

Selection of related presentations

1. Spetz J, Montelius M, Ljungberg M, Helou K, Nilsson O, Forssell-Aronsson E: **^{177}Lu-octreotate induces tumor volume regression and suppresses invasive potential in small intestine neuroendocrine tumors**. *Cancerfondens planeringsgrupp för onkologisk radionuklidterapi, Höstmöte,* Uppsala, Sweden, November, **2016**.

2. Spetz J, Montelius M, Ljungberg M, Helou K, Nilsson O, Forssell-Aronsson E: **Temporal proteomic responses to ^{177}Lu octreotate therapy in GOT1 human small intestine neuroendocrine tumors indicate suppressed invasive potential**. *4th Swedish Cancer Research Meeting*, Gothenburg, Sweden, November, **2016**.

3. Montelius M, Spetz J, Ljungberg M, Helou K, Forssell-Aronsson E: **Multiparametric MRI (mpMRI) for spatiotemporal characterization of tumor tissue response to radionuclide treatment**. *62nd Annual Meeting of the Radiation Research Society*, Waikoloa, USA, October, **2016**.

4. Spetz J, Montelius M, Ljungberg M, Helou K, Forssell-Aronsson E: **Spatial proteomic analysis of GOT1 human small intestine neuroendocrine tumor in nude mice following ^{177}Lu octreotate therapy**. *62nd Annual Meeting of the Radiation Research Society*, Waikoloa, USA, October, **2016**.

5. Spetz J, Rudqvist N, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E: **Hedgehog inhibitor Sonidegib potentiates ^{177}Lu-octreotate therapy of GOT1 human small intestine neuroendocrine tumors in nude mice**. *Cancerfondens riksplaneringsgrupp för onkologisk radionuklidterapi, Höstmöte*, Linköping, Sweden, November, **2015**.

6. Spetz J, Dalmo J, Rudqvist N, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E: **Transcriptional effects of ^{177}Lu-octreotate therapy on GOT1 tumor in nude mice using conventional and priming treatment schedules**. *15th International Congress of Radiation Research*, Kyoto, Japan, May, **2015**.

7. Spetz J, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E: **Hedgehog inhibitor LDE225 increases efficacy of ^{177}Lu-octreotate therapy on GOT1 tumors in nude mice**. *60th Annual Meeting of the Radiation Research Society*, Las Vegas, USA, September, **2014**.

8. Spetz J, Dalmo J, Rudqvist N, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E: **Transcriptional response of GOT1 midgut carcinoid in nude mice following ^{177}Lu-octreotate treatment**. *27th Annual Congress on European Association of Nuclear Medicine*, Gothenburg, Sweden, October, **2014**.

9. Forssell-Aronsson E, Spetz J, Langen B, Dalmo J, Larsson M, Montelius M, Rudqvist N, Parris TZ, Arvidsson Y, Ljungberg M, Helou K, Nilsson O, Wängberg B: **Optimization of ^{177}Lu-octreotate treatment of neuroendocrine tumours**. *3rd Swedish Cancer Research Meeting*, Stockholm, Sweden, September, **2014**.

10. 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**. *59th Annual Meeting of the Radiation Research Society*, New Orleans, USA, September, **2013**.

11. Spetz J, Dalmo J, Langen B, Parris TZ, Wängberg B, Nilsson O, Helou K, Forssell-Aronsson E: **Combination therapy of GOT1 tumours in nude mice using ^{177}Lu-octreotate and the hedgehog inhibitor LDE225**. *Swedish Radiation Research Association for Young Scientists (Swe-Rays) workshop*, Uppsala, Sweden, August, **2013**.

12. Spetz J, Langen B, Parris TZ, Rudqvist N, Helou K, Nilsson O, Ahlman H, Forssell-Aronsson E: **Regulation of gene expression in GOT1 midgut carcinoid in nude mice following injection with ^{177}Lu-octreotate**. *25th Annual Congress on European Association of Nuclear Medicine*, Milano, Italy, October, **2012**.

13. Spetz J, Langen B, Parris TZ, Rudqvist N, Helou K, Nilsson O, Ahlman H, Forssell-Aronsson E: **Effects of internal irradiation from ^{177}Lu-octreotate on gene expression in GOT1 midgut carcinoid in nude mice**. *58th Annual Meeting of the Radiation Research Society*, San Juan, Puerto Rico, October, **2012**.

Table of Contents

Abbreviations

2D	Two-dimensional
A	Activity
Bq	Becquerel
C	Activity concentration
Cd	Cadmium
Cl	Chloride
CM	Cellular membrane
D	Absorbed dose
DMSO	Dimetylsulfoxid
DNA	Deoxyribonucleic acid
DOTA	Dodecanetetraacetic acid
DTPA	Diethylenetriaminepentaacetic acid
EC-cell	Enterochromaffin cell
eV	Electron volt
GO	Gene Ontology
Gy	Gray
Hf	Hafnium
Hh	Hedgehog
IA	Injected activity
IKB	Ingenuity Knowledge Base
IHC	Immunohistochemical
In	Indium
IPA	Ingenuity Pathway Analysis
ITLC	Instant thin layer chromatography
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
$l\!N_2$	Liquid nitrogen
Lu	Lutetium
MIRD	Medical Internal Radiation Dose Committee
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NaCl	Sodium Chloride
NaI(Tl)	Thallium-activated sodium iodine
NET	Neuroendocrine tumor
PRRT	Peptide receptor radionuclide therapy
RARE	Rapid acquisition with relaxation enhancement

RNA	Ribonucleic acid
SEM	Standard error of the mean
SI-NET	Small intestine neuroendocrine tumor
SSTR	Somatostatin receptor
T	Tesla
T/N	Tumor-to-normal-tissue activity concentration ratio
Tc	Technetium
TMT	Tandem mass tag
Tyr	Tyrosine
UPR	Unfolded protein response
Y	Yttrium
Zr	Zirconium

Background

Despite many years of research regarding the treatment of patients with cancer, curative therapeutic options are in many cases still not available [1, 2]. Radiopharmaceuticals – utilizing high-affinity molecules as carriers of radionuclides to tumor cells – may be a useful option in the treatment of cancer, especially considering metastatic disease [3]. These pharmaceuticals are often injected intravenously, and subsequently circulate in the blood stream. The radiopharmaceuticals have the potential to reach target molecules on the surface of tumor cells throughout the body of the patient, thus delivering a locoregional irradiation in close proximity to the tumor [4]. This treatment option is, however, still in need of optimization in order to reach its full potential.

The Neuroendocrine system

Neuroendocrine cells facilitate a link between the nervous and endocrine systems. Neurotransmitters released from the nervous system communicate signals to neuroendocrine cells in endocrine glands (*e.g.* adrenal glands, hypothalamus, ovaries, pancreas, pineal gland, pituitary gland, testes, thyroid gland, and parathyroid gland, and gastrointestinal tract) to regulate hormone synthesis, storage and secretion. Somatostatin, serotonin, histamine, cholecystokinin and gastrin are examples of hormones released from neuroendocrine cells in the gastrointestinal tract [5]. The most common neuroendocrine cell type in the gastrointestinal tract is the enterochromaffin cell (EC-cell), which regulates blood flow, motility and hormone secretion. EC-cells comprise the majority (>90 %) of the production of serotonin (important in regulation of *e.g.* bowel motility) in the body [6], and contains storage vesicles for neuroendocrine secretory protein chromogranin A and synaptic vesicle glycoprotein synaptophysin [7]. EC-cells also express G protein-coupled seven transmembrane receptors called somatostatin receptors (SSTRs), which occur in five different subtypes (SSTR1-5) [8, 9]. Binding of the ligand somatostatin to SSTR inhibits the release of serotonin and many other hormones from EC-cells [10-12].

Small intestine neuroendocrine tumors (SI-NETs)

Neuroendocrine tumors (NETs) represent many different malignancies that arise from neuroendocrine cells in different parts of the body, and are frequently associated with the synthesis and secretion of peptides and amines causing hormone overproduction symptoms (*e.g.* carcinoid syndrome, which is caused by endogenous secretion of mainly serotonin and kallikrein).

SI-NETs are rare, but have shown significantly increasing incidence rates during recent decades [13]. SI-NETs occur most frequently in the ileum, and are thought to originate from EC-cells [14, 15]. They have retained many of the neuroendocrine characteristics, *e.g.* abundant expression of serotonin, chromogranin A, synaptophysin and SSTR (mainly subtype 2 and 5) [16]. SI-NETs grow invasively in the wall of the small intestine and often metastasize to the liver and abdominal lymph nodes. SI-NETs are slowly proliferating tumors, and symptoms are seldom evident until the disease is in an advanced stage, due to the fact that hormones from the gastrointestinal tract are released into the hepatic portal circulation and degraded in the liver [17]. Hence, symptoms are generally not presented until metastatic spread to the liver has subdued the hormone metabolism capacity of the liver. Therefore, disseminated disease is usually present at the time of diagnosis [18, 19].

Genetic alterations in SI-NETs

During carcinogenesis, cells undergo several genetic and epigenetic alterations to acquire new capabilities that ultimately lead to uncontrolled growth, tissue invasion and metastasis. There are three classes of genes involved in this process: oncogenes (genes that promote cell proliferation and inhibit apoptosis, *e.g. MYC*, *PDGFB*, the Wnt gene family, *EGFR*, and *BCL2*), tumor suppressor genes (genes that inhibit cell proliferation, *e.g. TP53*, *RB*, *APC*, and *VHL*) and DNA repair genes (genes that decrease mutation rates in oncogenes and tumor suppressor genes, *e.g. BRCA1, BRCA2,* and *RAD51*) [20-22]. Accumulation of mutations affecting these three classes of genes enables the normal cell to become cancerous. The molecular alterations leading to the development of SI-NETs have not been fully characterized. However, gene expression profiling of SI-NETs has provided some information on

the molecular changes underlying cancer initiation and progression. For example, *TP53* (encoding p53) – the most commonly mutated gene in human cancers – is rarely inactivated in NETs [23-26]. However, epigenetic and regulatory aberrations interfering with the p53 network activity have been described in NETs, which might restrict its function [27-29]. Furthermore, the oncostatic regulator TGFß has been reported to be inactivated in some NET cell lines but not in others [30, 31]. Several genetic biomarkers for diagnosis and/or therapy in SI-NETs have been proposed, *e.g. GRIA2, RET, FGFR1/3, PDGFRB, FLT1, SPOCK1, PNMA2, APLP1, SERPINA10, MTA1, GPR112* and *OR51E1* [32-35]. Furthermore, loss of chromosome 18 occurs in >60 % of SI-NETs (reported in both primary tumors and metastases) [36-38], and gain of chromosomes 4, 5, 7, 14 and 20 is frequently observed [37-40].

Peptide receptor radionuclide therapy (PRRT)

Surgery is currently the only curative treatment for patients with localized SI-NET, but high expression of SSTR can be exploited for palliative treatment of metastases by administration of somatostatin analogs (*e.g.* octreotide) [17, 41]. Radiolabeling of somatostatin analogs (*e.g.* octreotide or octreotate) offers an option for both imaging and therapy in patients with SSTR-overexpressing SI-NETs. ^{111}In-[DTPA]-octreotide (^{111}In-pentetreotide Octreoscan™, Mallinckrodt Pharmaceuticals) is routinely used for diagnosis and staging of patients with SSTR-positive NETs [42-44]. Due to more favorable therapeutic characteristics (*cf.* **Table 1**), Lu-177-[DOTA^0, Tyr^3]-octreotate (^{177}Lu-octreotate or ^{177}Lu-DOTATATE, illustrated in **Figure 1**) and ^{90}Y-[DOTA]-octreotide are frequently used for PRRT [42, 45]. Successful results in terms of tumor regression, increased overall survival, and improved quality of life have been reported from ^{177}Lu-octreotate and ^{90}Y-[DOTA]-octreotide therapy of patients with different types of NET, with response rates of about 50 % [46-52]. These results are superior compared with chemotherapy, where response rates seldom reach 20 % [53-55].

Table 1: Physical properties of the radionuclides ^{90}Y, ^{111}In and ^{177}Lu, including physical half-life, daughter nuclide, decay mode, and average energy per decay emitted as electrons and photons, respectively, and total energy emitted per decay [56]

Radio-nuclide	Half-life	Daughter	Decay mode	Energy per decay [keV]		
				Electron	Photon	Total
^{90}Y	2.7 d	^{90}Zr	β^-	934	0.00	934
^{111}In	2.8 d	^{111}Cd	EC	32.3	406	438
^{177}Lu	6.6 d	^{177}Hf	β^-	147	33.4	180

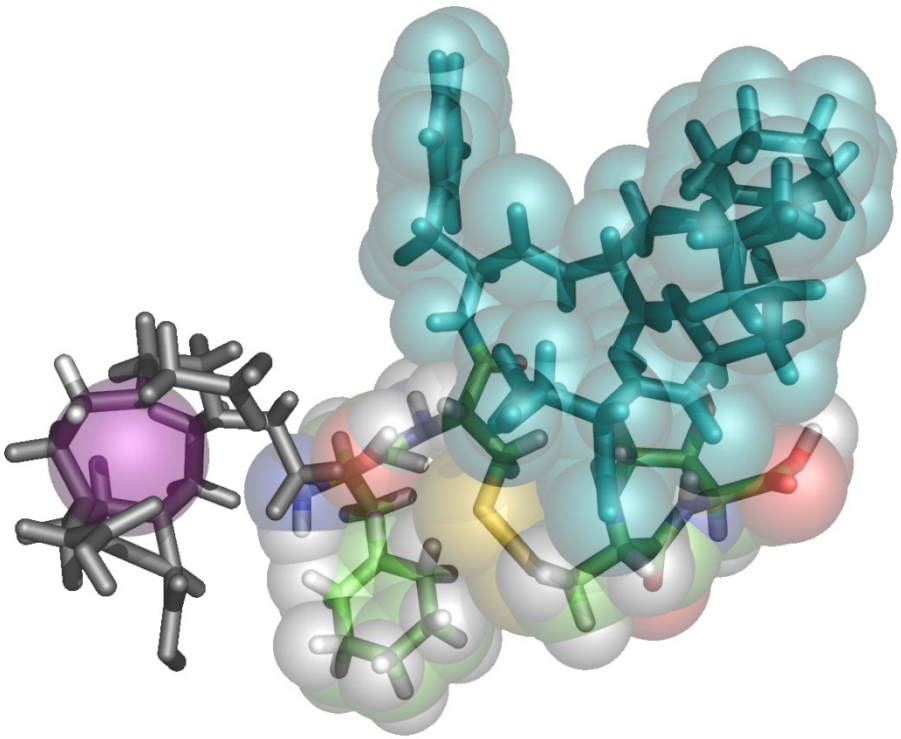

Figure 1: Illustration of the radiolabeled somatostatin analogue ^{177}Lu-[DOTA^0-Tyr^3]-octreotate (^{177}Lu-octreotate) showing a potential conformation built with 1YL8.pdb (Tyr^3-octreotate) and 1NC2.pdb (adapted DOTA) using PyMOL. The DOTA is colored with black and encloses ^{177}Lu (in lilac). In turquoise color is the part of octreotate that binds to the receptor. Reprint from [57], with kind permission from Johanna Dalmo and Britta Langen.

NET animal models

Several different NET models have been established and are used as *in vitro* models or are xenotransplanted to mice or rats as *in vivo* models. Studies in animal models are usually required before clinical trials of new radiopharmaceuticals are allowed to be conducted. Clinically relevant models are needed to study biodistribution and dosimetric data, tumor characteristics (e.g. size, growth rate, and radiosensitivity), and normal tissue characteristics and toxicity. There are many differences between humans and animals, and results from animals may be difficult to translate to the clinical situation. Two major types of models are used: (1) tumors (human or animal) growing on immunosuppressed animals (xenogeneic models), and (2) tumors growing on animals of the same species (syngeneic models). A selection of available NET models is presented in **Table 2**. The biodistribution data of ^{177}Lu-octreotate, especially the uptake in different types of SSTR-expressing tumor tissues, varies between the animal models described (**Table 3**).

Table 2: Animal models and tumor cell lines/types used with radiolabeled somatostatin analogs

Cell line/type	Origin	Tumor type	Animal species	Study
GOT1	human	SI-NET	nude mouse	[58]
KRJ-1	human	SI-NET	nude mouse	[59]
GOT2	human	medullary thyroid carcinoma	nude mouse	[60]
TT	human	medullary thyroid carcinoma	nude mouse	[61]
BON	human	pancreatic NET	nude mouse	[62]
IMR-32	human	neuroblastoma	nude mouse	[63]
CLB-BAR	human	neuroblastoma	nude mouse	[64]
CLB-GEMO	human	neuroblastoma	nude mouse	[65]
NCI-H727	human	bronchial NET	nude mouse	[66]
NCI-H69	human	small cell lung cancer	nude mouse	[67]
ZR-75-1	human	Invasive ductal carcinoma	nude mouse	[68]
AR42J	rat	hyperplastic exocrine pancreatic nodule	rat, nude mouse	[69]
CA20948	rat	pancreatic acinar tumor	rat, nude mouse	[70]

Table 3: Biodistribution, given as ^{177}Lu activity concentration [%IA/g] in various tumor-bearing animal models 1 and 7 days after injection of ^{177}Lu-octreotate

Tumor type	**GOT1**		**GOT2**		**NCI-H69**		**AR42J**	**CA20948**		**CA20948**	**IMR-32**	**CLB-BAR**	**CLB-GEMO**
Animal	**n.m.**		**n.m.**		**n.m.**		**n.m.**	**rat**		**rat**	**n.m.**	**n.m.**	**n.m.**
Study	[71, 72]		[73]		[67]		[69]	[70]		[74]	[75]	[75]	[75]
Injected activity (MBq)	7.5		5		3.3		0.74	1.3		3	15	15	15
Amount of peptide (µg)	0.25		0.2		0.7		1	0.67		0.5	0.6	0.6	0.6
	^{177}Lu activity concentration, %IA/g												
Time after injection (d)	1	7	1	7	1	7	1	1	7	1	1	1	1
Adrenals	-	-	0.87	0.43	0.34	0.43	2.1†	0.21	0.11	8.6	1.1	1.2	1.5
Blood	0.35	0.024	0.02	0.0027	0.008	0.001	0.06	0.03	0.01	0.002	0.039	0.041	0.044
Heart	-	-	0.054	0.027	0.034	0.011	0.1	0	0.07	-	-	-	-
Kidneys	4.6	0.78	5	0.62	2.2	0.27	4.4	1.7	0.94	1.6	18	15	15
Liver	0.2	0.11	0.14	0.048	0.1	0.068	0.4	0.28	0.18	0.032	0.34	0.21	0.41
Muscle	-	-	0.012	0.0024	0.011	0.001	0.03	0.012	0	0.002	-	-	-
Pancreas	-	-	2	0.28	0.41	0.032	1.6	2.3	1.1	3.6	-	-	-
Spleen	-	-	0.12	0.058	0.12	0.032	0.3	0.01	0.01	0.023	0.30	0.41	0.31
Tumor	18	7.2	0.37	0.094	3.7	1.2	0.8	6.1	0.65	2.2	11	4.0	12
Dosimetry	**D/IA (Gy/MBq)**												
	1.6–4.0		0.013		0.29		-	-		0.097	-	-	-

Data are corrected for physical decay. Mean absorbed dose to tumor per injected activity, D/IA (Gy/MBq). n.m. indicates nude mouse, - indicates non-reported value, † indicates the value is given as %IA/organ.

The GOT1 model

The GOT1 human SI-NET cell line was derived from a surgically removed liver metastasis of a patient with metastatic SI-NET (*cf.* **Table 2**) [58]. The GOT1 cells have retained characteristic properties of NETs, such as abundant expression of SSTR2 and SSTR5, and a relatively slow growth rate (doubling time 14–16 d) [58, 76]. GOT1 can be successfully xenotransplanted to nude mice [76], and it has previously been shown that ^{177}Lu-octreotate induces cell cycle arrest, apoptosis and dose dependent tumor volume reduction in GOT1 tumors, with a maximum apoptosis response at 1 and 3 d after injection [71, 72].

Molecular radiation biology

While it is assumed that the genetic background of an organ or tissue has a major role in the response to radiation, the radiation effects on cells at the molecular level are still largely unknown. Ionizing radiation is known to induce damage to the DNA, either directly via charged particles or indirectly via free radical production, but can also modulate intra- and intercellular signaling pathways [77-79]. For example, radiation exposure can result in activation of the p53 signaling pathway which, depending on the extent of DNA damage, promotes cell survival (by cell cycle arrest and DNA damage repair), or activates cell death mechanisms such as apoptosis [80]. The cellular mechanisms involved in radiation responses vary between different tissues, and depend on absorbed dose, dose rate, and type of radiation [79-87]. In agreement with results in normal cells, activation or inhibition of certain signaling pathways and cellular response mechanisms may also vary between different tumor types. The activation and/or inhibition of cellular mechanisms leading to radiation-induced cell death often involve apoptosis, including both intrinsic (mitochondria-mediated) and extrinsic (death-receptor-mediated) pathways [80, 88]. Senescence, autophagy or mitotic catastrophe may also contribute to radiation-induced cell death mechanisms In fact, mitotic catastrophe is today considered to be the major radiation-induced cell death mechanism in solid tumors after radiation therapy, owing to the frequent inactivation of p53 and loss of apoptotic activity [80, 89, 90]. A better understanding of the molecular mechanisms underlying responses to radiation in SI-NETs is needed to establish strategies for treatment optimization. Studies in an *in vivo* setting are necessary to determine radiation-induced effects in a

systemic environment with regards to, e.g., hypoxia, cell-to-cell communication, and induction of immune response.

Gene expression regulation and protein abundance alterations are complex dynamic processes which fluctuate over time. To assess the effect of radiation on cellular functions and mechanisms, it is of interest to investigate the effects on networks of genes and proteins, which are associated with one or several specific biological functions or processes.

Gene ontology (GO)

The GO consortium is a large bioinformatics initiative in which over 100 000 scientific papers have been assessed to catalogue biological processes involving different genes and proteins, with the goal of creating a common terminology in the analysis of biological systems [91]. The GO database is constructed as an ancestor chart, with specialized biological processes in one end and more general biological processes in the other, interconnected via GO terms with a wide spectrum of specificity. To calculate the significance of an enrichment of regulated genes in the data associated with a certain biological process, Fisher's exact test is used to compare two different ratios. The first ratio relates to the present study: the number of identified genes related to a certain GO term divided by the total number of identified genes. The second ratio is the total number of genes related to a certain GO term divided by the total number of genes in the human genome.

Ingenuity pathway analysis (IPA)

The IPA software (Ingenuity Systems, USA) utilizes the Ingenuity Knowledge Base (IKB) to associate transcriptional or proteomic response patterns with biological information [92]. IPA contains several different tools for analysis of the biological impact of the observed responses. The p-value of overlap between the experimental data and the IKB is calculated with Fisher's exact test and used to rank the statistical significance of each prediction. The resulting z-score (a measure of prediction strength) is used to determine activation state; $z>2$ indicates activation, while $z<-2$ indicates inhibition.

^{177}Lu-octreotate treatment optimization

The main goal of PRRT is to deliver the highest possible absorbed dose to tumor tissue, while avoiding side effects on non-tumor tissues. In the treatment protocols that are routinely used in the clinics, the uptake in the kidneys, which are one of the dose limiting organs, is reduced with infusion of amino acids (often lysine and arginine) [93, 94]. Administration of the radiopharmaceutical is also fractionated to enable recovery of normal tissues from acute radiation effects. However, these treatment regiments are inadequate for the majority of patients because few patients show complete remission [95, 96], while results from animal models show high cure rates [71, 97]. These findings, together with overall mild toxicity in normal tissues [49, 52, 98], indicate that patient treatment can be further optimized to increase the anti-tumor effects and therapeutic window.

Aims

Patients with NET often have metastatic spread at the time of diagnosis, and surgery is then usually no longer a curative option. The moderate cure rate observed after PRRT together with few potentially beneficial alternative treatment options indicates that an optimization of PRRT treatment protocols is needed to enhance therapeutic results. The overall aim of this work was to study the possibilities to optimize the therapeutic effects of SSTR-mediated PRRT on neuroendocrine tumors in animal models.

The specific aims were:

- to summarize data from previous experimental animal studies on SSTR-mediated PRRT and define directions for future research to enhance the therapeutic results for NETs using radiolabeled somatostatin analogs (**Paper I**)
- to characterize the effect of ^{177}Lu-octreotate therapy on the transcriptome and proteome in GOT1 small intestine NET in nude mice, in order to identify and elucidate possible venues for treatment optimization (**Papers II-III**)
- to determine the effect of priming on the biodistribution and dosimetry of ^{177}Lu-octreotate in GOT1-bearing nude mice to evaluate the effect on the therapeutic window (**Paper IV**)
- to examine if a priming administration of ^{177}Lu-octreotate 24 h before a subsequent ^{177}Lu-octreotate administration increases the anti-tumor effect of ^{177}Lu-octreotate in GOT1 tumor tissue in mice, compared with a single administration of the total amount of ^{177}Lu-octreotate (**Paper IV**)
- to determine the transcriptional response in GOT1 tumor tissue from mice treated with a priming administration of ^{177}Lu-octreotate 24 h before a second ^{177}Lu-octreotate administration (**Paper V**)
- to determine the anti-tumor effect and study the transcriptional response profiles from combination therapy using ^{177}Lu-octreotate and the Hedgehog signaling pathway inhibitor sonidegib in GOT1 tumors in nude mice (**Paper VI**)

Strategies

To define suitable directions for the optimization of the therapeutic results for SI-NETs using ^{177}Lu-octreotate, a number of strategies for potential enhancement of therapeutic results for NETs in animal models using radiolabeled somatostatin analogs were proposed (**Paper I**). The basis of this work was a summarizing review of the available data on experimental animal studies in the literature. Three major venues for optimization were identified: (1) general methods including individualized treatment performance, (2) methods to increase the treatment effect on tumor tissue, and (3) methods to reduce the toxic effects on normal tissues. Many methods have been tested in animal studies, but some studies can only be performed in patients. None of the strategies has been fully optimized for clinical use.

Individualized treatment planning

Treatment planning should focus on delivering the highest therapeutic effect to tumor tissue, while avoiding acute and severe late effects in risk organs. This could be accomplished by *e.g.* obtaining treatment planning data with the same radiopharmaceutical which is used for therapy, using optimized fractionated treatment schedules to allow restitution of side effects between the fractions [99-102], administering an optimal amount of activity [71, 103-105], determining and applying radionuclide-specific tolerance doses in normal tissues, and accounting for differences in individual radiation sensitivity [79, 106, 107]. Attention should also be given to the choice of somatostatin analog (in terms of *e.g.* tumor SSTR subtype expression, newly developed somatostatin analogs, and new radiolabeling techniques) [108-113], and radionuclide (in terms of *e.g.* half-life *vs.* biokinetics, particle range *vs.* tumor size, and SSTR affinity) [71, 114-117].

Increased anti-tumor effect

Methods to increase the anti-tumor effect of radiolabeled somatostatin analogs were divided into two branches of strategies: (1) methods to

increase tumor uptake and retention of the radionuclide, and (2) methods to increase the radiobiological effect on tumor tissue.

Increased tumor uptake and retention of radionuclide

Binding of somatostatin analogs to the tumor depends on the number of peptide molecules that reach the tumor, and the number of SSTRs available. Achieving an increased tumor uptake and retention of radionuclide could be accomplished by *e.g.* using an optimal amount of injected peptide [73, 104, 118], up-regulating SSTR expression in the tumor [119-121], or increasing tumor perfusion [122, 123].

It has previously been demonstrated that tumor cells with neuroendocrine features increase their expression of *SSTR1, 2* and *5* after exposure to ionizing radiation *in vitro* (0.12-8 Gy, X-rays) [119, 120]. Studies in the GOT1 model in mice have shown that the uptake of a subsequent injection of 0.5 MBq ^{111}In-octreotate in tumor was higher following an injection of 7.5 MBq ^{177}Lu-octreotate (a non-curative "priming" amount), than following an injection of 30 MBq ^{177}Lu-octreotate (a curative amount) [121, 124]. Furthermore, the optimal time between administration of ^{177}Lu-octreotate and elevated concentration of ^{111}In-octreotide in the tumor was 1 day, or 3–13 days in GOT1 tumors in nude mice. The increased uptake of ^{111}In-octreotate in tumor tissue may be due to *SSTR* up-regulation.

Increased radiobiological effect on tumor tissue

While ionizing radiation is known to induce DNA damage, it can also affect intra- and intercellular signaling pathways [79]. In radiotherapy, tumor cell death is the desired end-point, and beside direct effects on tumor cells other radiation-induced mechanisms can influence curative potential, *e.g.* tumor angiogenesis and protein integrity, but also invasiveness and metastatic potential [79, 125]. The cellular mechanisms involved in radiation responses vary between different tissues, and depend on absorbed dose, dose rate, and type of radiation [79-87, 126-133]. Achieving an increased radiobiological effect on tumor tissue could be accomplished using combination therapy with other radiopharmaceuticals, systemic anti-tumor agents, or radiosensitizing agents [134-144].

The Hedgehog (Hh) pathway is a major developmental signaling pathway, which regulates both proliferation and differentiation of

various types of stem cells during embryogenesis [145]. It is involved in, *e.g.* cell cycle regulation, cell adhesion, signal transduction, angiogenesis, and apoptosis [138, 146]. Defective Hh signaling has been implicated in various types of human cancers [147], and several components of the Hh pathway have been studied and proposed as targets for cancer treatment [138, 146, 148]. Hh signaling has been shown to be activated in NETs and treatment with Hh inhibitors have resulted in reduced cell viability [149-151]. Since the Hh pathway is important in cancer initiation and development, it may also be important for tumor radioresistance and regrowth after treatment with ionizing radiation. Hh signaling has been shown to promote radiation resistance, and increased anti-tumor effects have been found when combining ionizing radiation and Hh inhibitors [137, 138, 152].

Reduced normal tissue toxicity

The major side effects after therapy with radiolabeled somatostatin analogs are acute effects on bone marrow (usually reversible) and late effects on kidneys [48, 153, 154]. Achieving a reduced nephrotoxicity could be accomplished by: (1) reducing uptake and retention in the kidneys [155-157], and (2) reducing toxic effects of radiation in the kidneys [93, 94, 158, 159].

Materials and methods

Tumor and animal model (Papers II-VI)

Pieces of GOT1 tumors were transplanted subcutaneously in the neck of 4-week-old female BALB/c nude mice (Charles River, Japan and Germany) [58]. Drinking water and autoclaved food were provided ad libitum. The studies were approved by the Ethical Committee on Animal Experiments in Gothenburg.

Pharmaceuticals (Papers II-VI)

$^{177}LuCl_3$ and [DOTA^0, Tyr^3]-octreotate were purchased from the Nuclear Research & Consultancy Group (IDB Holland, the Netherlands). Preparation and radiolabeling were conducted according to the manufacturer's instructions. Instant thin layer chromatography (ITLC™ SG, PALL Corporation, USA) was used for quality control, with the mobile phase consisting of 0.1 M sodium citrate (pH 5; VWR International AB, Sweden). The fraction of peptide-bound ^{177}Lu was >98 % and the specific activity was approximately 26 MBq/µg octreotate. Saline solution was used to dilute the ^{177}Lu-octreotate stock solution to the desired activity concentration for administration.

Sonidegib (an Hh inhibitor used in **Paper VI,** also known as Odomzo®, erismodegib or NVP-LDE225) was purchased from Active Biochemicals Co., Limited (Hong Kong, China) and dissolved in DMSO as per manufacturer's instructions.

Study design (Papers II-VI)

In total, 103 GOT1-bearing nude mice were included in the experiments contained within this thesis. The workflow of the experiments is shown in **Figure 2**. Control animals were injected with saline solution.

To study the radiobiological effects of ^{177}Lu-octreotate on GOT1 SI-NET in nude mice and determine promising strategies for optimization of the anti-tumor effects among those detailed in **Paper I**, global transcriptional and proteomic response profiles were determined (**Papers II-III**). This

was performed at different time-points (1, 3, 7, and 41 d in **Paper II**, and 1 and 13 d in **Paper III**) after injection of 15 MBq ^{177}Lu-octreotate. The time-points were chosen to represent immediate early (1 d) and early (3 and 7 d) responses, as well as responses during tumor regrowth (13 and 41 d). The non-curative activity (15 MBq) was chosen to induce moderate anti-tumor effects and enable analysis of tumor tissue during regression and re-growth, and to better reflect results observed in the clinic.

In **Paper IV**, GOT1-bearing animals were treated with a priming administration of ^{177}Lu-octreotate followed by a second injection of ^{177}Lu-octreotate 24 h later. Biodistribution and dosimetry studies were performed for 5+10 MBq and 15 MBq (at 1, 3, and 7 d after the last injection), and therapeutic studies were performed for 0.5+14.5 MBq, 2.5+12.5 MBq, 5+10 MBq, 10+5 MBq, 15 MBq, and 30 MBq, evaluating the tumor volume response until 41 d after the last injection. The groups receiving 5+10 MBq were further studied in **Paper V.**

In **Paper VI**, GOT1-bearing mice were treated with either sonidegib (80 mg/kg twice a week via oral gavage), or a combination of sonidegib and 30 MBq ^{177}Lu-octreotate. Tumor volume responses were studied until 41 d after treatment start.

During the study period, tumor volume measurements were performed twice-a-week using calipers (assuming an ellipsoidal shape, **Papers II**, and **IV-VI**) and/or magnetic resonance imaging (MRI, **Papers III-IV**). All tumor volume measurements for each group were expressed as the mean value and standard error of the mean (SEM). Student´s t-test was used to compare data between groups using a two-tailed unpaired t-test, and $p<0.05$ was considered statistically significant.

At the end of experiments, tumor tissue from all animals was excised and divided into two pieces: one piece was instantly frozen in liquid nitrogen for transcriptomic (**Papers II**, and **IV-VI**) or proteomic analysis (**Paper III**), and the remaining piece was weighed and placed in neutral buffered formaldehyde for radioactivity measurements and/or subsequent paraffin embedding.

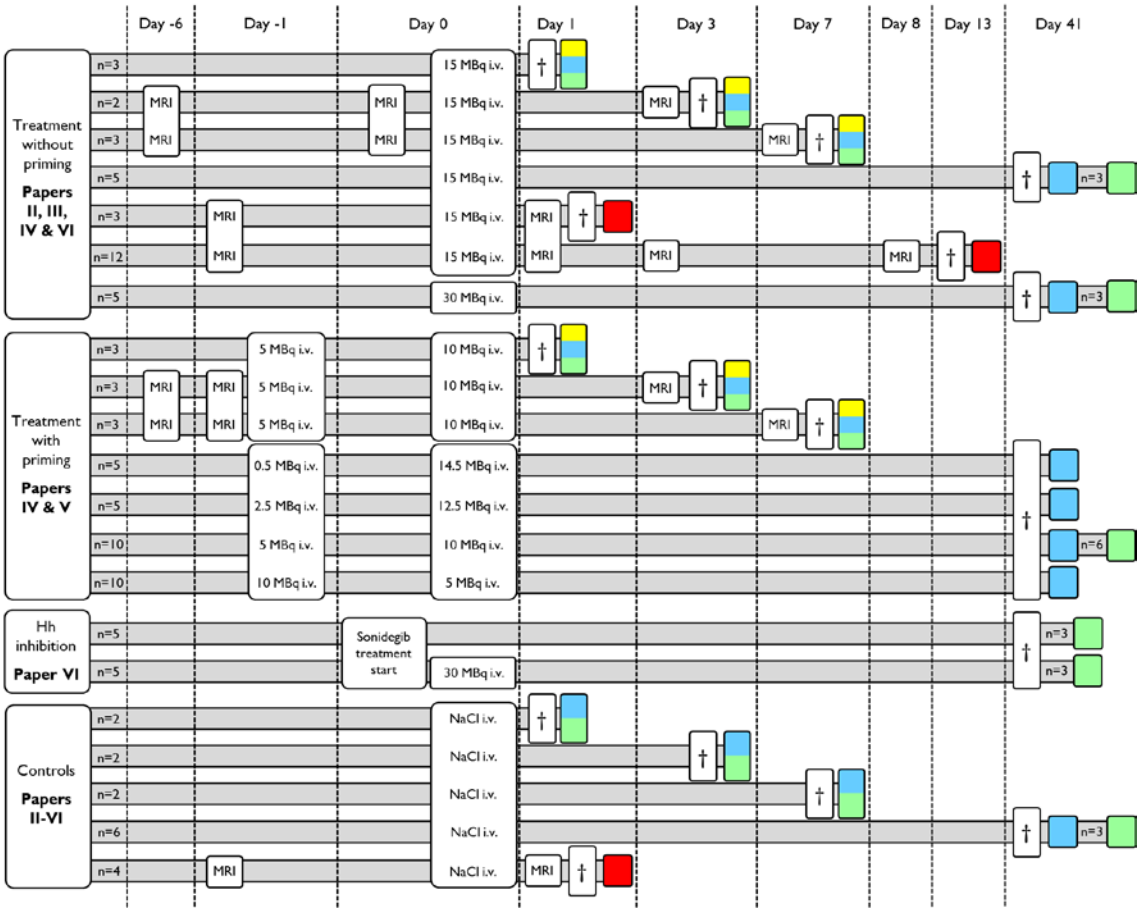

Figure 2: Overview of the animal experiments included in **Papers II-VI.** GOT1-bearing BALB/c nude mice were treated with different amounts of ^{177}Lu-octreotate (with or without a priming injection of ^{177}Lu-octreotate), sonidegib (80 mg/kg body weight twice a week via oral gavage) or saline solution (NaCl). MRI, biokinetics, mean absorbed dose, tumor volume, tumor morphology, gene expression of tumors, and protein expression of tumors, were analyzed. † indicates that animals were killed and dissected; yellow indicates that radioactivity measurements and dosimetric calculations were performed on samples from adrenals, blood, kidneys, liver, lungs, pancreas, spleen, and tumor; blue indicates that tumor samples were fixed in formaldehyde, embedded in paraffin, and subjected to morphological and immunohistochemical (IHC) analyses. Tumor samples from each group were snap frozen in *l*N_2 followed by either RNA extraction and gene expression analysis (indicated by green), or peptide extraction and protein expression analysis (indicated by red).

Radioactivity measurements and dosimetry (Papers II-VI)

^{177}Lu activity during the labeling process and in syringes (before and after injection) was measured using a direct reading well-type ionization chamber (CRC-15R; Capintec, USA). The ionization chamber consists of an aluminum chamber wall and uses argon (high pressure) as the filling gas, and has a measuring range up to 200 GBq (of ^{99m}Tc) [160].

A 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 [161]. A ±10 % energy window over the 208 keV photon peak was used to measure ^{177}Lu activity in tissue samples. The efficiency of the gamma counter was determined against the efficiency of the CRC-15R ionization chamber. Correction for detector background, dead-time loss, and radioactive decay was performed.

The ^{177}Lu activity concentration in each tissue was calculated as percent of injected activity per gram tissue (%IA/g):

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

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.

The tumor-to-normal tissue activity concentration ratio (T/N) was determined according to

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

For animals receiving two injections of ^{177}Lu-octreotate (5+10 MBq, **Papers IV** and **V**), the ^{177}Lu activity concentration and T/N-ratios were estimated for the second administration (10 MBq) only (in order to determine the biokinetics for the second administration, since the 5 MBq administration was supposed to act as a priming administration and not as a therapeutic activity), based on measured ^{177}Lu activity concentration (MBq/g) after 5+10 MBq and subtraction of the contribution from the priming administration. This contribution was estimated by assuming

that the 5 MBq priming activity had the same biokinetics as the single administration of 15 MBq, but extrapolated to the time-points 48 h, 96 h and 192 h after injection, corrected for radioactive decay.

The mean absorbed dose to the tissue was calculated according to the Medical Internal Radiation Dose Committee (MIRD) pamphlet 21 formalism [162]:

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

where $\tilde{A}(r_S,\, T_D)$ is the time-integrated activity in source tissue, r_S, over dose-integration period, T_D ($\tilde{A} = \int_0^{T_D} A(r_s, t)\ dt$), and $M(r_T, T_D)$ is the mass of the target tissue, r_T. The mean energy emitted per nuclear decay i, $\sum_i E_i Y_i$, was approximated to 147.9 keV/decay [56], including β-particles, Auger and conversion electrons. The absorbed fraction, $\phi(r_T \longleftarrow r_S,\, E_i, T_D)$, was set to 1 for all tumors, and $r_T$was set to be the same as r_S in all calculations.

MRI examinations (Papers III and IV)

Tumor volume determination was performed using a dedicated small animal 7T MRI system (Bruker BioSpin MRI GmbH, Germany; software: ParaVision 5.0) with a 72 mm transmit volume coil and a 4 channel array rat brain receiver coil (RAPID Biomedical GmbH, Germany) as previously described [163]. Briefly, a T2-weighted, 2D RARE (rapid acquisition with relaxation enhancement) sequence was used with respiration triggering and fat suppression (repetition time: 4200 ms, effective echo time: 30 ms, signal averages: 3, turbo factor: 4) [164]. Voxel size was ~160*160*700 μm^3 (contiguous slices). Images were processed using software developed in MATLAB (R2011b, The MathWorks, USA). All images were subjected to histogram equalization and a 3*3 median filter, and each tumor was manually delineated on all image slices to calculate the volume by multiplying the total number of voxels with the voxel volume.

Morphological and IHC analyses (Papers II and IV)

Tissue samples in direct proximity to the samples subjected to transcriptomic analysis were fixed in formaldehyde, paraffin-embedded, and processed for histological examination. Sections (3-5 microns) were stained with hematoxylin-eosin or Masson's trichrome to examine tumor morphology. For IHC analysis, sections were placed on positively charged microscope slides and treated with EnVision™ FLEX Target Retrieval Solution (high pH) using a PT-Link (Dako, Denmark). IHC staining for chromogranin A (MAB319; MerckMillipore, Germany), synaptophysin (SY38, M0776, Dako, Denmark), serotonin (M0758, Dako, Denmark), Ki-67 (AB9260; Merck Millipore) and BAX (B-9; sc-7480; Santa Cruz Biotechnology, CA) was performed in an Autostainer Link (Dako, Denmark) according to the manufacturer's instructions. Positive and negative controls were included in each run.

Transcriptional analyses (Papers II, IV-VI)

Frozen tumor tissue was homogenized using the TissueLyser LT (Qiagen, Germany) and total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quantity was determined using an ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA) and RNA quality was determined with the RNA 6000 Nano LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). RNA samples with RNA Integrity Numbers above 6.0 were used for gene expression analysis.

Hybridization of the RNA samples was performed at the Swegene Center for Integrative Biology (SCIBLU, Lund University, Sweden) on Illumina HumanHT-12 v4 Whole-Genome Expression BeadChips (**Papers II** and **IV-VI**, Illumina, CA, USA), containing 47 231 probes per array, or Illumina MouseRef-8 v2 Whole-Genome Expression BeadChips (**Paper II**, Illumina, CA, USA), containing 25 435 probes per array. The BeadChips were analyzed using the Illumina iScan N240 microarray scanner (Illumina, CA, USA). Data pre-processing and quantile normalization were performed on the raw signal intensities using the BioArray Software Environment (BASE) system. Further processing was then performed using Nexus Expression 3.0 (BioDiscovery, CA, USA) as

previously described [165, 166]. Differentially regulated genes (treated *vs.* control) were identified using an adjusted p-value cut-off of <0.01 (Benjamini-Hochberg method [167]) and |fold change|≥1.5 (|$\log_2$-ratio|≥0.58). The gene expression data presented in this thesis have been deposited at the NCBI's Gene Expression Omnibus (GEO accession GSE80024).

Proteomic analyses (Paper III)

Frozen tumor tissue samples were homogenized and 30 µg total protein per sample was taken for trypsin digestion using the filter-aided sample preparation modified from Wisniewski *et al.* [168]. Peptides were labeled with TMT 10plex (Thermo Scientific) for relative quantification, pooled and fractionated using high pH reversed-phase chromatography (Waters XBridge BEH C18 3.0 x 150 mm, 3.5 µm).

Proteomics data were acquired using liquid chromatography tandem-mass spectrometry (LC-MS/MS) with an Easy nanoLC1000 coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). During a 60 min acetonitrile gradient in 0.2 % formic acid, positive ions with an m/z range of 380-1200, and a resolution of 120 000 were scanned. Mass spectrometry (MS) scans were selected for fragmentation (MS2) by collision induced dissociation for identification in the ion trap, while MS3 fragments produced by high energy collision dissociation were detected in the Orbitrap. MS ions were isolated in the quadrupole with a 1.6 m/z window, and a dynamic exclusion of 30 seconds for already identified m/z values.

Protein identification and quantification was performed using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) with the integrated Mascot search engine (Matrix Science, Boston, MA, USA) using the *Homo sapiens* SwissProt database version March 2015 (Swiss Institute of Bioinformatics, Switzerland). Resulting proteins passing a false discovery rate of 1 % and containing at least one unique peptide were further analyzed. For significance testing of the treatment response, a t-test according to Welch was performed using the R statistical computing environment (http://www.r-project.org). For this, data were log transformed, treatment groups were selected, and significance values for protein regulation were calculated using a p-value cut-off of 0.01, and a fold change (treated *vs.* control) cut-off of 1.5.

Bioinformatics analyses (Papers II-VI)

Hierarchical clustering of genes or proteins was performed in the R statistical computing environment, based on regulation patterns using the function hclust {stats} (with the complete linkage algorithm and Lance-Williams dissimilarity update formula [169], version 3.1.1).

Nexus Expression 3.0 (**Papers II-V**) and the DAVID bioinformatics resource tool (**Paper VI**, http://david.abcc.ncifcrf.gov/) [170] were used for functional annotation of regulated genes or proteins with altered levels, and affected GO terms were identified with a p-value cut-off of 0.05. Identified GO terms were classified according to higher level cellular function using an in-house developed categorization model (created using parental GO terms, http://geneontology.org) [128]. Main and subcategories are shown in **Table 4**.

Three of the IPA tools were used in this thesis: (1) the canonical pathway analysis tool (**Papers III**, **V** and **VI**) was used to relate response profiles with signaling pathways, (2) the upstream regulator analysis tool (**Papers II-VI**) was used to identify upstream regulators that could potentially explain changes in mRNA or protein levels, and (3) the diseases and functions analysis tool (**Papers III** and **V**) was used to predict altered biological functions from changes in mRNA or protein levels.

Table 4: Categories of biological processes. Reprint from [171], with kind permission from Britta Langen.

Category	Biological processes that…
DNA integrity	
Damage and repair	*…recognize damage or initiate or facilitate repair pathways*
Chromatin organization	*…maintain the structural integrity of DNA on the chromatin level*
Gene expression integrity	
Transcription	*…are involved in transcription or its regulation*
RNA processing	*…are involved in processing immature or mature RNA or its regulation*
Translation	*…are involved in translation or its regulation*
General	*…are valid for any of the above subcategories*
Cellular integrity	
Physico-chemical environment	*…are associated with e.g. regulation of ion homeostasis or transport*
Cytoskeleton & motility	*… establish or regulate cytoskeleton integrity, chemotaxis or cellular motility*
Extracellular matrix & CM	*…regulate biogenesis of the cellular membrane (CM), maintain the extracellular matrix, regulate cell adhesion, etc.*
Supramolecular maintenance	*…are involved in or regulate e.g. protein (re)folding, protein oligomerization or modification, general transport of molecules or vesicles, etc.*
General	*…are valid for any of the above subcategories*
Cell cycle and differentiation	
Cell cycle regulation	*…are involved in e.g. cell growth, regulation of growth arrest, etc.*
Differentiation & aging	*…regulate e.g. cellular development, proliferation, or aging*
Apoptotic cell death	*…are involved in regulating pro-apoptotic or anti-apoptotic pathways*
Cell death	*…in non-apoptotic cell death, e.g. cytolysis*
General	*…are valid for any of the above subcategories*
Cell communication	
Intercellular signaling	*…facilitate communication between cells, e.g. synaptic or hormone signaling*
Signal transduction	*…regulate or effect signal transduction, e.g. signal processing within a cell*
Metabolism	
Proteins, amino acids	*…regulate or facilitate anabolic or catabolic processes for proteins or amino acids*
Lipids, fatty acids	*…regulate or facilitate anabolic or catabolic processes for lipids or fatty acids*
Carbohydrates	*…regulate or facilitate anabolic or catabolic processes for carbohydrates*
Signaling molecules	*…regulate or facilitate anabolic or catabolic processes for signaling molecules*
Nucleic acid-related	*…regulate or facilitate anabolic or catabolic processes for nucleic acid-related*
Other	*…are part of metabolism but not associated with other specific subcategories*
General	*…are valid for any of the specific subcategories*
Stress responses	
Oxidative stress response	*…respond to e.g. superoxide, hydrogen peroxide, or other reactive oxygen species*
Inflammatory response	*…regulate or facilitate pro-inflammatory or anti-inflammatory responses*
Immune response	*…regulate or facilitate e.g. the acute-phase response, responses to pathogens, phagocytosis, or concern immune-specific biosynthesis*
Other	*…are part of stress responses but not associated with other specific subcategories*
General	*…are valid for any of the specific subcategories*
Organismic regulation	
Behavior	*…regulate behavioral responses of the organism*
Ontogenesis	*…regulate or facilitate developmental processes on the organ or organism level*
Systemic regulation	*…are involved in organismic regulations with systemic relevance*
Reproduction	*…regulate or facilitate e.g. germ cell development, parturition, or pregnancy*

Results

Biodistribution and dosimetry

The biodistribution and dosimetry of ^{177}Lu-octreotate using conventional single injection (15 MBq) and using a priming treatment schedule (5+10 MBq) was studied in **Paper IV.** Mean ^{177}Lu activity concentration in GOT1-bearing mice at 1, 3 and 7 d after the last injection of 15 or 5+10 MBq ^{177}Lu-octreotate is presented in **Figure 3A**. Compared with the values at 1 d, the mean activity concentration in the tumor increased 3 d after priming but decreased for the single administration (15 MBq), with a statistically significant difference between the treatment schedules at 3 d. In the kidneys, the activity concentration decreased equally in both groups, as was observed for blood. For all normal tissues except spleen (with priming) and adrenals (without priming) the activity concentration decreased with time.

The mean T/N ratios for blood, kidneys, liver and spleen were higher when a priming administration was given (**Figure 3B)**. Pancreas, adrenals and lungs in the priming group had a peak in T/N ratio after 3 d and decreased thereafter, while the T/N ratio increased with time in the group without priming. There was a statistically significant difference in T/N ratios in kidneys and pancreas at 3 d in animals receiving treatment with and without priming.

Mean absorbed dose to the tumor per administered activity was 0.51 Gy/MBq after priming and 0.27 Gy/MBq without the priming administration (**Figure 3C**). The kidneys received a lower mean absorbed dose per administered activity with priming compared with no priming (0.25 Gy/MBq vs. 0.32 Gy/MBq).

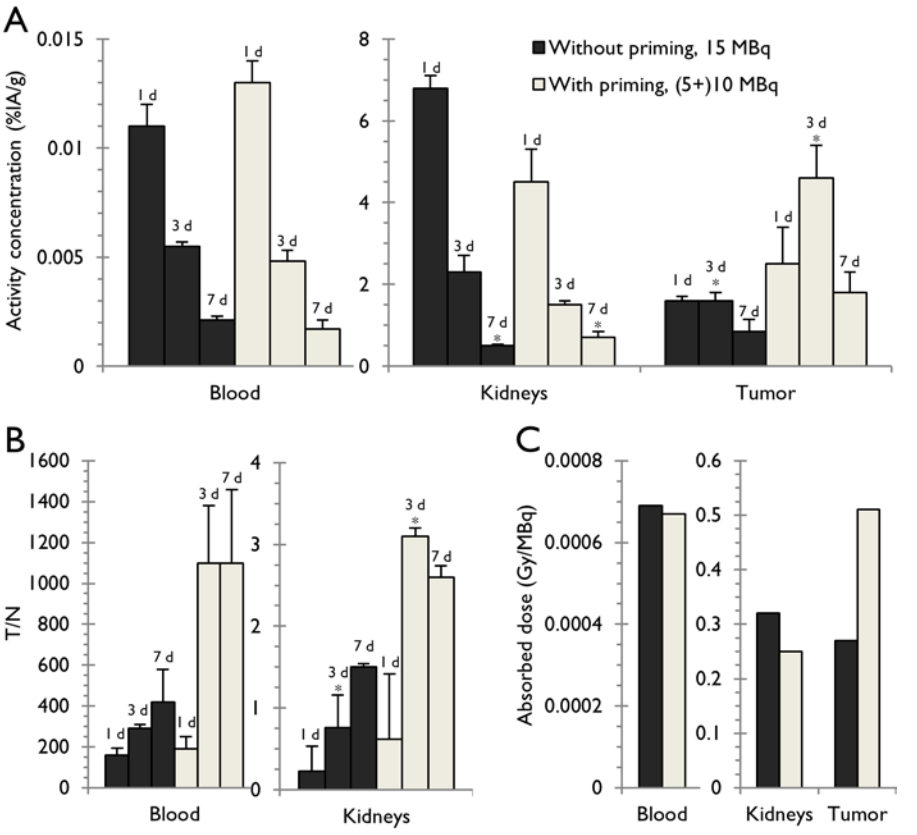

Figure 3: Biodistribution and dosimetry of ^{177}Lu-octreotate in GOT1-bearing nude mice. A: Mean ^{177}Lu activity concentration (%IA/g), B: Mean T/N, and C: Mean absorbed dose per administered activity (Gy/MBq) after treatment with 15 or 5+10 MBq ^{177}Lu-octreotate, respectively, in blood, kidneys and tumor from GOT1 tumor-bearing nude mice. Values for the 5+10 MBq group (activity concentration and T/N) were estimated based on data from the 10 MBq administrations only. Error bars indicate SEM. Statistically significant differences ($p<0.05$) between priming and single administration are indicated by *.

Tumor volume reduction

Single administration of 15 or 30 MBq ^{177}Lu-octreotate in GOT1-bearing mice resulted in a reduced tumor volume relative to the day of injection, with a minimum at 8-9 days for the 15 MBq injections (**Figure 4**, **Papers II-IV**) and at 14 d for the 30 MBq injections (*cf.* **Figure 5**, **Papers IV** and

VI). The tumor volume reduction was followed by regrowth in both groups.

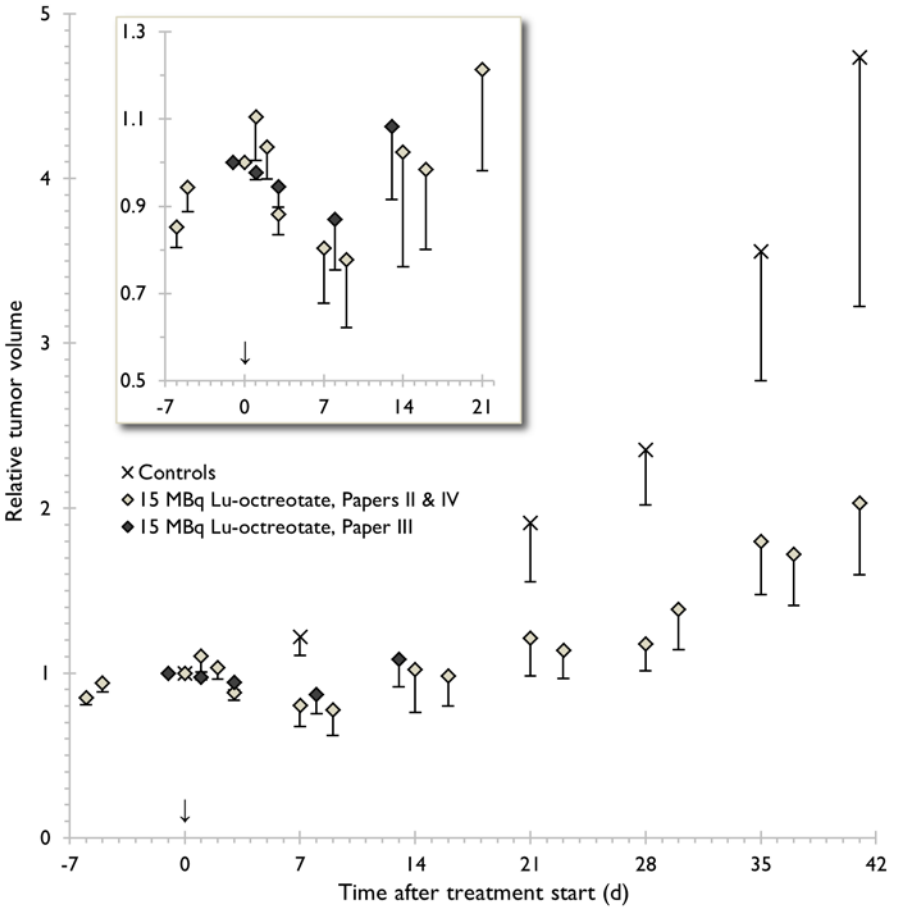

Figure 4: The mean relative tumor volume *versus* time after injection for mice treated with 15 MBq ^{177}Lu-octreotate or NaCl. ^{177}Lu-octretotate therapy resulted in three phases of treatment response; Growth: 6 d before injection to 2 d after injection, Regression: 2–9 d after injection, and Regrowth: 9–42 d after injection. The insert shows the early phase in the treated groups. Tumor volumes were normalized to day 0 (**Papers II** & **IV**) or day -1 (**Paper III**). Error bars indicate SEM. ↓ indicates the time for treatment start.

The anti-tumor effects of a priming treatment schedule (5+10 MBq) in comparison with 15 MBq monotherapy, is shown in **Figure 5A** (**Papers IV-V**). The 5+10 MBq treatment schedule showed the best anti-tumor effects among the evaluated priming schedules (in terms of minimum relative tumor volume and time to regrowth), followed by 2.5+12.5 MBq.

A statistically significant difference in minimum relative tumor volume was observed between these treatment groups and the 15 MBq group ($p<0.05$). In the groups receiving priming, the minimum relative tumor volumes were largest in the 0.5+14.5 MBq and 10+5 MBq groups, but the mean values were generally smaller than if a single administration was given (15 MBq and 30 MBq).

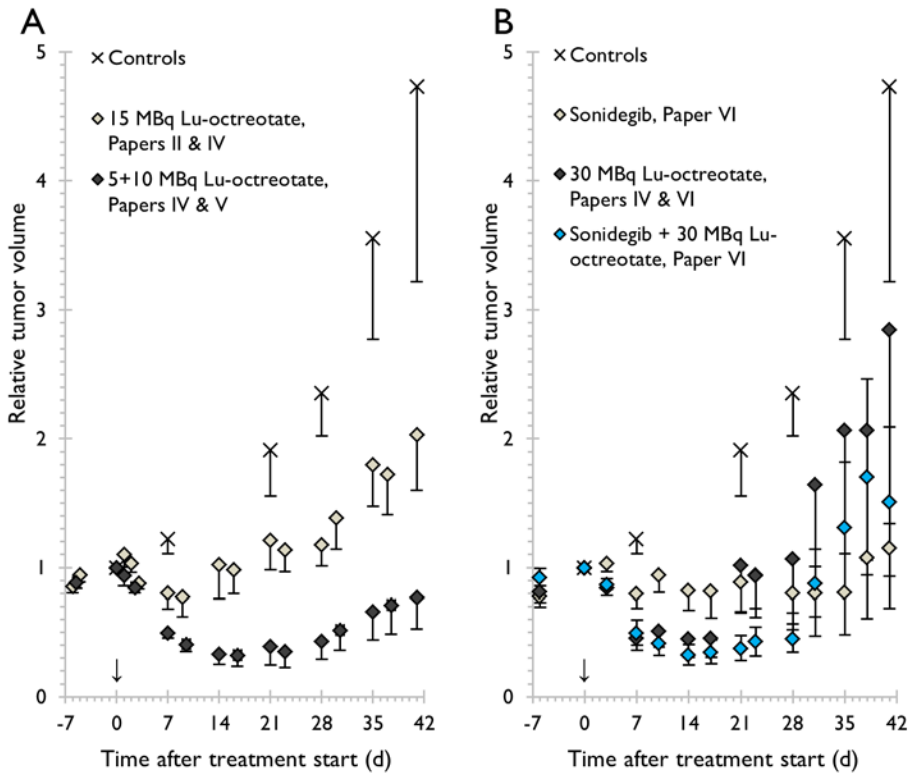

Figure 5: Anti-tumor effect of ^{177}Lu-octreotate using priming or combination therapy in GOT1 in nude mice. The mean relative tumor volume *versus* time after injection for mice treated with A: 15 or 5+10 MBq ^{177}Lu-octreotate or B: Sonidegib, 30 MBq ^{177}Lu-octreotate, or a combination of both pharmaceuticals, compared with controls. Error bars indicate SEM. ↓ indicates the time for treatment start.

Sonidegib monotherapy resulted in significant inhibition of tumor growth (**Figure 5B**, **Paper VI**). Statistically significant differences in mean relative volume between sonidegib-treated animals and controls were found at 7, 21, 28 and 35 d after treatment start. Combination treatment with sonidegib and ^{177}Lu-octreotate caused a reduction in mean relative

tumor volume (**Figure 5B**, **Paper VI**). The minimum relative tumor volume for tumors receiving the combination therapy was lower than in either monotherapy group (sonidegib or 30 MBq ^{177}Lu-octreotate, mean=0.33, SEM=0.09 at 14 d after injection). Furthermore, the combination therapy group had a prolonged time to progression, i.e. time from treatment start to progression of first tumor in the treatment group. There was a statistically significant difference between relative tumor volume in animals treated with a combination of sonidegib and ^{177}Lu-octreotate, and controls at 7, 21 and 28 d after treatment start. In addition, a statistically significant difference between combination treatment and sonidegib monotherapy was found at 10 and 14 d after treatment start. No symptoms of toxic effects were observed in the animals of either group receiving sonidegib.

Morphology of GOT1 tumors

Microscopic examination of xenografts confirmed NETs with a morphology typical of GOT1 (**Papers II** and **IV**). The percentage of tumor cells was in the 70-90 % range. Examination of xenografts from the therapeutic studies showed heterogeneity of tumors, with viable NET cells alternating with areas of fibrosis and inflammation, characteristic for tumor regression. Immunohistochemical staining for the neuroendocrine markers chromogranin A, synaptophysin and serotonin showed strong staining in the tumor cells. A reduction in number of Ki-67 positive tumor cells was found in xenografts from treated animals compared with untreated controls. BAX protein was found to be strongly expressed in all examined tumors.

Global transcriptional and proteomic responses to ^{177}Lu-octreotate therapy

A significant effect on gene- and protein expression levels was observed in GOT1 tumor after administration of 15 MBq ^{177}Lu-octreotate at all time-points studied (**Papers II-IV**). The number of regulated transcripts or proteins with altered expression levels varied with time after injection; of the detected transcripts, 43 (62%), 16 (52%), 50 (59%) and 35 (67%) were uniquely regulated at 1, 3, 7 and 41 d, respectively. Only three genes (*LY6H*, *RNU1A3* and *RNU1-5*) were significantly regulated at all time-points, and seven additional genes were regulated at three of

the four time-points. Hierarchical clustering of the transcriptional profiles for these ten commonly regulated transcripts revealed similarities and differences in gene expression over time. Notably, the response patterns of six non-coding RNAs were clustered together, while the transcriptional profiles for *LY6H*, *CDKN1A* (alias p21) and *EEF1A2* showed low similarity with the other recurrently regulated transcripts.

Annotation of the regulated transcripts to GO terms revealed a significant effect on biological processes associated with cell cycle and differentiation at all time-points studied. Specifically, cell cycle regulation was highly enriched immediate early and early after treatment start, while apoptosis was enriched at early and late time-points. Cellular integrity-associated processes were affected at all time-points, in part due to the immediate early down-regulation followed by up-regulation of *TGFBI* at early time-points. Genes associated with supramolecular maintenance were also differentially regulated at all time-points, e.g. by cytokine secretion via *ABCA1* at early and late time-points, and by *APOE*- and *BAX*-mediated protein complex assembly. The regulation of *CDKN1A* was responsible for the general stress responses seen at immediate early and early time-points. Pathway analysis identified activation of p53 (regulator of e.g. DNA damage response) as a significant up-stream regulator, activated at early time-points after ^{177}Lu-octreotate treatment. IL6 (pro-inflammatory cytokine), TGFß1 (regulator of epithelial-mesenchymal transition and metastasis), and HIF1α (regulator of hypoxic response) were also shown to be significant up-stream regulators at early time-points. At late time-points (41 d), NEDD9 (important in cell attachment, migration and invasion) and TNF (pro-apoptotic cytokine) were shown to be significant up-stream regulators.

In total, 3861 different proteins were quantified in the LC-MS/MS analysis. Among these, 155 proteins had significantly altered levels at either 1 or 13 d after injection, and 20 proteins had significantly altered levels at both time-points.

Analysis of affected biological functions using IPA showed that a variety of functions related to tumor invasiveness and migration were significantly inhibited both early after injection of ^{177}Lu-octreotate, and during regrowth, owing to the altered expression levels of a variety of proteins (*e.g.* CD166, K1C17, LEG1, LMNA and VTNC). Also inhibited early after injection were *e.g.* cytoskeleton organization, tumor

internalization, and tumor endocytosis, while *e.g.* vesicle formation and methylation of DNA were inhibited and activated during regrowth, respectively. Pathway analysis revealed a variety of significantly affected canonical signaling pathways. Several pathways were related to Rho family of GTPases (a subfamily of the Ras superfamily, involved in cytoskeletal cell morphology) both early after injection of ^{177}Lu-octreotate, and during regrowth, owing to the altered expression levels of *e.g.* ARHG1, ARPC4 and BORG4. Most of these Rho-related pathways were inhibited. Several other pathways were inhibited at both time-points, e.g. actin cytoskeleton-, integrin-, CXCR4-, and phospholipase C signaling. Up-stream regulator analysis identified inhibition of SYVN1 (E3 ubiquitin-protein ligase synoviolin, involved in apoptosis inhibition) and activation of RABL6 (RAB member RAS oncogene family-like 6a, involved in cell cycle regulation) early after injection of ^{177}Lu-octreotate. During regrowth, p53 was identified as a significantly inhibited up-stream regulator.

Transcriptional effects of ^{177}Lu-octreotate therapy using a priming treatment schedule

The transcriptomic analysis following ^{177}Lu-octreotate treatment with a priming treatment schedule also revealed a time-dependent response pattern (**Papers IV-V**). Notably, several of the transcripts associated with stress responses were significantly regulated at early time-points, and transcripts with a pivotal role in DNA integrity were only significantly regulated at 3 d after the last injection. Analysis of affected biological functions using IPA showed that tumor cell proliferation was significantly affected at immediate early and early time-points after the last injection of ^{177}Lu-octreotate, due to regulation of *e.g.* the *CDKN1A*, *GDF15*, and *SGK* genes. Apoptotic processes were activated at 3 d after the last injection, due to the regulation patterns of *e.g.* the *BAX*, *GADD45A* and *TNFRS10B* genes. Pathway analysis revealed a variety of significantly affected canonical signaling pathways, *e.g.* PI3K/AKT signaling at 1 d, unfolded protein response (UPR) at 3 d, p53 signaling at 3 and 7 d and Wnt/β-catenin signaling at 41 d. p53 was also identified as an activated upstream regulator at early time-points after the last injection of ^{177}Lu-octreotate. Other upstream regulators with predicted activation states were ANXA2 (Annexin A2, involved in in the regulation of cellular growth and in signal transduction, predicted to be inhibited) and KDM5B (Lysine Demethylase 5B, a histone demethylase involved in

the transcriptional repression of certain tumor suppressor genes, predicted to be activated) at 3 d and PARP1 (Poly(ADP-Ribose) Polymerase 1, involved in DNA strand break repair, predicted to be inhibited) at 7 d after the last injection of ^{177}Lu-octreotate.

Transcriptional effects of combined sonidegib and ^{177}Lu-octreotate treatment

Genome-wide transcriptional analysis of total RNA revealed a large variation in total number of regulated genes between treatment groups. Altogether, 7, 106 and 496 transcripts were significantly regulated in the sonidegib, ^{177}Lu-octreotate, and combination treatment groups, respectively. The number of uniquely regulated transcripts in each group was 4, 7, and 397, respectively, while two genes (corresponding to three transcripts), *BCL11A* (involved in negative p53-regulation) and *CXCR7* (encoding a chemokine receptor), were regulated in all treatment groups. The *EVC2* and *PDGFRA* genes involved in the Hh pathway were among the four uniquely regulated transcripts in the sonidegib treatment group.

GO analysis revealed regulation of several genes related to apoptotic cell death and cell cycle regulation (including several genes related to cell cycle arrest or DNA replication) in the ^{177}Lu-octreotate and combination therapy groups, but none in the sonidegib group. Pathway analysis showed that the NF-KB signaling pathway was significantly affected in the group treated with sonidegib monotherapy – owing to the unique up-regulation of the *PDGFRA* gene (encoding the platelet-derived growth factor receptor, alpha polypeptide). The Wnt/ß-catenin signaling pathway was significantly affected in both the ^{177}Lu-octreotate and combination therapy groups, where the *SOX2*, *TLE4*, and *WNT11* genes were down-regulated in both groups. However, a larger number of genes in the Wnt/ß-catenin pathway were affected in the combination therapy group. The PI3K/AKT-, G-protein coupled receptor-, and Notch-signaling pathways were also affected in the combination therapy group.

Discussion

The biodistribution and dosimetry studies in **Paper IV** showed that the activity concentration in the tumor was higher using a priming administration, and increased until 3 d after the last injection. In several *in vivo* studies with ^{177}Lu-octreotate treatment of NETs, rapid tumor shrinkage has been associated with a long retention time or even increasing tumor activity concentration over time [121, 172, 173]. This type of uptake and retention of ^{177}Lu-octreotate was also found in the present study. However, the decrease in activity concentration in the tumor was nearly 60 % between 3 d and 7 d in the 5+10 MBq group compared with a 25 % decrease for the 15 MBq group, indicating that ^{177}Lu-octreotate probably was degraded and released faster from the cells after 5+10 MBq than after 15 MBq administrations. Despite these results, the activity concentration (in %IA/g) was still higher in the 5+10 MBq group at 7 d.

In non-tumor tissue, the mean activity concentration after 1 d was higher in the SSTR-expressing tissues after priming (5+10 MBq group). These findings are not in accordance with previous results, which did not show any increased ^{177}Lu-octreotate uptake in normal tissues [124]. An up-regulation of *SSTR* was not observed in the tumors at the time-points studied. An alternative explanation to the higher activity concentrations found in tumor and SSTR-expressing non-tumor tissues might be that the priming administration influences vascular perfusion. Previous studies have shown an increase in perfusion in tumor tissue shortly after irradiation with X-rays, followed by a decrease in perfusion at later times after irradiation, and a subsequent increase to control levels at 7 to 11 days after irradiation [122, 123]. This indicates that priming might increase perfusion in the tissue, resulting in an increased tumor uptake of the second injection of ^{177}Lu-octreotate. Since the activity concentration was retained to a higher extent in the tumor tissue than in the kidneys and in blood, the T/N ratios for the kidneys and blood were higher after priming, which is reflected by differences in mean absorbed dose. These findings indicate that the risk of renal toxicity would be lower or similar if priming was used, which would expand the therapeutic window.

In **Papers II-III,** a non-curative treatment schedule (15 MBq/injection) was chosen to better reflect the clinical situation and enable analysis of tumor tissue during regression and re-growth. In this model, all tumors showed growth before treatment, and ^{177}Lu-octretotate therapy initially resulted in a reduction of tumor volume, followed by regrowth.

The therapeutic studies utilizing a priming treatment schedule (**Papers IV-V**) showed that the best overall therapeutic effect was found for the 5+10 MBq group. To further optimize ^{177}Lu-octreotate therapy, and increase the cure rate, the long-term anti-tumor effects of priming must be examined in more detail, as well as the mechanisms responsible for the increased tumor uptake of ^{177}Lu-octreotate. Furthermore, studies on the difference in long-term adverse effects between different treatment schedules are needed, especially concerning adverse effects on *e.g.* the kidneys.

Sonidegib is a selective and orally bioavailable antagonist of SMO [174], which has previously shown an anti-tumor effect in NET models [175]. It has received FDA approval for treatment of basal cell carcinoma, and is currently being investigated as a potential treatment for various cancer forms (*e.g.* small cell lung cancer) [176]. Common side effects include neutropenia, anemia and loss of taste sensation [176, 177]. Treatment with Hh inhibitor sonidegib (administered orally two times/week, **Paper VI**) resulted in inhibition of GOT1 tumor growth over time. The initial tumor volume response in the animals treated with a combination of sonidegib and ^{177}Lu-octreotate mimicked that of the ^{177}Lu-octreotate monotherapy. However, the time to progression was longer in the combination therapy group, resulting in the lowest mean tumor volume at the time of study end. This indicates a potential benefit when using Hh inhibitors in combination with ^{177}Lu-octreotate for treatment of SI-NETs. However, further studies on the difference in adverse effects between different treatment schedules are needed, especially concerning risk organs (*e.g.* kidneys and bone marrow).

The global transcriptional and proteomic analyses of GOT1 responses to 15 MBq ^{177}Lu-octreotate treatment (**Papers II-III**) resulted in the prediction of several up-stream regulators, *e.g.* IL6, FOXO3/4, TNF, TGFβ1, HIF1α, p53, SYVN1 and RABL6, suggesting radiation-induced apoptosis, followed by activation of pro-survival mechanisms in the tumor cells [89]. This was also evident in the remainder of the transcriptional studies (in **Papers IV-VI**). p53 activation can promote cell

survival or activate cell death mechanisms, depending on the extent of DNA damage [80]. Several genes involved in p53-signaling were regulated in **Papers II**, and **IV-VI**, corresponding to both growth arrest (*e.g. ACTA2*, *BTG3, CDK6*, *CDKN1A*, *CDKN1B, CDKN2A, CTGF, DDT4* and *SGK*) and apoptosis (*e.g. APOE*, *BAX*, *BHLHE40*, *BIK, BNIP3*, *GADD45A* and *PBK*) [178-184]. Up-regulation of the *APOE* and *BAX* genes at 3 d after injection suggests an activation of the intrinsic apoptotic pathway during tumor regression, while up-regulation of *ADORA2A*, *BNIP3*, *BNIP3L* and *HSPB1* at 41 d after injection is compatible with the inhibition of the intrinsic apoptotic pathway during tumor regrowth [185-187]. However, the target genes for the prediction of apoptosis activation suggest that both the intrinsic (via *e.g.* the *BAX*, *GADD45A* and *PBK* genes [188-190]) and extrinsic (via *e.g.* the *TNFRSF10B* gene [191]) apoptotic pathways are implicated in the response to 5+10 MBq ^{177}Lu-octreotate (**Papers IV-V**). This is in contrast to the observed effects of 15 MBq monotherapy where only the intrinsic apoptotic pathway was affected (**Paper II**). In comparison with the results from the study using 15 MBq monotherapy, no anti-apoptotic functions were affected during regrowth following treatment with 5+10 MBq ^{177}Lu-octreotate. This may account for the slower regrowth observed with the priming treatment schedule.

The proteomic response of intravenous injection of 15 MBq ^{177}Lu-octreotate (**Paper III**) revealed alterations of multiple biological functions and signaling pathways including suppression of invasive potential in GOT1 tumor xenografts (*e.g.* CXCR4-, Phospholipase C-, ILK-, integrin-, paxillin-, calpain-, Rho-, and protein kinase A signaling) [192-200]. This may indicate that PRRT could have an advantage over conventional photon radiotherapy due to possible preventive effects on invasiveness and metastatic formation in irradiated malignant tumor cells.

UPR was affected at early time-points in **Paper V**. UPR is a stress response pathway, which is caused by endoplasmic reticulum stress. Protein folding occurring in the endoplasmic reticulum is extremely sensitive to environmental changes regarding e.g. reactive oxygen species or inflammatory stimuli, and studies have shown that endoplasmic reticulum stress can induce apoptosis (mediated by e.g. JNK-signaling) and enhances the radiosensitivity of tumor cells by degradation of RAD51 and subsequent reduction of DNA double strand break repair [201, 202]. Furthermore, the prediction of PARP1 as an inhibited upstream regulator at 7 d also suggests an impaired ability to repair

DNA double strand breaks. This response was not seen in the study of 15 MBq ^{177}Lu-octreotate monotherapy, and may be a contributing factor in the increased anti-tumor effect of a priming treatment schedule. This suggests that the use of radiosensitizers to enhance the anti-tumor effects of ^{177}Lu-octreotate may be even more efficient when combined with a priming treatment schedule.

In the combination therapy study presented in **Paper VI,** *CXCR7* and *BCL11A* were regulated in all groups. Treatment with a CXCR7 antagonist has been shown to delay tumor growth and increase survival rates in human breast cancer models [203]. *CXCR7* has also been identified as a possible downstream target of Hh pathway members GLI1 and GLI2 [146]. The *BCL11A* gene was down-regulated in all three treatment groups. It negatively regulates p53 by directly regulating the *BCL2*, *BCL-xL*, *MDM2* and *MDM4* genes. Consequently, down-regulation of the *BCL11A* gene might result in apoptotic and proliferative defects [204]. The *EVC2* and *PDGFRA* genes regulated in the sonidegib monotherapy group have been associated with Hh signaling. The EVC2 protein binds to SMO after it accumulates in cilia in response to Hh ligands, and up-regulation of the *EVC2* gene can activate the Hh pathway downstream of SMO, but upstream of GLI transcription factors [148]. The *PDGFRA* gene is a transcriptional target of GLI1, and down-regulation of *PDGFRA* has previously been associated with decreased GLI1 levels despite Hh pathway activation [205]. The observed up-regulation of the *EVC2* and *PDGFRA* genes in the sonidegib group may therefore correspond to activation of the Hh pathway downstream of SMO, countering the effect of the SMO antagonist sonidegib.

Several of the affected pathways detected in **Paper VI** are known to be involved in cancer development (e.g. Wnt/ß-catenin-, PI3K/AKT-, G-protein coupled receptor-, and Notch signaling) [21]. Down-regulation of several key components of the Wnt/ß-catenin pathway (*e.g.* FZD9 and *WNT11*) in the combination therapy group suggests that evasive radioresistance may be reduced following this treatment regimen [206]. The PI3K/AKT signaling pathway has previously been recognized as a possible candidate for combination therapy with PRRT, since the mTOR signaling pathway is often up-regulated in NETs and the mTOR inhibitor everolimus has shown promising anti-NET results [207]. However, a previous study found that a combination treatment with everolimus and ^{177}Lu-octreotate promotes metastasis formation in a pancreatic NET model in rats [207]. G-protein coupled receptor signaling

is a major factor in many cellular functions in cancers [208]. These diverse biological functions complicate an interpretation of the predicted effect on G-protein coupled receptor signaling. However, the unique up-regulation of the *GNAS* gene in the combination therapy group indicates a possible inhibition of the Hh pathway. The *GNAS* gene encodes the heterotrimeric Gs-protein α subunit (Gαs), which transmits various G-protein coupled receptor signals regulating, *e.g.* cell growth and survival. Previous *in vivo* studies have shown that the *GNAS* gene can act as a tumor suppressor in Hh-driven medulloblastomas [209]. Notch has a direct role in DNA damage response and Notch inhibitors have been considered for treatment of various cancers in combination with radiotherapy. Inhibition of Notch has been shown to prevent up-regulation of Notch ligands, *e.g. DLL1*, after radiotherapy in breast cancer cells, and the down-regulation of *DLL1* in the combination therapy group in the present study may indicate a possible explanation of the mechanism involved in the enhanced anti-tumor effects in this treatment group [210, 211].

Conclusions

Much work is still needed to obtain optimal treatment results using radiolabeled somatostatin analogs. This thesis summarizes different strategies, some focused on enhanced treatment effects on tumor tissue and others on reduced side effects of normal tissues. Furthermore, two different strategies to enhance the therapeutic effects of ^{177}Lu-octreotate in nude mice bearing GOT1 human small intestine neuroendocrine tumors were evaluated. The conclusions related to the specific aims of this thesis are summarized as follows:

- the literature study in **Paper I** resulted in three major venues for optimization of SSTR-mediated PRRT: (1) individualized treatment planning, (2) increased anti-tumor effects, and (3) reduced toxic effects on normal tissues. None of the identified strategies within these venues has been fully optimized for clinical use

- the global transcriptional and proteomic responses of intravenous injection of 15 MBq ^{177}Lu-octreotate in GOT1 tumors (**Papers II-III**) revealed time-specific response in terms of biological processes. This indicates that timing has to be considered in treatment modalities using multiple administrations of therapeutic agents. Treatment effects on p53-related cell cycle regulation, angiogenesis, ER-stress, and oxidative stress suggest that combination therapy with ^{177}Lu-octreotate and other anti-cancer agents such as angiogenesis inhibitors, unfolded protein response inhibitors, or proteasomal inhibitors may have favorable effects for treatment of NETs

- a priming administration of ^{177}Lu-octreotate 24 h before a subsequent administration of ^{177}Lu-octreotate revealed a higher uptake and an almost two-fold higher mean absorbed dose to tumor tissue compared with a single administration schedule (**Paper IV**). The mean absorbed doses to the dose limiting organs – i.e. the kidneys and bone marrow – were lower or similar between the groups

- tumor volume measurements revealed that the groups receiving 5+10 MBq and 2.5+12.5 MBq ^{177}Lu-octreotate showed better anti-

tumor effects than the 15 MBq single injections (**Paper IV**). The best overall therapeutic effect was found for mice injected with 5 MBq followed by 10 MBq ^{177}Lu-octreotate

- the transcriptional responses in GOT1 tumors from the group receiving a priming treatment schedule revealed increased cellular stress compared with those seen after ^{177}Lu-octreotate monotherapy (**Paper V**). Effects on unfolded protein response indicated oxidative stress in the tumors, which may account for the increased anti-tumor effects by impairing DNA double strand break repair

- combination therapy of GOT1 tumors in nude mice using sonidegib and ^{177}Lu-octreotate (**Paper VI**) resulted in a profound reduction in tumor volume shortly after treatment start, similar to the effect of ^{177}Lu-octreotate monotherapy. In contrast to the ^{177}Lu-octreotate monotherapy, a prolonged time to progression (tumor regrowth) was observed in the combination therapy group. Gene expression analysis revealed an interaction between sonidegib and ^{177}Lu-octreotate, affecting several cancer-related signaling pathways (*i.e.* Wnt/ß-catenin, PI3K/AKT, G-protein coupled receptor, and Notch) not affected by either monotherapy.

Future aspects

The work presented in this thesis has revealed several aspects which should be examined further.

Individualized treatment planning, including optimal choice of radiopharmaceutical and somatostatin analog, is a relatively straightforward way to increase the absorbed dose to the tumor. To achieve this, there is a need for further research in animal models and eventually in patients, comparing biodistribution and dosimetry, anti-tumor effects, and adverse effects of different radionuclides bound to different somatostatin analogs. For tumors with relatively low SSTR-expression, where receptor saturation may become a dose-limiting factor, there might be a potential benefit in combining several radiolabeled somatostatin analogs, with high affinity for different SSTR-subtypes. In the patient situation, the choice of somatostatin analog should then be made based on individual SSTR-subtype-expression, considering that the expression might vary between primary tumor and metastases in the same patient.

By combining radiolabeled somatostatin analogs with other compounds, treatment effects can be enhanced. There are a variety of different compounds that may be interesting in this setting. For example, combination with other radiolabeled compounds (*e.g.* ^{131}I-MIBG or radiolabeled anti- or affibodies) could be useful, provided that the risk-organs for these compounds are not the same as for the radiolabeled somatostatin analog. Other choices for combination therapy are *e.g.* radiosensitizing agents (which act to potentiate the effects of the radiation), or to use the radiolabeled somatostatin analog as a "chemosensitizer" (to potentiate the effect of the other compound).

Compounds with treatment effects on signaling pathways affecting apoptosis or cell cycle regulation in the tumors may also be of interest, to increase cellular stress in tumors by different mechanisms than those related to radiation response. Future studies on combination therapies should also focus on determining optimal dosage of radiolabeled somatostatin analog and the combination-compound, together with the time schedule for administration of the two compounds in order to optimize anti-tumor effects.

Although there is a known toxicity-profile for many of the anti-tumor compounds proposed in the literature, the mechanisms that increase the anti-tumor effects in the combination therapy setting may also increase unwanted side-effects. The studied combination therapies (including the use of sonidegib or other Hh inhibitors) need to be examined more thoroughly in animal models, with focus on adverse effects on healthy organs and tissues, before studies on patients can be considered.

Considering the promising results from the studies using priming the study design should be used in other animal models (*e.g.* GOT2 and CLB-BAR) to investigate if these results can be repeated in other NETs, Future studies should also aim to determine the mechanisms responsible for the increased tumor uptake of ^{177}Lu-octreotate when using a priming treatment schedule. Furthermore, studies on the difference in long-term adverse effects between different treatment schedules are needed, especially concerning adverse effects on *e.g.* the kidneys, even though observations in this thesis suggest an increased therapeutic window. A translation to clinical studies in this setting is feasible, considering that the total activity administered to the patient would initially be the same. In the patient situation, the optimal size relation of the priming and subsequent administrations would need to be studied as well as the optimal time between administrations.

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