Improvement of ^{177}Lu-octreotate treatment of small-intestine neuroendocrine tumors by hyperfractionation

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Gothenburg, Sweden, 2023

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Cover illustration by Joakim Hanner

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ISBN 978-91-8069-471-1 (Print)
ISBN 978-91-8069-472-8 (PDF)

Printed by Stema Specialtryck AB, Borås, Sweden 2023

“Nothing's ever the same,” she said.
“Be it a second later or a hundred years.
It's always churning and roiling.
And people change as much as oceans.”

— Neil Gaiman, The Ocean at the End of the Lane

Abstract

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Neuroendocrine tumor incidence is steadily rising, and the late diagnosis often results in metastatic disease and current treatments mostly prolong life and increase quality of life without increasing cure rate. ^{177}Lu-octreotate was recently approved for treatment of neuroendocrine tumors, but the dosage scheme should be optimized. The aim of this thesis was to study the effects of fractionated administration of ^{177}Lu-octreotate in the human GOT1 tumor mouse model.

GOT1 bearing mice were given ^{177}Lu-octreotate as a single or fractionated administration. The biodistribution and tumor volume response was followed with time. DNA and RNA were extracted from tumor tissue. DNA methylation was evaluated, and expression of genes involved in apoptosis determined.

Hyperfractionated administration gave a more pronounced anti-tumoral effect and longer progression-free survival than single administration with the same total amount of ^{177}Lu-octreotate. The methylation analysis of genes and promoters revealed ^{177}Lu-octreotate treatment specific responses across the groups. Altered expression of apoptosis related genes in regrown tumors was modest, with varying commonalities between the groups. Hyperfractionation generally resulted in a different apoptotic gene expression pattern compared with single-administration in regrown tumors. Hyperfractionation led to higher absorbed dose to tumor and lower to kidneys than single-administration. Expression of genes related to apoptosis in tumors were similar between groups early after high dose level of ^{177}Lu-octreotate, but sometimes with a trend towards higher gene regulation for the hyperfractionated groups. The pro apoptotic genes *BAX*, *FAS*, *GADD45A* and *TNFRSF10B* were significantly regulated at several early time-points for both high dose groups.

In conclusion, hyperfractionation of ^{177}Lu-octreotate shows promise compared with single administration, and should be tested clinically.

Keywords: Peptide receptor radionuclide therapy, PRRT, somatostatin receptor, SSTR, apoptosis, gene expression, epigenetic effects

Sammanfattning på svenska

Cancer är en av våra vanligaste sjukdomar idag och en av våra vanligaste dödsorsaker i Sverige (omkring 25%). Idag är de huvudsakliga behandlingsformerna för cancer kirurgi, kemoterapi och extern strålbehandling. En tidigt upptäckt cancer har oftast en god chans till bot. För en sent påkommen cancer som har spritt sig, kan det vara svårt att bota patienten med de huvudsakliga behandlingsformerna. En mindre vanlig behandlingsform är målsökande radioaktiva läkemedel, som består av en radioaktiv atom som är bunden till en bärarmolekyl som kan binda sig till speciella mottagare (t ex receptorer) på tumörcellernas yta. Det radioaktiva läkemedlet injiceras oftast direkt in i blodet i patienten, cirkulerar med blodet och binder till receptorer på tumörcellerna. Det radioaktiva ämnet kan sedan bestråla alla tumörer i kroppen lokalt.

^{177}Lu-oktreotat (även kallat ^{177}Lu-DOTATATE och Lutathera®) är ett exempel på ett radioaktivt läkemedel. Det är idag ett godkänt läkemedel som används kliniskt för behandling av patienter med neuroendokrina tumörer som har receptorer för det kroppsegna hormonet somatostatin. Läkemedlet består av en radioaktiv atom av grundämnet lutetium (^{177}Lu) som är kopplad till en bärarmolekyl som imiterar somatostatin. ^{177}Lu-oktreotat binder till somatostatinreceptorerna och transporteras sedan in i tumörcellerna där bestrålningen skadar tumörcellerna som förhoppningsvis dör. Begränsningen för denna behandlingsform är biverkningar i friska vävnader, de så kallade riskorganen. Idag skänker behandlingen med ^{177}Lu-oktreotat ökad livslängd och livskvalitet, men patienterna blir sällan botade.

Målet med denna avhandling var att undersöka om behandlingseffekten av ^{177}Lu-oktreotat i neuroendokrina tunntarmstumörer kan ökas genom att dela upp en behandlingsomgång (fraktion) i flera omgångar (hyperfraktionering). Detta studerades i möss som var transplanterade med en mänsklig typ av neuroendokrin tunntarmscancer. Vid behandlingarna undersöktes effekten av olika dosnivåer och med olika tidsintervaller mellan fraktionerna.

Resultaten visar att behandling med hyperfraktionering leder till kraftigare minskning av tumörstorlek samt en längre tid till återväxt av tumörerna. En större ansamling av det radioaktiva läkemedlet upptäcktes i de tumörer som fick hyperfraktionerad behandling, gentemot andra organ i kroppen. Upptag i friska vävnader ökade också med hyperfraktionering, med undantag för njurarna (som är ett av riskorganen i dessa behandlingar) där upptaget blev något lägre. Detta tyder på att man kan öka stråldosen till tumörvävnaden vid hyperfraktionering och då få en större behandlingseffekt på tumörvävnaden utan att öka risken för biverkningar på njurarna. Kanske kan hyperfraktionering även leda till fler behandlingsomgångar innan gränserna för vad riskorganen klarar är uppnådda. Studierna av de underliggande (biologiska) mekanismer av behandlingen gav information som kan användas för att ytterligare förbättra behandlingen i framtiden.

Sammanfattningsvis visar dessa studier att hyperfraktionering med ^{177}Lu-oktreotat har potential att ge en förbättrad behandling av patienter med vissa neuroendokrina tumörer, och metoden bör testas kliniskt.

List of papers

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

I. Elvborn, M., Shubbar, E., & Forssell-Aronsson, E. (2022). *Hyperfractionated Treatment with ^{177}Lu-Octreotate Increases Tumor Response in Human Small-Intestine Neuroendocrine GOT1 Tumor Model.* Cancers, 14(1), 235.

II. Elvborn, M., Rassol, N., Pettersson, D., Shubbar, E., Spetz, J., Helou, K., Forssell-Aronsson, E.
Biological effects in regrown tumors in GOT1 mouse model after hyperfractionated ^{177}Lu-octreotate treatment. Manuscript.

III. Elvborn, M., Rassol, N., Pettersson, D., Spetz, J., Shubbar, E., Helou, K., Forssell-Aronsson, E.
Biodistribution and early effects after hyperfractionated administration of ^{177}Lu-octreotate in GOT1 tumor-bearing mice. Manuscript.

IV. Elvborn, M., Rassol, N., Pettersson, D., Shubbar, E., Spetz, J., Helou, K., Forssell-Aronsson, E.
Late apoptotic effects after treatment with ^{177}Lu-octreotate in small-intestine neuroendocrine GOT1 tumor model. Submitted manuscript.

Content

Abbreviations

^{111}In	Indium-111
^{177}Lu	Lutetium-177
^{90}Y	Yttrium-90
Bq	Becquerel
cDNA	Complementary DNA
CgA	Chromogranin A
DNA	Deoxy ribonucleic acid
DOTA	Dodecane tetraacetic acid
EBRT	External beam radiotherapy
ER	Endoplasmic reticulum
GO	Gene Ontology
GOT1	Human small-intestine neuroendocrine tumor cell line
GPCR	G protein-coupled receptor
Gy	Gray
H&E	Haematoxylin & Eosin
IHC	Immunohistochemistry
i.v.	Intravenous
keV	Kilo electron volt
MIRD	Medical Internal Radiation Dose Committee
mRNA	Messenger RNA
NaCl	Sodium chloride
NET	Neuroendocrine tumor
OS	Overall survival
PET	Positron emission tomography
PCR	Polymerase Chain Reaction
PFS	Progression Free Survival
PRRT	Peptide Receptor Radionuclide Therapy
RNA	Ribonucleic Acid
RT-qPCR	Quantitative reverse transcription PCR
SI	Small intestine
SPECT	Single photon emission tomography
SST	Somatostatin
SSTA	Somatostatin analog
SSTR	Somatostatin receptor
T/N	Tumor-to-normal-tissue activity concentration ratio
T/B	Tumor-to-blood activity concentration ratio
UPR	Unfolded protein response

Background

Radionuclide therapy for patients with NETs

Neuroendocrine tumors

Neuroendocrine tumors (NETs) are malignant neoplasms whose attributes are gradual progression and non-specific clinical presentation. Diagnosis usually occurs at a late stage with distant spreading from the primary tumor. The most common origin of gastro-entero-pancreatic-NETs (GEP-NETs) is the bronchopulmonary system or the gut [1-3]. NETs are regularly spread to the liver and is known to be found metastasized even when the location of the primary tumor is unknown [4, 5].

GEP-NET annual occurrence is estimated to range between 2.5 and 5 cases per 100 000 individuals [3, 6]. Historically, there has been a discrepancy of NET incidence in different parts of Europe [7] and in recent decades, there has been a considerable escalation of the incidence of NETs in a large part of the world [8-11]. The increase can partly be attributed to diagnostic advancements and implementations, but not completely [6]. The incidence has also been reported to differ between populations. As an example, the overall NET incidence has been reported to be from almost 50% and up to 100% higher in the African American population, compared to its Caucasian counterpart [1, 4].

Neuroendocrine system and somatostatin

In the early 1970s, a study was performed to isolate a hypothesized release factor for the growth hormone somatotropin –a study that lead to finding and characterizing its counterpart, the somatotropin-release inhibiting factor that was later given the name “somatostatin” [12]. Somatostatin (SST) binds to receptors (SSTRs), part of the family of G protein-coupled receptors, GPCRs, located in the cell membranes. Somatostatin acts as a secretion inhibitor of many hormones in the body, including most hormones in the gastrointestinal tract [13-15]. After binding to the

SSTR, somatostatin internalizes through endocytosis, mediated by the protein β-arrestin [16, 17].

There are essentially five subtypes of somatostatin receptors, SSTR 1–5, that display some differences in function [18]. The subtype receptor SSTR2 exists in two isoforms, SSTR2A and SSTR2B, as a result of alternative splicing of the transcript [19, 20]. The location of the SSTR genes differ between humans and mice, e.g., SSTR2 is located in chromosome 17 for humans and chromosome 11 for mice [21, 22]. SSTR2 is expressed in a wide range of human tumors, including adenomas, meningiomas, breast tumors and GEP-NETs [23]. There are several analogues to somatostatin, including octreotide, lanreotide and octreotate, which have different affinities to the different SSTRs and longer half-time in the circulation than somatostatin, whose half-time is too short to be used clinically [24].

Excessive release of signaling substances from well-differentiated NETs are connected to a paraneoplastic syndrome resulting in cutaneous flushing [25, 26], diarrhea and other ailments, commonly referred to as carcinoid syndrome (CS) [27-29]. Several factors seem to be connected to CS, e.g. high serotonin levels and rates of metastases in patients, especially in the liver [28, 29]. Prolonged secretion of serotonin and other blood vessel affecting substances can lead to forming of fibrotic tissue in the body and even carcinoid heart disease, CHD, (also referred to as Hedinger's syndrome) or fibrosis [30]. The CS symptoms can be relieved and the growth of NET can be inhibited by treatment with somatostatin analogues (SSTAs) [31-33]. The somatostatin analog lanreotide, is administrated intramuscularly or subcutaneously every 2 or 4 weeks, respectively [34], and has been reported to be more effective than short-acting octreotide in terms of relieving CS [35].

Somatostatin analogues can also be labeled with radionuclides for targeting malignant tissues overexpressing SSTRs, called peptide receptor radionuclide therapy (PRRT). One such combination is ^{177}Lu-octreotate (^{177}Lu-[DOTA^{0},Tyr^{3}]-octreotate, ^{177}Lu-DOTATATE, Lutathera®), where the ^{177}Lu is bound to an SSTA via a chelate [36]. In addition to antitumoral attributes, PRRT with ^{177}Lu-octreotide is reported to also ease symptoms like fatigue, nausea and diarrhea [37].

Binding and internalization of ^{177}Lu-octreotate

After binding of an SST or SSTA agonist molecule to an SSTR, the complex may be internalized, and the SSTR is either degraded or recirculated to the membrane [16, 38]. The actual time for receptors to recirculate to the cell membrane is not clearly established. However, restoration of receptors on the cell mem-brane has been reported to occur within 24 hours [39]. SST is degraded in the early endosomes of the cell, while analogs like octreotide are to a high extent released intact and are suggested to be able to recirculate as well and re-interact with the SSTRs [16, 40, 41]. The compound ^{177}Lu-DOTA-octreotate is internalized in the same manner as SST and other SSTAs, but the peptide is degraded in lysosomes, and ^{177}Lu is retained within the cell for a longer time period, similar to ^{111}In-DTPA-octreotide [38, 42].

NET treatments

Surgery represents the exclusive curative approach for GEP-NETs today. Studies have indicated that survival might increase by resecting the primary tumor [43, 44]. However, even though resection of primary tumor and part of liver increases survival and control symptoms, the recurrence of NETS after debulking surgery is high, and over 60% of patients relapse within 5 years [5, 45-47]. When patients present with metastases, curation cannot easily be attained. Consequently, a demand arises for supplementary therapeutic approaches, comprising chemoembolization, mass-reduction surgery, and treatment with somatostatin analogs. The radiolabeled SST analog ^{177}Lu-octreotate is clinically approved by European Medicines Agency (EMA) and the American Food and Drug Administration (FDA). The indication for patient treatment is SSTR-overexpressing GEP-NETs, that are disseminated or surgically irremovable, well differentiated (Grade 1 or 2) and progressive [48, 49]. The recommended treatment schedule of Lutathera is 4 cycles á 7400 MBq, approximately every 8 weeks with an extension up to 16 weeks if needed to avoid toxicity [50]. It is clinically administered with prophylactic antiemetics to prevent nausea and vomiting, and co-administered with amino acid infusion (e.g. lysine and arginine) as a renal protective agent (e.g., VAMIN®), starting before the treatment and continued for some time after [50-54]. Excretion of ^{177}Lu is mainly made via the urine [53], with the vast majority of ^{177}Lu excreted within the first 6 hours [55, 56].

For ^{177}Lu-octreotate treatment, bone marrow and the kidneys are considered to be the main organs at risk, with early effects as transient blood cell count drop and late effects as renal function loss and blood malignancies, e.g., myelodysplastic syndrome and leukemia. [57, 58]. Enough time between cycles is scheduled for the bone marrow to recover from the early effects, commonly 10 ± 2 w [50, 59]. It has been reported that early effects, such as thrombocytopenia and leucocytopenia are more frequent in patients with previous chemotherapy [58]. The total dose limit for the bone marrow is estimated to be 2 Gy [53]. In addition to PRRT, patients often receive systemic treatment in between, such as chemotherapy or Everolimus that could further affect the hematological system [60].

The kidney is a late responding organ that takes many months to present complications [61]. The proposed limit for absorbed dose to kidneys for late nephrotoxicity is 28 Gy, or 23 Gy if the limit is conservative, both values taken from experience from external radiation therapy [62]. Biokinetics for kidneys has been shown to be dependent on the individual, and the absorbed dose do differ up to eight times between patients as a result [54]. With a calculated absorbed dose limit of 27 Gy, one study concluded that several patients would be able to receive more cycles than four [63], and another reported a mean absorbed dose to kidneys of almost 24 Gy after a median of 6 cycles [60]. PRRT with ^{177}Lu-octreotate has been monitored through dosimetry, where the kidneys' uptake are continuously measured after treatment to calculate the absorbed dose of each cycle. This dosimetry-based PRRT is aiming to elucidate the variability of patient treatment response and individualization potential of the treatment [51, 63, 64].

Radionuclides used in NET imaging and treatment

Throughout the years, many SSTAs and radionuclides have been utilized for imaging and therapy of NETs. ^{111}In-octreotide has been used since the 1990ies for diagnosis and intraoperative tumor localization of patients with NETs overexpressing SSTRs using scintigraphy and SPECT [65, 66]. Later, positron emission tomography (PET)-radionuclides, such as ^{64}Cu and ^{68}Ga, were introduced enabling imaging with high resolution. However, the choice of method for diagnosis can be of importance. Tumors have been reported to be undetected with ^{68}Ga-octreotate (and ^{18}F-

FDG) and then shown intense uptake with ^{177}Lu-octreotate, since the choice of radionuclide influences the affinity of the radiopharmaceutical to the different SSTR subtypes [67].

For therapy, ^{90}Y has been used since the 1990s, but due to a high rate of side-effects with ^{90}Y-[DOTA]-octreotide [61, 68, 69] ^{177}Lu-octreotate is favored [70]. ^{161}Tb has been proposed as an alternative to ^{177}Lu, emitting Auger and conversion electrons with somewhat shorter range and thus more suitable for smaller tumors [71]. A suitable combination of two radiolabeled SST analogues for imaging and therapy, respectively should have small differences in biokinetics, which is easiest obtained with isotopes from the same element. Then, several terbium isotopes have been proposed together. The positron-emitting ^{152}Tb and the SPECT-compatible isotope ^{155}Tb could be used for diagnostics and treatment planning, while the α-emitting ^{149}Tb and electron-emitting ^{161}Tb and could be used for therapy [72, 73]. ^{64}Cu-octreotate has been clinically approved for by FDA for PET-imaging since 2020 [74]. ^{61}Cu is proposed for PET imaging due to recent increased availability with trials in 2024 [75], and ^{67}Cu is proposed for therapeutic counterpart to ^{61}Cu/^{64}Cu [76, 77]. A selection of proposed and used radionuclides for imaging and therapy of NETs are presented in **Table 1.**

Table 1: Properties of different radionuclides used/proposed for therapy and imaging (SPECT or PET) of NETs. Decay mode and half-lives sampled from ICRP 107 [78]

Radionuclide	Decay mode	$T_{1/2}$	E_α [keV]	$E_{\beta,ave}$ [keV]	E_γ [keV]	E_{tot}	Application	Study
^{61}Cu	EC(β^+)	3.3h	-	309.0	823.7	1 133	PET	[75]
^{64}Cu	EC(β^+)(61%) β^- (39%)	12.7h	-	124.8	185.5	310.2	PET	[79]
^{67}Cu	β^-	61.8	-	150.4	115.4	265.7	Therapy	[80]
^{68}Ga	EC(β^+)	67.7min	-	737.9	948.7	1 687	PET	[81]
^{86}Y	EC(β^+)	14.7h	-	217.9	3 578	3 796	PET	[70]
^{90}Y	β^-	64.1h	-	931.1	<E-04	931.1	Therapy	[82]
^{111}In	EC	2.8h	-	34.8	406.1	440.9	SPECT	[83]
^{149}Tb	α(16.7%), EC(β^+)(7.1%)	4.1h	681.0	87.1	1 361	2 129	Therapy	[73]

^{152}Tb	EC(β^+)(17%)	17.5h	-	250.3	149.3	174.4	PET	[73]
^{155}Tb	EC	5.3d	-	43.4	177.7	221.1	SPECT	[73]
^{161}Tb	β^-	6.9d	-	202.5	36.5	239.0	Therapy	[71]
^{177}Lu	β^-(100%)	6.6d	-	147.9	35.1	183.0	Therapy	[53]
^{213}Bi	β^-(97.9%) α(2.1%)	45.6min	124.5	444.0	127.7	696.3	Therapy	[84]
^{211}At	β^-(58.2%), α(41.8%)	7.2h	2 500	5.9	36.7	2 542	Therapy	[85]
^{225}Ac	α	10.0d	5 892	24.8	17.1	5 934	Therapy	[86]

Biological responses to radiation

The classical paradigm of DNA damage is that the radiation interacts with the DNA indirectly or directly, causing breaks of the DNA or base pair damage. While direct DNA damage occurs when ionizing radiation interact with the DNA, indirect DNA damage occurs through a sequential process: radiation interact with the water molecules within the nucleus, leading to formation of Reactive Oxygen Species (ROS) that damage the DNA through oxidation of bases, inducing single- and double-strand breaks [87]. Oxidative stress may induce additional effects, notably a decline in cloning efficiency due to damage to mitochondria and the emergence of genomic instability [88]. The classical paradigm of DNA damage has shifted to a new radiobiological paradigm also including non-targeted and systemic response and requires a better knowledge of biological processes [89, 90]. One way to broaden the knowledge base is to study biological effects at different stages after irradiation, i.e., utilize omics approach to study e.g., genome, transcriptome, proteome and metabolome.

Analyses of comparisons between cellular biomarkers originating from in vitro studies and those arising from live mice in vivo demonstrate a significant reduction in the detected biomarkers (transcripts) in the latter, alongside with distinct expression patterns [91, 92]. These observations highlight intricacies and extensive knowledge deficit prevalent in radiobiology research, thereby emphasizing the necessity to incorporate systemic effects into the analysis of radiobiological impacts on cellular structures. The use of a single protein/gene would not be sufficient for biodosimetry in an exposure scenario, instead a group of proteins/genes

needs to be involved [93]. Time- and dose-dependent levels of blood proteins and gene transcripts have been suggested as exposure related biomarkers, using a panel of genes or proteins [94-96].

Apoptosis

There are several types of cell death, including uncontrolled and controlled cell death, with the uncontrolled/chaotic cell death (necrosis) leading to the release of cellular content in the interstitial fluid and resulting in stress activation of neighboring cells. One type of controlled (programmed) cell death is apoptosis. Apoptosis is a natural and required process to uphold homeostasis of cells in the tissues and organs. Apoptosis can also occur as a response to damage, caused by ionizing radiation, either via damage to the DNA and its repair mechanisms (intrinsic pathway) or via damage to death receptors (extrinsic pathway) [97].

The apoptotic process typically includes the steps blebbing and cell shrinkage, followed by chromatin condensation and fragmentation. Involved in apoptosis are 7 of the caspases. Apoptosis-facilitating caspases are divided into initiator and executioner (effector) caspases, where caspases 2, 8, 9 and 10 belong to the initiators, and caspases 3, 6 and 7 to the effectors. Effector caspases, after being activated by initiators, degrade intracellular proteins and results in apoptosis. [98]

Apoptosis can be induced via several pathways in the cell, for instance, via activation of a family of proteins, called death receptors, located in the cellular membrane (extrinsic pathway) or through several pro-apoptotic proteins in the cell (intrinsic pathway). A part of the intrinsic pathway is initiated by the leakage of cytochrome c from the mitochondrion and that form a complex, sometimes referred to as apoptosome, together with apoptotic protease activating factor-1 (Apaf-1) and the procaspase-9. This will activate caspase-9 that activates caspases 3, 6 and 7. However, the process can be interrupted by inhibitors of apoptosis (IAPs), e.g., the X-linked inhibitor of apoptosis (XIAP) or cellular inhibitors of apoptosis (c-IAPs). [98]

The release of cytochrome c, as a part of the intrinsic pathway of apoptosis, can be initiated by apoptotic proteins, such as pro-apoptotic proteins of the BCL-2 family, BID, BAK and BAX. When activated, the BAK and

BAX proteins can induce mitochondrial outer membrane permeabilization (MOMP). The BID protein acts as a precursor that activates the BAX protein. The BCL-2 family also contains anti-apoptotic proteins, such as BCL-2 and BCL-Xl, that can inhibit MOMP and altogether the predisposition of an apoptotic event is determined by the balance of pro- and anti-apoptotic proteins. [98]

Death receptors of the tumor necrosis factor (TNF) superfamily, e.g., FAS (APO-1 or CD95), TRAIL-R1 (TNFRSF10A or death receptor 4, DR4) and TRAIL-R2 (TNFRSF10B, or death receptor 5, DR5), are a primary part of the extrinsic apoptotic pathway. Upon activation from e.g., radiation, the receptors' death domains will form a complex called death-receptor-induced signaling complex (DISC), that recruit pro-caspases 8 or 10 that, when activated, will be released into the cytosol and activate effector caspases 3 and 7 and results in further cascades. The caspase 8 also has the possibility to cleave the truncated BID (tBID) that will reposition to the mitochondrion and activate the intrinsic pathway by activating BAX. [98]

Epigenetics

Epigenetic changes are changes in the chromatin that does not alter the DNA sequence, but that regulate the expression of genes. DNA methylation, one of the most common epigenetic alterations, occurs most often in areas rich on cytosine/guanine pairs (CpG). Methylation of a gene promoter region in the DNA may cause gene silencing that can be hereditary. Likewise, hypomethylation of a promoter would mean an overexpression of the gene. Hypermethylation of specific genes is sometimes attributed to tumor development, and atypical DNA methylation status is considered a hallmark of neoplasms. Histone modification is a term including different processes that either make the DNA more, or less, active. Histone acetylation commonly result in increased transcription, whereas both active and inactive transcription states can be implied through histone methylation. Micro-RNAs, miRNAs, (a form of non-coding RNA) is also involved in epigenetics alterations, and downregulate expression of genes, e.g., through cleaving of mRNA or inactivating protein translation. [99]

Epigenetics can cause persisting changes on the DNA that can cause illness long after the alteration, e.g., in children from procreation during times of hunger that in their adult life have persisting changes of a gestational marker gene, the *IGF2* [100]. Epigenetic changes can occur rapidly after a stress-event, caused by a multitude of factors in environment, including chemicals and radiation [101, 102]. After radiation exposure, DNA methylation, histone modification and non-coding transcripts (miRNAs) are the main epigenetic events [103]. However, epigenetic changes can also be reversed [99, 100].

Optimization of MRNT

The most prominent effects of ^{177}Lu-octreotate treatment in the clinic today is prolonged survival of the patients, with about 35% stable disease (SD), up to 30% partial remission (PR) and a few percent complete remission (CR) [58, 59]. Animal studies with NET models have reported more prominent outcomes, achieving 96% and even 100% CR from a single administration of ^{177}Lu-octreotate [104, 105]. The absorbed dose to kidney per cycle has been reported to differ among patients, up to a factor of 8, and many patients could undergo additional treatment after 4 fractions [54, 63]. The contrast between clinical and pre-clinical findings points to distinct opportunities for refining the treatment of NET patients [106].

New SSTAs are continuously being developed and are labeled with various radionuclides and tested. An example is the antagonist DOTA-JR11, which in a preclinical setting showed some promise when labeled with ^{177}Lu, but resulted in a higher absorbed dose to kidneys when labeled with the alpha-emitter ^{225}Ac [86]. An Evans blue analog, EB, has been used to modify ^{177}Lu-DOTATATE to create ^{177}Lu-DOTA-EB-TATE that uses the blood-ubiquitous albumin for transportation [107]. Biokinetic study have shown promising results in terms of uptake in tumor tissue, but with a significant higher uptake in kidneys and bone marrow [108]. Late-stage NET patients that received escalating doses to further determine the tolerability of the compound, demonstrated moderate hematotoxicity and low nephrotoxicity [109].

Another approach is to use pharmaceuticals to improve the radiation response, commonly called radiosensitizers. These agents work e.g., by elevating reactive oxygen species (ROS) generation, enhance tumor oxygenation, impede the repair of DNA double-strand breaks (DSBs), and induce cell retention within the more sensitive G2/M-phases of the cell cycle [110]. Utilization of sensitizers in PRRT rises the complete remission rate in patients, e.g., elevating them to 13% from the typical 2% [42]. There is also the option of optimizing the treatment by decreasing the toxicity in risk organs, while not affecting the efficacy of the treatment. An example of this is to prevent the kidneys from damage by addition of the antioxidant rA1M, that is proposed to reduce oxidative stress [111].

A clear majority on studies of therapy and biodistribution in NET-bearing rodents have been performed as mono-injections with different dosage levels. Fractionation, with daily treatments in a succession of days, is common practice in external beam radiotherapy but is less common in the field of radiopharmaceutical treatments. In our group, this have been studied to some extent previously. Effects of increased uptake of ^{177}Lu-octreotate in tumor tissue was observed when giving low activity prior to the main administration, occasionally called ‘priming’ [112, 113], where a small preceding dose acted up-regulatory on the SSTR expression [114-116]. Priming resulted in about doubled absorbed dose to tumor, and no changes in absorbed dose to kidneys [113]. Moreover, increased tumor reduction was also observed when administering a lower total dose of radiopharmaceutical as two administrations, compared to a higher single-injection [105]. A patient case study reported that there was an almost twofold increase in the absorbed dose to the tumor, attributed to higher uptake and longer retention of ^{111}In-DTPA-D-Phe1-octreotide when dividing a single administration into two daily administrations [117].

By dividing an amount of SSTR-targeting radiopharmaceutical into two or more fractions, henceforth here called hyperfractionation, the uptake of the radiopharmaceutical to tumor, and also the tumor treatment effect, could be increased and should be further studied.

Aims

The overall aim of this thesis was to study the effect of fractionation scheme in radionuclide therapy using ^{177}Lu-octreotate in the GOT1 tumor mice model, both in terms of tumor growth response and in terms of biological responses after treatment.

The specific aims were to

- Compare the response of single and hyperfractionated administration of ^{177}Lu-octreotate in the human GOT1 mouse model in terms of tumor volume reduction and tumor regrowth (Paper I)
- Compare epigenetic changes in the human GOT1 mouse model between single and hyperfractionated administration of ^{177}Lu-octreotate late after treatment, (Paper II)
- Compare the biodistribution of ^{177}Lu-octreotate in the human GOT1 mouse model between single and hyperfractionated administration at early time-points after treatment (Paper III)
- Assess and compare the expression of apoptosis regulatory genes in the human GOT1 mouse model early and late after single and hyperfractionated administration of ^{177}Lu-octreotate (Papers III-IV)

Methods and Materials

Tumor model (Papers I-IV)

The GOT1 cell-line was established from a resected tumor from a patient with SI-NET and converted to a cell-line [118]. A first generation of GOT1 mouse model is established by subcutaneous (s.c.) inoculation of tumor cells in the T-cell deficient mouse strain BALB/c. After growth, tumors are divided in small pieces and propagated by s.c. transplantation to get new generations of tumor bearing mice. Occasionally, the cycle is restarted with an inoculation from an early passage of cells.

Radiopharmaceutical (Papers I–IV)

DOTA^{0}-Tyr^{3}-octreotate was labeled with ^{177}Lu according to the manufacturer's instructions (I.D.B. Holland, Baarle-Nassau, Netherlands). The peptide-bound fraction was >93% at all administrations.

Animal cohorts and experiments (Papers I-IV)

Altogether, 64 female mice, transplanted with GOT1 tumors were included. The mice were divided into groups and i.v. injected with either saline for control or with ^{177}Lu-octreotate according to one of the various selected treatment schedules. The mice were kept under standard day/night-cycle, i.e., dark between 18:00–06:00, and received standard laboratory chow and water *ad libitum*. Tumor volumes were in general measured twice weekly with calipers during the experiments, starting from one week before the treatment start. The tumor volume was calculated assuming ellipsoid geometry, using three perpendicular measurements of height, width and length.

Long term effects (Papers I, II, IV)

In the experiments focused on long term effects (**Papers I, II & IV**), mice were divided into groups of 3–5 and given saline for control or ^{177}Lu-octreotate. Treatment schedules were a total 30 or 60 MBq of ^{177}Lu-

octreotate in 1–2 administrations, 7 or 14 days between, or 120 MBq of ^{177}Lu-octreotate in 1–3 administrations, 1 day between. The mice were followed regularly and killed when they had a too large tumor, poor health condition or lost too much weight. At killing, one part of the tumor was fixed in formalin and one part snap-frozen and stored in a -80 °C freezer. An area under curve (AUC) was calculated, using the trapezoid rule, from the tumor volume curve for each individual mouse. Statistical analysis for AUC was performed using Kruskal Wallis test between data from three groups and Mann-Whitney U test between data from two groups.

Short term effects (Paper III)

In the experiments with short-term effects and biodistribution examination (**Paper III**), mice were divided into groups of 2–3 and received either saline only for control or 120 MBq of ^{177}Lu-octreotate as 1 or 3 administrations, 1 day between. The mice were killed after 1, 3 or 7 days, when the tumor and the majority of the organs were excised and snap-frozen and stored in -80 °C, and/or measured for radioactivity.

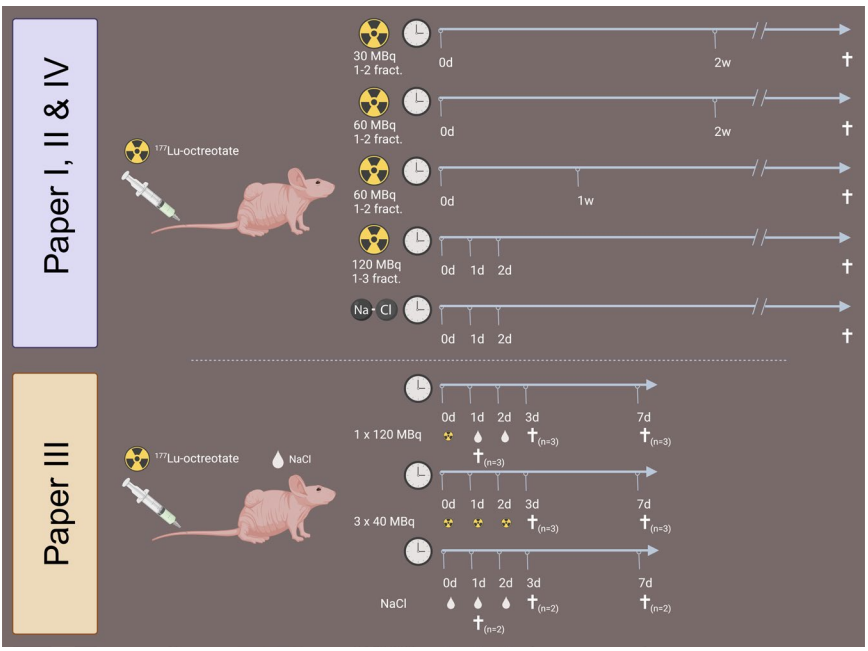


Figure 1: Overview of the performed treatment schedules in the studies of this thesis. The illustration was created with BioRender.com.

Radioactivity measurements and biodistribution analyzes (Paper III)

To determine the injected amount of ^{177}Lu-octreotate to each mouse, the syringes were measured by a well-type ionization chamber (CRC-15R; Capintec) before and after injection.

Tissue samples were measured for ^{177}Lu activity, $A_{tissue}(t)$, in a 2480 WIZARD^2 automatic gamma counter (PerkinElmer), with a 20% energy window centered at 208 keV. Cross-calibration between the gamma counter and the ionization chamber was made. Data were corrected for dead time loss and background signal.

Activity concentration in a tissue sample, $C_{tissue}(t)$ (expressed as %IA/g), was calculated as the percentage of injected activity ($A_{injected}$) per mass of tissue sample, $m_{tissue}(t)$, corrected for physical decay to the time of injection:

$$C_{tissue}(t) = \frac{A_{tissue}(t)}{A_{injected} \cdot m_{tissue(t)}} \cdot 100\%$$

where $A_{tissue}(t)$ is the activity of the tissue sample.

Tumor-to-normal-tissue activity concentration ratios were determined:

$$\frac{T}{N}(t) = \frac{C_{tumor}\,(\mathrm{t})}{C_{normal\ tissue}(\mathrm{t})}$$

Dosimetric analyzes (Paper III)

Mean absorbed dose for each tissue was calculated according the Medical Internal Radiation Dose (MIRD) formalism [119]:

$$D = \frac{\tilde{\mathrm{A}}_{tissue} \cdot \mathrm{n} \cdot \mathrm{E} \cdot \Phi}{m_{tissue}},$$

where $\tilde{\mathrm{A}}_{tissue}$ is the time-integrated activity for each tissue. Only electrons were considered and the emitted mean energy per nuclear transition, $\mathrm{n} \cdot \mathrm{E}$, was set to 147.9 keV per decay [78]. Absorbed fraction, Φ, for each

tissue was estimated to be 1, i.e., only self-absorbed fractions were included.

Immunohistochemical analyzes (Papers I)

Protein expression was studied in **Paper I**. Tumor samples were fixed in formalin at excision, subsequently embedded in paraffin and sliced. The sections were stained with hematoxylin and with antibodies for chromogranin A and somatostatin receptor 2 and evaluated by certified pathologists.

Epigenetic analyzes (Paper II)

Long term epigenetic effects were studied after fractionation of ^{177}Lu-octreotate. Methylation analysis of extracted DNA was performed using Illumina Methylation EPIC DNA analysis BeadChip (EPIC), including 865 918 methylation sites, at the SNP&SEQ Technology Platform in Uppsala (www.genotyping.se).

The data were analyzed in the R environment/Bioconductor using the package RnBeads. Frequencies of methylation in DNA sites was investigated. The level of DNA methylation was read out from the β-value density across genomic regions.

Expression of apoptosis related genes (Papers III, IV)

Early and late effects on expression of 84 genes related to apoptosis in GOT1 tumors were studied using real-time quantitative PCR (RT-qPCR). Fold change, compared to controls, was calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$ method) [120]. Gene with $|FC| \geq 1.5$ was considered regulated. Pathway analysis was conducted on the regulated genes, utilizing the Reactome Pathway database. Gene expression data have been deposited to the NCBI Gene Expression Omnibus database.

Results and Discussion

Tumor volume response (Papers I, III)

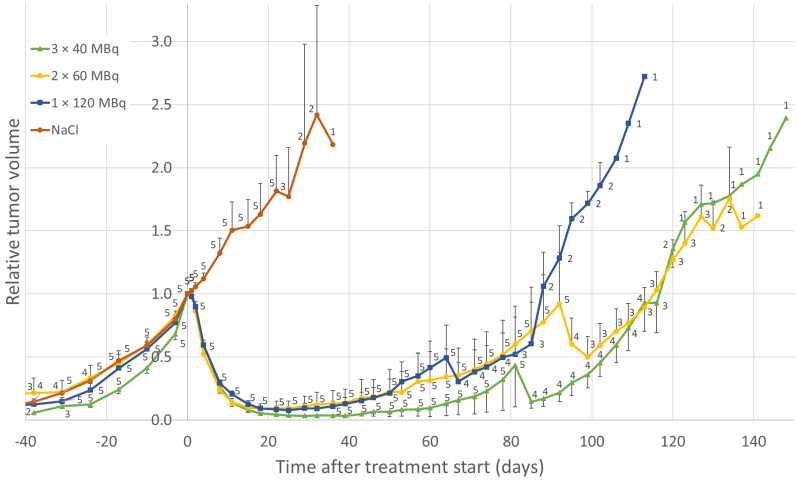
The tumor volume response after single and hyperfractionated administration of ^{177}Lu-octreotate was studied. In **Paper I** the total amounts of activity were 120, 60 and 30 MBq of ^{177}Lu-octreotate, referred to as high, medium and low dose. In **Paper III**, focused on early time-points after treatment, the activity was 120 MBq, given as either single or triple administration.

Effects of high dose (120 MBq)

Treatment with high with high dose of ^{177}Lu-octreotate in the long-term study (**Paper I**) was followed by tumor reduction and later followed by regrowth for all tumors. Single administration was followed by a lesser tumor volume reduction than hyperfractionated administrations that showed longer tumor volume response and later tumor regrowth, as shown in **Figure 2A**. Minimum volume was 8% of treatment starting volume for single administration and 9% and 3% double and triple administration, respectively. The hyperfractionated groups received longer time to regrowth and overall survival compared to single administered group.

The short-term study (**Paper III**) displayed similar volume reduction pattern, with the onset of the deviation in tumor reduction at day 2–3, between the groups, whereafter 7 days the difference was 38% compared to 12% of starting volume for single and hyperfractionated administration, as shown in **Figure 2B**.

A



B

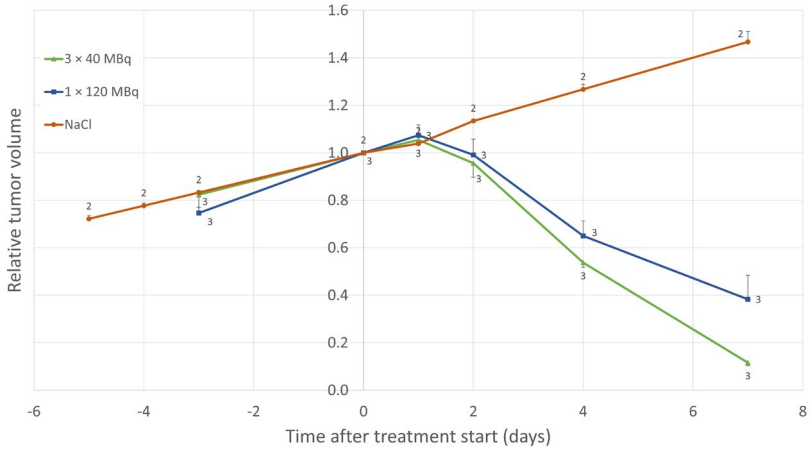


Figure 2: Relative tumor volume *versus* time after treatment start (day 0). **(A)** Tumor volume before and after start of long-term study with high total dose (120 MBq) of ^{177}Lu-octreotate (**Paper I**), reprinted with permission from original copyright holders. **(B)** Tumor volume before and after start of short term biodistribution study with high total dose (120 MBq) of ^{177}Lu-octreotate. Green triangles indicate groups given an activity of 3x40 MBq, yellow circles 2x60 MBq, blue squares 1x120 MBq of ^{177}Lu-octreotate, and brown circles saline as mock treatment (controls). Group size is indicated close to data label at the time-point. Error bars indicate standard error of the mean (SEM).

Effects of medium and low doses (60 and 30 MBq)

Treatment with medium and low activities of ^{177}Lu-octreotate was followed by tumor reduction for all tumors except one. After reduction, regrowth followed for all tumors except two, where only fibrotic tissue remained (2x30 MBq). For single administered groups minimum tumor volume was 48% and 56% of volume at study start for 1x60 and 1x30 MBq, while for the fractionated groups the minimum volume was 9% and 39% of volume at study start for the medium (2x30 MBq) and low (2x15 MBq) dose groups, respectively (**Figure 3**). Statistically significant differences in the AUC for tumor volume curve were observed between the 1x60 and 2x30 MBq groups.

Interestingly, the tumor volume response pattern was similar for the single and fractionated groups, respectively. The groups receiving single administered treatments had a shorter time to regrowth than the fractionated, and where the medium dose was not more efficacious than the low dose.

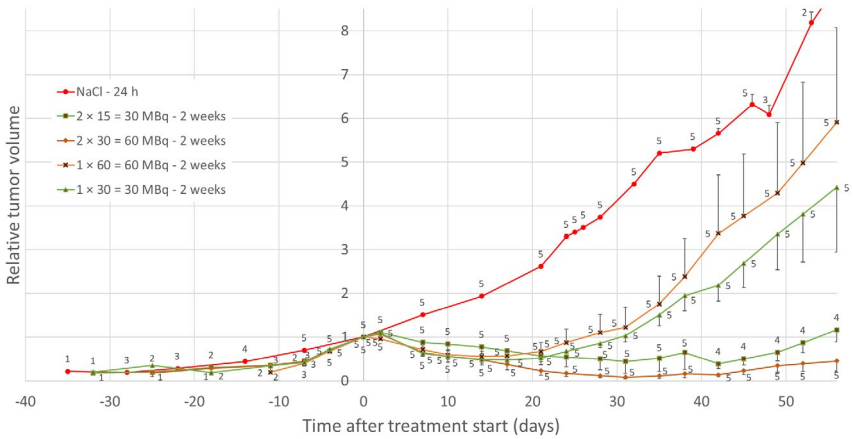


Figure 3: Relative tumor volume *versus* time after treatment start (day 0) from the long-term study with medium and low total activity (60 and 30 MBq) of ^{177}Lu-octreotate (**Paper I**), reprinted with permission from original copyright holders. Brown diamonds and crosses indicate groups given a total activity of 60 MBq, green squares and triangles 30 MBq of ^{177}Lu-octreotate. Red circles indicate saline as mock treatment (controls). Group size is indicated close to data label at the time-point. Error bars indicate standard error of the mean (SEM).

Increased anti-tumoral effects and prolonged survival after fractionated treatment have been noticed in a study on the exocrine pancreatic AR42J tumor mouse model, both for ^{177}Lu-octreotate and ^{67}Cu-sartate [80]. Furthermore, in a study on a human prostate cancer mouse model, a higher tumor growth delay was found for hyperfractionated groups (compared to single dose), treated with the alpha-emitter ^{149}Tb bound to PSMA-617 [121].

Biodistribution and dosimetry (Paper III)

Hyperfractionated administration resulted in higher tumor-to-normal-tissue activity concentration ratios for most organs and time-points evaluated, compared to single administration (**Table 2, Paper III**). The absorbed dose to tumor tissue was 2.6 times higher after hyperfractionation than after single administration, and corresponding value for bone marrow was 1.8 times higher (**Table 3, Paper III**). On the contrary. the mean absorbed dose to kidneys after hyperfractionation was 0.9 times that for single administration. Kölby et al, reported no significant change in kidney uptake of ^{177}Lu-octreotate after administration of various amounts of ^{177}Lu-octreotate, however, there were increased levels in the tumors with lower injected amounts [104]. Lower absorbed dose to kidneys was also reported for consecutive administrations of ^{177}Lu-octreotate compared with single administration [113]. The resulting absorbed dose to kidneys in **Paper III** is in agreement with the aforementioned studies and indicates that there will not be worse side effects in kidney for hyperfractionation of ^{177}Lu-octreotate.

The observed higher absorbed dose to tumor in the work of this thesis could be contributed by prolonged retention, higher uptake, or a combination of both. Longer retention of ^{177}Lu-octreotate after therapeutic amounts has previously been reported in the GOT1 tumors in mice [104], and is similar to the elevated tumor dose for hyperfractionated group reported in this thesis. The higher uptake and absorbed dose to tumor from ^{111}In-octreotide after fractionation in a patient small intestinal NET tumor [117] further support the results of **Paper III**.

Epigenetic effects in tumor tissue (Paper II)

Effects on DNA was studies in **Paper II**, on regrown tumor tissues collected from the long-term study described in **Paper I**, where mice bearing GOT1 tumors were treated with saline or a total of 30 (low), 60 (medium) or 120 (high) MBq of ^{177}Lu-octreotate, given as 1–3 fractions.

Methylation status

Multidimensional scaling (MDS) typically revealed 2–3 main clusters (**Paper II, Figure 2**). MDS dimension 1 separated the control group, receiving only mock-treated with saline, to one side of the plot. Although most treated individuals tended to cluster away from the controls in dimension 1, some clustered closer to the controls in terms of methylation profile.

The five most statistically significant enriched GO-processes related to the differentially methylated genes and promoters showed several similarities and differences among the groups compared to control (**Paper II, Table 2–3**). Genes linked to G protein-coupled receptor signaling pathway were hypomethylated in most hyperfractionated groups (2x30, 2x60(1w) and 3x40MBq), while hypermethylated in 1x120 MBq group. Promoters linked to G protein-coupled receptor signaling pathway were also hypomethylated in most hyperfractionated groups (2x15, 2x30 and 3x40 MBq).

Estrogen catabolic process arose as the top five most statistically significant hypermethylated in promoters in all groups, and in genes in two of the groups. In the full lists, containing all statistically significantly differentiated processes (**Paper II, Table S1–2**), estrogen catabolic process was affected in the genes of all groups, with exception of the 1x120 and 3x40 MBq groups.

This study was, to our knowledge, the first to evaluate epigenetic response in tumor tissue after ^{177}Lu-octreotate treatment, and also after fractionated radionuclide therapy. We therefore had no references to compare our results with.

Transcriptional response (Papers III, IV)

The long-term and short-term transcriptional responses with focus on apoptotic genes were studied in **Paper IV** and **Paper III**, respectively. In **Paper IV**, the transcriptional effects were studied on the tumors saved from the tumor response study of **Paper I**, with single and hyperfractionated groups ranging 30-120 MBq of ^{177}Lu-octreotate. In **Paper III**, the transcriptional analysis was performed on tumor tissues saved during the biodistribution study.

Long-term studies (Paper IV)

The PCR data for the regrown tumors (**Paper IV, Figure 1–2**) presented a moderate gene expression. The medium and high dose groups only presented upregulated gene expression, while the low dose group of 30 MBq presented mostly downregulated genes. The single-administered groups mostly presented divergent gene regulation-pattern compared to their hyperfractionated corresponding groups at the same total dosage levels. The low dose single administered group had mostly downregulated genes while the medium and high ones only had upregulated genes. The *FASLG* gene was regulated in the tumors from the single-administered groups and hyperfractionated low dose group. In the short-time study of **Paper III**, the FASLG was observed to be regulated (ns).

The hyperfractionated high dose-group (3x40 MBq) shared in general fewer regulated genes with groups with the same total dose levels than the others did. The 3x40 MBq-group, with its somewhat divergent gene expression, also showed dissimilarities in the methylation patterns in the study in **Paper II**, compared to groups with the same total dose. However, the similarities found were almost only with the 2x60 MBq-group, with *RIPK2* and *TNFSF8* as upregulated and *TNFRSF10A* as downregulated genes. Further, these two groups showed also similar overall survival among the high dose-groups (**Paper I**).

The pathway analysis for the long-term studies, presented in **Table S2, Paper IV**, showed activation of the *TP53 Regulates Transcription of Death Receptors and Ligands pathway* for all groups except the group receiving 2x30 MBq ^{177}Lu-octreotate. For the 2x30 group, pyroptosis pathway was the only pathway connected to programmed cell death. However, the 2x30 group presented the pathway *TP53 Regulates Tran-*

scription of Cell Death Genes via the downregulated caspase 1 gene (CASP1). The involvement of TP53 in all the groups emphasizes the significance of the gene in the mechanisms of radiation-induced cell death.

Short-term studies (Paper III)

The pro-apoptotic genes *BAX*, *GADD45A*, *FAS*, *TNFRSF10B* and the anti-apoptotic gene *BIRC5* were found significantly regulated (adjusted p-value<0.05) (**Paper III, Table 4**). The *BAX*, *FAS* and *GADD45A* genes showed significant regulation at day 3 for both groups and at day 7 for the single-administered group, where the expression also showed a time-dependent decrease, with larger difference for the *FAS* (also known as *CD95* and *APO-1*) gene. *TNFRSF10B* (death receptor 5 or *DR5*) was significantly regulated at all time-points studied for the single-administered groups. Significant regulation of *DR5* has been reported in previous radiobiological studies published by the group, together with the pro-apoptotic genes *BAX*, *FAS* and *GADD45*, on the same animal tumor model, similar time-points and the same radiopharmaceutical as in the present study [113, 114, 122].

Most significant regulations occurred at day 3 among the single-administered groups, although the number of analyzed samples was low due to high therapeutic effects. In future studies, the cohort sizes should benefit from being increased or the ^{177}Lu activity reduced, as it would simplify analyzes and conclusions. Time-points of special note for larger cohort sizes would be from day 3, as the tumor volume reduction, especially after hyperfractionation, could lead to difficulties in analyzing the tissue due to heavy volume loss, low cell count and large amount of fibrotic tissue in the tumor tissue after treatment with curative amounts of ^{177}Lu-octreotate [104, 112].

The regulation of *FAS* and *TNFRSF10B*, described above, is pointing to a recurring involvement of the extrinsic apoptotic pathway [123, 124] in the effects of ^{177}Lu-octreotate on GOT1 tumors. The FAS/FASLG system is an important part of the immune system that is expressed by a large number of cell types, such as B- and T-cells [125-127]. It also plays a crucial part of tissue homeostasis [125, 127]. Upon binding with its ligand, the FAS receptor initiates apoptosis through a series of caspase activations [123]. However, even though FAS/FASLG is a well-recognized re-

ceptor pathway for cell death, it has been suggested that the FAS ligand can bestow immunogenic benefits, that can drive tumor progression [128-130]. It has also been suggested that the loss of FAS in liver and ovarian cancer models can prevent oncogenesis and that suppression of FAS rather than promotion should be considered in therapy [131, 132]. The higher expression of *FAS* and *FASLG* and the more pronounced anti-tumor effect for the hyperfractionated group in **Paper III** could indicate that the FAS/FASLG system acted for apoptosis after treatment. However, if the higher expression of *FASLG* in the single-administered groups and the low dose fractionated group in the regrown tumors could be a tumor-promoting effect would need to be studied in more depth.

The regulation of genes *BAX and GADD45A* in the studies of GOT1 tumors points toward an involvement of the intrinsic apoptotic pathway [133, 134]. BAX protein activates through oligomerization and induce mitochondrial outer membrane permeabilization (MOMP) [135] and GADD45A acts through the release of the protein Bim from the cytoskeleton, that will cause the mitochondria to release cytochrome C into the cytosol and induce apoptosis [136, 137].

Effects on tumors of hyperfractionation

Tumors have been proposed to be constantly stressed due to their harsh surrounding and speed of cellular proliferation, especially when the high cell growth can easily cause nutrient deficiency and hypoxia in the tumor's microenvironment [138-141]. Stressors in cells can lead to an accumulation of unfolded proteins in the cell's endoplasmic reticulum [142, 143]. Furthermore, it has been reported that unfolded protein response (UPR) is a set of pathways, onset by stress in the endoplasmic reticulum (ER) and that could result in apoptosis [143-145]. It has also been reported that tumors produce heat shock proteins and chaperones as a way to mitigate the stress and to prevent UPR to result in apoptosis [144, 146-148]. It is possible that the additional number of stress events (i.e., number of radiation decays due to increased uptake) could cause more stress in terms of UPR. This could partly explain why the response in the tumors was more pronounced after hyperfractionated administration. Further investigations of effects from fractionated administrations at early time-points, and analysis of the produced proteins, could elucidate the mechanics behind the pronounced treatment effect.

The results presented in this thesis strongly indicate that hyperfractionation of ^{177}Lu-octreotate will be beneficial on the anti-tumoral effect. The results are supported by previous studies in which increased anti-tumoral effects were demonstrated by dividing a dose into more than one administration [105, 113]. We believe SSTR number, receptor saturation and SSTR recycling time to be important factors in these effects. SSTR amplification and higher binding of ^{177}Lu-octreotate has been shown after various doses of external radiation of a small cell lung cancer, in vitro [115, 116]. Brief (up until 24h) lowered uptake of a small dose of ^{111}In-octreotate in GOT1 tumor have been attributed to down-regulation of SSTR expression in vivo, after previous injection of low therapeutic amounts of ^{177}Lu-octreotate, but with an up-regulation at day 3-13 by up to 2 times [149]. The use of a first injection of curative amounts in the same setting demonstrated a lower subsequent uptake of a second small injection of ^{111}In-octreotate during the time-span investigated (4h to 13 days) [112]. The reason as to why the decreased uptake in the GOT1 tumor at all time points after preceding curative amounts and the low uptake at 4h after initial injection is unclear. It is of note here that the reported increased uptake after a preceding amount was limited to the GOT1 tumor and not to any other organ studied, such as kidneys, adrenal glands or pancreas [149]. Saturation effects of SSTR have been demonstrated in several studies. In a GOT1 mouse model, saturation effects were shown in both tumor and normal tissues in a dose escalation study with ^{111}In-octreotide [150]. Saturation effect has also been demonstrated in rat normal tissues expressing somatostatin receptors, where the uptake per mass organ of ^{111}In-octreotide increased up until a certain point and then decreased with increasing dose [151]. The time needed for receptor recycling is, to our knowledge, not well known. Receptor recycling has been demonstrated in the rat pancreas tumor model AR42J, where receptor recycling occurred between 6 and 24 hours after injection of an octreotate compound (where internalization was detected after 2.5 min post injection) [39].

Research on GOT1 tumors in mice are beneficial in many ways, partly since the tumor originates from a patient and therefore is of human origin and have preserved many properties as when it was in the patient. It is also beneficial since systemic effects are present that would not be involved in in-vitro-studies. However, there are challenging factors with the GOT1-model that is not effortless to overcome. Firstly, the GOT1-tumor

is very slow-growing [118]. The first generation of tumor bearing mice is made by subcutaneous inoculation of tumor cells in mice. The mice are then housed while the tumors are allowed to develop. After the tumors have grown, they are then removed from the mice, cut into small tumor pieces and transplanted subcutaneously to new mice, to form the second generation of tumor-bearing mice. The wait for the tumor to grow from inoculated cells could be up to a year, and the time is reduced to approximately 3–4 months [118] when transplanted with tumor pieces. However, the growth rate of the second and later generations differ between animals. This leads to a size discrepancy between mice in a generation. The generations of tumors can also differ between each other, making the use of several tumor generations simultaneously complicated. ^{177}Lu-octreotate has previously been reported to be curative above the amounts of 30 MBq [104]. In **Paper I**, there was only a small percentage of mice cured, even though the amounts were 30–120 MBq of ^{177}Lu-octreotate. The reason is most probably a reduced SSTR expression with the number of generation. In addition, the radiopharmaceutical ^{177}Lu-octreotate has not consistently been available for research. There is therefore always a chance-element when setting up these kinds of studies. In order to maximize the number of groups when executing the short-time study, which primary function was to scope the biodistribution of ^{177}Lu-octreotate, the cohort size in each time-point was thus low. ^{177}Lu-octreotate was not available for a long time-period due to production problems of ^{177}Lu, and the limited amount available was reserved for clinical use. Therefore, supplementing the studies with more mice to obtain a larger cohort was not feasible.

Ethical commentary and reflections

Scientific research with animals is centered on the 3R-principles, first published by Russel & Burch in 1959 [152]: Reduce, Refine and Replace. The implication of these is to minimize the number of animals used to achieve the aims of the study, refine the experimental setup to minimize the distress, pain and suffering of the animals, and lastly to avoid animals in the research when possible. As the systemic responses in our studies are of significant importance, a model without animals is not feasible. We have focused on the principles “Reduce” and “Refine” to make the experiments as humane as possible for the animals. We keep the number of animals at a reasonably low amount in each group and we ease the mice

into the studies by letting them acclimatize after arrival from the producer. We also try to minimize the pain and suffering of the animals by working with great care and practiced movements to reduce the stress of the mice at injections and measurements. The mice are kept with unlimited food and water as well as nesting material to keep them active. The refinements may also increase the quality of the results of the study as sensitive gene and protein expression levels otherwise can be affected by stress in animals. By reusing data and tissue samples when possible, we also reduced the number of groups and animals in the studies. All animal experiments conducted in this thesis were approved by the Ethical Committee for Animal Research in Gothenburg, Sweden [no. 107-2015].

Conclusion

The work in this thesis includes preclinical studies on the GOT1 tumor mouse model, where effects of hyperfractionated administration compared to single-injection of ^{177}Lu-octreotate was assessed.

The results of **Paper I** demonstrated that hyperfractionation of ^{177}Lu-octreotate have advantageous effects on tumors volume reduction and delays the time until tumor regrowth. The effects could be attributed to either a higher uptake of the radiopharmaceutical (most probably due to lower SSTR saturation or radiation-induced increased SSTR expression), a higher stress response or a combination of both.

Paper II showed ^{177}Lu-octreotate treatment specific responses across the groups, considering affected biological processes based on methylated genes and promoters, compared to controls. Furthermore, the study revealed hypomethylated genes and promoters linked to G-protein coupled signaling pathway, only seen in hyperfractionated groups.

Paper III showed that the uptake and absorbed dose increased in tumor and most organs after hyperfractionated administration of ^{177}Lu-octreotate, but that the tumor-to-normal tissue activity concentration ratios increased considerably. The only exception was found for the kidneys, where the absorbed dose did not increase and even decreased after hyperfractionation. Time-specific gene response of the pro-apoptotic genes *BAX*, *FAS*, *GADD45A* and *TNFRSF10B* were discovered, suggesting these genes to be potential indicators at early time-points after treatment with high doses of ^{177}Lu-octreotate.

The long-term study in **Paper IV** showed that hyperfractionation generally resulted in a different apoptotic gene expression pattern compared with single-administration in regrown tumors. A modest regulation of genes compared to controls was noted overall, with *FASLG* consistently affected in all single-injected treatments. The TP53 pathway was involved in almost every group, underlining its importance in tumor cell death after ^{177}Lu-octreotate therapy.

Future Perspective

The aim of this work was to increase our understanding of the processes and mechanisms behind effects of hyperfractionation. The studies clearly demonstrated that hyperfractionated administration of ^{177}Lu-octreotate resulted in much higher uptake and absorbed dose to tumor tissue than normal tissues.

In future animal biodistribution studies, single photon emission computed tomography, SPECT, and positron emission tomography, PET, should be tried. In animals, the advantage would be that the biodistribution is followed in each mouse over time. This procedure will most probably give a more accurate time-dependent data in tumor and major organs, due to the possibility to follow each mouse over time. This would also decrease the number of animals in the studies and further follow general ethical principles. Moreover, having consecutive biodistribution data from the same individual would allow for statistical analyzes that are not possible when sacrificing the mice at each time point, and could aid drawn conclusions.

The focus on the molecular radiobiological studies in the performed work was on apoptotic effects in tumor tissue after radionuclide therapy. Then the regulation of a limited number of genes related to apoptosis was evaluated. Other genes related to apoptosis (e.g., the pro-apoptotic and p53-induced *PUMA)* would also be interesting to examine. Furthermore, the utilization of multi-omics-studies could be a good strategy in order to identify biomolecular change patterns between different treatment schemes with ^{177}Lu-octreotate. To extend the analysis of the extracted RNA in the performed studies and compare it to DNA and proteomic response could perhaps further elucidate the responses of the treatment regiments presented in this thesis.

This thesis was focused on the tumoral changes in the mice. However, many normal tissue samples were also collected in the performed studies and stored in -80°C. These should be analyzed, and data compared to the results in this thesis. Tissues of primary interest to compare would then be bone marrow and kidneys. Also analyzing components in the blood would be valuable, since blood is the most readily available tissue in the clinic and could reveal interesting data on systemic effects.

Further experiments including different dose-levels and times between administrations would be interesting to evaluate in order to find an optimal dosage level and time interval between fractions. Such studies should then include analyses of effects on both tumors and normal tissues as kidneys and bone marrow, to evaluate the extent of the implications of hyperfractionation.

The full potential of hyperfractionation in the clinic remains to be investigated. If clinical results in terms of biodistribution in patients are similar to the findings in this thesis, there would not be additional stress or side effects on the late responding kidneys after hyperfractionation, while increasing the effects on tumor tissue. Furthermore, hyperfractionation of ^{177}Lu-octreotate as radiopharmaceutical compound Lutathera could readily be performed in the clinic, since the shelf-life is guaranteed for 72h enabling up to three 24h-administrations using the same batch.

Acknowledgement

What now feels like a lifetime ago, I set out on a journey, and I have since traveled with many extraordinary individuals who, in their own ways, have supported and helped along the way. I want you to know that I am truly grateful for all that you have done for me, and I want to recognize the efforts you have made to aid. Now, to specify each and every thing all of you have contributed with, and all the laughs, I would have needed several more pages, most likely a book. This acknowledgement is going to have to be brief, and it is not going to do you the justice to the extent you deserve, but here it goes:

My supervisor, Prof. Eva Forssell-Aronsson. From you I have learned many things that will be useful in the future. Thank you for your unwavering commitment to finding creative solutions to challenges.

My co-supervisor, Assoc.Prof. Khalil Helou. Your generosity with knowledge and resources is a much-needed asset in this world and I am grateful for having you with me in my project.

I've shared office space with many phenomenal people throughout the years: Amin Al-Awar, Arman Romiani, Julia Johansson, Nishte Rassol and Viktor Sandblom. Thank you for all the weird topics you have endured, and for all the shenanigans, especially Arman –thank you for making me expect the unexpected. Thank you also, Nishte, for all your help and for being relentless in the sharing of yourself.

My present and former group members of the Forssell-Aronsson Lab, not already mentioned: Ann Wikström, Anja Schroff, Britta Langen, Charlotte Ytterbrink, Daniella Pettersson, Emman Shubbar, Hana Bakr, Johan Spetz, Klara Insulander Björk, Lukas Lundholm, Malij Larsson, Martin Andersson, Mikael Montelius, Torgny Lundberg and Sofia Saadati with whom I have had countless of hours of interesting and developing discussions. Special thanks to Johan Spetz. You were there at the beginning of this academic endeavor, and you came back in time for the end. Your dedication and attention to detail is truly remarkable, both in terms of science but also people, and you inspire to great things!

Alexandra Hansson, Jennie Bengtsson and Maria Andersson for being great friends and taking care of important things so we can get anything done.

Everyone in the PhD-group (formerly known as DräG, formerly known as DoG). I've had many laughs and stimulating conversations with you! I hope you someday come up with a suitable name for the group that is both inclusive and descriptive.

My present and former colleagues and friends at and around Radiofysikhuset, previously unadressed. Thank you for all the wonderful things we have shared throughout the years! Special thanks to Tom Bäck, for all the heartwarming kindness, and Martin Hjellström and Rimon Thomas for being so wonderfully friendly and helpful in times when I least expected but needed it.

All the great people at the Central Radionuclide Pharmacy, Experimental Biomedicine, Sahlgrenska Centre for Cancer Research, especially Ella, Hugo and Roberta for all the laughs and discussions.

Corpsen. Were to even begin? When I had lost faith in what community was, you restored it. And that was just the beginning. Had it not been for Corpsen, I undoubtedly would have missed some great friends and great things! SFBH!

My family and my dear friends. Thank you for your inspiration, joy, encouragement, and for helping me picking up the pieces when I fell apart. Without you, I never would have finished this. Especially my sister, Linda, who always tries to help if she can –you're a rock! Special thanks to Elin and John for helping, listening, and counseling in times of need. Also, thank you Joakim Hanner, for illustrating the cover of this thesis, I hope you had as much fun with is as I had.

And Dad. Even though you are no longer among us, you will never really be gone. Thank you for all the great things.

The studies of this thesis were supported by grants from several benefactors. Thank you: Adlerbertska Research Foundation, Assar Gabrielsson Cancer Research Foundation, BioCARE –a National Strategic Research Programme at the University of Gothenburg, Herbert & Karin Jacobsson Foundation, the King Gustav V Jubilee Clinic Cancer Research Foundation, the Royal Society of Arts and Sciences in Gothenburg (KVVS), the Sahlgrenska University Hospital Research Funding, The Swedish Cancer Society, The Swedish Research Council, Swedish Society of Radiation Physics, the Swedish state under the agreement between the Swedish government and the country councils –the ALF-agreement, and Wilhelm and Martina Lundgren Research Foundation.

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