UNIVERSITY OF GOTHENBURG

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APOPTOTIC EFFECTS IN RENAL CORTEX AFTER TREATMENT WITH ^{177}LU-OCTREOTATE

M.Sc. Thesis

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Thesis:	30 HP
Program and/or course:	Medical Physicist Programme
Level:	Second Cycle
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Supervisors:	Britta Langen, Charlotte Andersson and Eva Forssell-Aronsson
Examiner:	Magnus Båth

Abstract

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Purpose: The purpose of this project was to investigate gene regulation of a predetermined panel of apoptotic genes in murine renal cortex after treatment with ^{177}Lu-octreotate after one day and seven days.

Theory: The kidneys and bone marrow are the risk organs in ^{177}Lu-octreotate treatment, but by fractioning the treatment the bone marrow recovers. This leads to the kidneys being the dose-limiting risk organ. Apoptosis is a known effect after irradiation of cells and can result in nephrotoxicity after treatment. Whether apoptosis is initiated or inhibited after irradiation depends on a balance of pro-apoptotic and anti-apoptotic signals in the cells and is controlled by many genes divided into gene families. By analysing the expression of apoptotic genes, information about the induction or inhibition of apoptosis can be obtained. The absorbed dose is calculated in order to relate the responses in the tissue to irradiation by ^{177}Lu. The renal cortex receives a higher absorbed dose after treatment with ^{177}Lu-octreotate and is therefore of high interest when studying apoptosis.

Method: RNA from the kidney cortex of 12 different mice was used for the analysis. The mice were divided into two irradiated and two control groups and killed at one or seven days after administration of 150 MBq ^{177}Lu-octreotate or physiological saline, respectively. A panel of both pro-apoptotic and anti-apoptotic genes was investigated with a quantitative real-time polymerase chain reaction (QPCR) array. Prior to this, the RNA-concentration was determined with the Qubit ® assay in order to use the same amount of RNA in the QPCR assay. The absorbed dose in the kidney cortex was calculated for each mouse in the irradiated groups and the transcriptional response was related to the absorbed dose and time of irradiation.

Result: The group that received a lower absorbed dose and was killed after one day (group 1) showed a higher increase in transcriptional response to irradiation than the group killed after seven days (group 2). The regulation in group 1 indicated pro-apoptotic responses in the renal cortex, wheras in group 2, a shift to anti-apoptotic responses was observed.

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Introduction

Neuroendocrine cells regulate, store and release hormones through signals from the central nervous system. When malignant, neuroendocrine tumours (NET) have an overexpression of somatostatin receptors on the cell membrane compared to normal tissue.

These tumours are often treated with the radiopharmaceutical ^{177}Lu-octreotate, which is a form of targeted radiotherapy. Treatment with targeted radiotherapy is designed so that the carrier molecule of the radionuclide has characteristics that leads to the radiopharmaceutical binding to the tumour in greater extent than to normal tissue cells. Octreotate is a somatostatin analogue, i.e. octreotate is structurally similar to somatostatin. This will lead to the radiopharmaceutical to bind to the receptors and deliver a radiation dose. Because of the therapy being designed to target the characteristics of the tumour cell, and being administrated systematically, it is possible to treat metastases.

^{177}Lu is a suitable radionuclide for NET therapy due to its relatively low beta energy (mean energy of 147 keV per decay) and low emission of gamma photons in comparison to other radionuclides [1, 2]. When using a radionuclide for therapy, the aim is to have a high emission of electrons and low emission of photons. The electrons deposit energy within a certain range, leading it to being of the same proportions as the average size of metastases, which gives a local absorbed dose [2]. This is the purpose of the therapy due to the carrier molecule being targeted to the tumour cell. Photons have no range and will thus expose all tissues of the body. The photon emission from ^{177}Lu can be used in imaging with single-photon emission computed tomography (SPECT) after treatment.

The kidneys and the bone marrow become the primary risk organs with ^{177}Lu-octreotate therapy [3]. Fractionated therapy allows the bone marrow to recover which makes the kidneys the dose limiting organ. The objective is to give the tumour as high radiation dose as possible, but at the same time to spare the kidneys from radiation induced renal toxicity [4-6]. When injecting a radiopharmaceutical to the bloodstream, the molecule will accumulate in different organs apart from the tumour to a different extent. This is depending on the characteristics of the organ and the radiopharmaceutical. A fraction of the administered activity stays in the kidneys in ^{177}Lu-octreotate treatment. The amount of radioactivity accumulating in the kidneys varies between individuals [1, 3, 7, 8]. The kidneys become a risk organ when treating tumours with ^{177}Lu-octreotate because of its role in the body, which is cleaning the blood from waste products by filtrating the blood. When the primary urine is reabsorbed in the proximal tubuli, an amount of ^{177}Lu will stay in the kidney and deliver a radiation dose [6, 8].

It is necessary to estimate the absorbed dose from ^{177}Lu in a tissue in order to relate biological effects to the treatment such as gene expression changes or other clinical parameters. Since the amount of ^{177}Lu-octreotate that remains in the kidneys differs between each individual, the individual absorbed dose calculation is preferable.

Several studies of irradiation of the kidney through treatments with radiolabelled somatostatin analogues showed that the dose distribution within the kidney is heterogeneous, leading to a higher uptake of the radiopharmaceutical in the cortex of the kidney compared to medulla [9, 10]. This makes the cortex a tissue of interest in the kidney when studying apoptosis as an effect of irradiation. There is also an interest in studying the impact that time has on the apoptotic burden, due to the differential biodistribution of the radiopharmaceutical in the kidney and increased absorbed dose (protracted irradiation). The absorbed dose in an organ after injection of a radiopharmaceutical is dependent of the retention in the organ, but also on the half-life of the radionuclide.

When irradiated, cells can undergo apoptosis as a major response to irradiation [9]. This happens when a cell's DNA is damaged to an extent when it cannot be repaired. Depending on the type of cell, it can be one of the most common cell death mechanism after irradiation [8]. Apoptosis in the kidneys has been shown to correlate with kidney damages resulting in critical loss of kidney functions [4, 5, 10].
Apoptosis, in comparison to necrosis, is a process that involves a large number of genes, divided into gene families. The genes play different roles in producing proteins that activate caspases. Caspases are enzymes that fragmentate the cell, causing no inflammation or leakage outside of the cell [11, 12], see figure 1 for example.

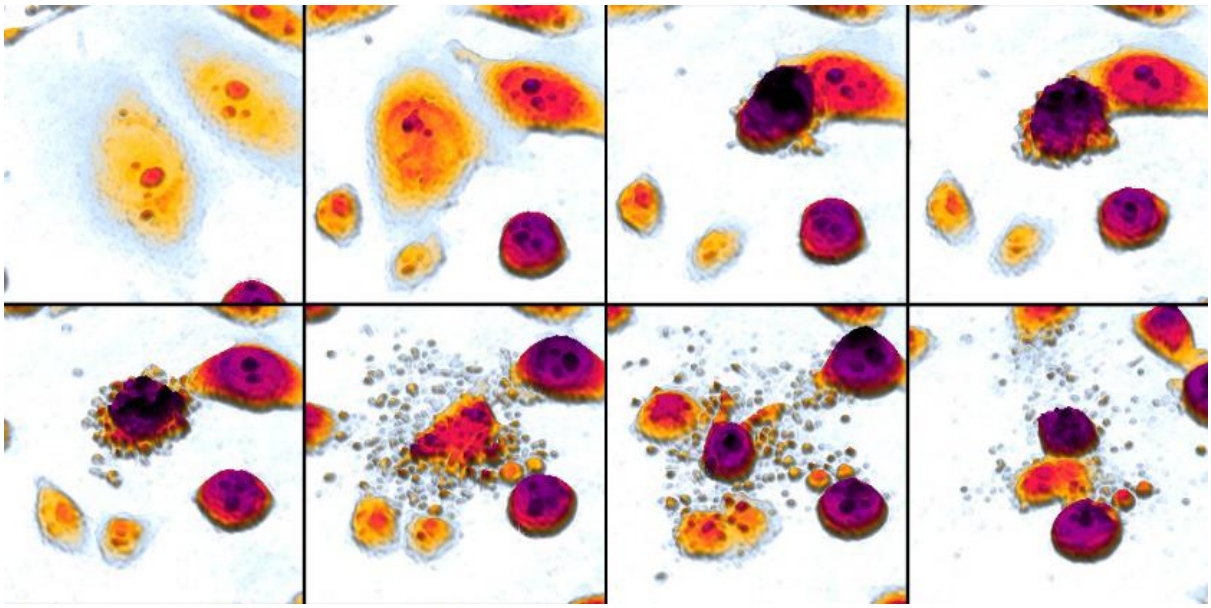


Figure 1: A prostate cancer cell undergoing apoptosis and being fragmentated into apoptotic bodies. Image from Egelberg's own work [13].

Whether the cell lives or dies through apoptosis is a balance between signals inhibiting apoptosis and activating it. Apoptosis can be triggered either from intrinsic or extrinsic pathways, by activation of pro-apoptotic genes or lack of anti-apoptotic genes [9, 11]. Apoptosis is initiated through the extrinsic pathway by signalling molcules from outside of the cell that bind to death receptors. These death receptors belong to a gene superfamily called tumour necrosis factor superfamily (TNF). The activation of these death receptors occurs via their death domains which activates pro-caspase 8. This can lead to the activation of *Bid*, a member of the

Bcl-2 gene family, in turn activating the mitochondrial pathway, which also plays a role in apoptosis through intrinsic stimuli.
Caspase 8 can also activate *effector caspases* (caspase 3, 6 and 7), which directly fragment the different parts of the cell [9, 11]. Another class of caspases are *initiator caspases* that have the function of activating the effector caspases, which creates a chain reaction resulting in the execution of apoptosis.
Caspases normally exist in cells as an inactive form (procaspases) and are cleaved to their active form following release of different pro-apoptotic proteins.

Intrinsic activation of apoptosis can be triggered by several different processes within the cell; either by the activation of pro-apoptotic factors through e.g. radiation, or the lack of anti-apoptotic factors, activating the effector caspases [11]. When irradiated, the DNA of the cell is damaged either through direct or indirect damage to the molecule, the ratio of which depending on the type of radiation and other physical properties. Radiation through charged particles causes mainly direct damage by single or double strand breaks. Photon irradiation creates free radicals, which in turn damages the DNA [14]. DNA damage can be repaired, but if the lesions are too complex or repair is error-prone, the cell may accumulate deleterious effects (e.g. mutations, loss of genetic material or chromosomal rearrangement) which can lead to carcinogenesis. If the damage burden is too high, the cell usually undergoes apoptosis instead of initiating repair. Whether or not a cell's damages are repaired is controlled to a great extent by the gene p53.

When the intrinsic pathway is activated, the mitochondria are stimulated through a change in the trans-membrane potential, disturbing its function, which in turn makes the mitochondria release cytochrome c, activating the effector caspases [9, 11]. The genes involved in the intrinsic pathway, controlling the mitochondrial release of pro-apoptotic signals are mainly from the Bcl-2 family and p53, which activates certain gene groups in the Bcl-2 gene family [9, 11]. P53 is an important gene in regulation of apoptosis, since its activation plays a crucial role in the processes following irradiation. It activates transcription of genes for DNA repair, or of certain pro-apoptotic genes and inhibits transcription of anti-apoptotic genes.

When either of the two means of activation of apoptosis occurs, the mitochondria are responsible for releasing apoptosis-inducing proteins into the cell, such as cytochrome c, which in turn activate caspase 9 and then the effector caspases [9, 11, 12].
These different types of activation of apoptosis are controlled and balanced through different gene families, where an activation or suppression of these genes can give information about the balance between pro-apoptotic and anti-apoptotic signals following irradiation. By analysing genes that give signals for suppressing or activating apoptosis, information about the processes that take place in the cell related to apoptosis can be obtained.

When a gene gets expressed, DNA is first transcribed to RNA. This messenger RNA (mRNA) serves as a template for the translation to a specific protein. The occurrence of mRNA related to a specific protein gives an indication of the amount of protein being expressed in the cell.

A method of investigating differential gene expression is the quantitative real-time polymerase chain reaction (QPCR) assay. QPCR uses complementary DNA (cDNA) which is synthesized from RNA to analyse whether a gene is up- or down-regulated, compared to a control sample. The target genes are normalised with reference genes, which should show no up- or down-regulation irrespective of treatment [15].

Reverse transcription is the process of creating cDNA from RNA samples. A primer (a short sequence of nucleotides) binds to the mRNA, which enables the enzyme reverse transcriptase to bind to the location of the double strand. Reverse transcriptase adds nucleotides- creating the complementary DNA sequence to the mRNA. After reverse transcription, the RNA is denaturised, and the single-stranded cDNA is used for the QPCR assay [16]. By analysing the differential regulation of genes involved in apoptosis using QPCR, considering both pro-apoptotic and anti-apoptotic genes, information about the induction or inhibition of apoptosis in a tissue can be obtained after irradiation with ^{177}Lu. Analysis with QPCR shows early responses to irradiation as well as the balance between pro-apoptotic and anti-apoptotic genes.

Aims

The purpose of this study was to investigate the expression of a panel of genes involved in apoptosis in the kidney cortex of mice that had been treated with ^{177}Lu-octrotate at two different times after injection. The absorbed dose to the kidney cortex was also calculated in order to relate the gene expression to the absorbed dose.

The aim of this work is to enhance the knowledge of the renal apoptotic burden after ^{177}Lu-octreotate treatment.

Materials and methods

Animal experiment

Prior to this study, an animal experiment was performed to obtain mRNA-samples and activity data for this project. For the experiment, 12 female C57BL mice were used, divided into four groups, two control groups and two irradiated groups. The irradiated groups were i.v. injected with 0.1 ml 150 MBq ^{177}Lu-octreotate and the other two control groups with the same volume of a physiological saline solution. Two of the groups were killed (treated and control) after one day and the other two groups after seven days.

After killing the mice, one of the kidneys was excised and the activity in the entire kidney was measured in a gamma counter (2480 Wizard Automatic Gamma Counter, PerkinElmer). The other kidney was frozen in liquid nitrogen. Cortical and medullary tissue samples were dissected on dry ice and respective samples subjected to extraction of total RNA. RNA samples from the kidney cortex were used for this study, see figure 2.

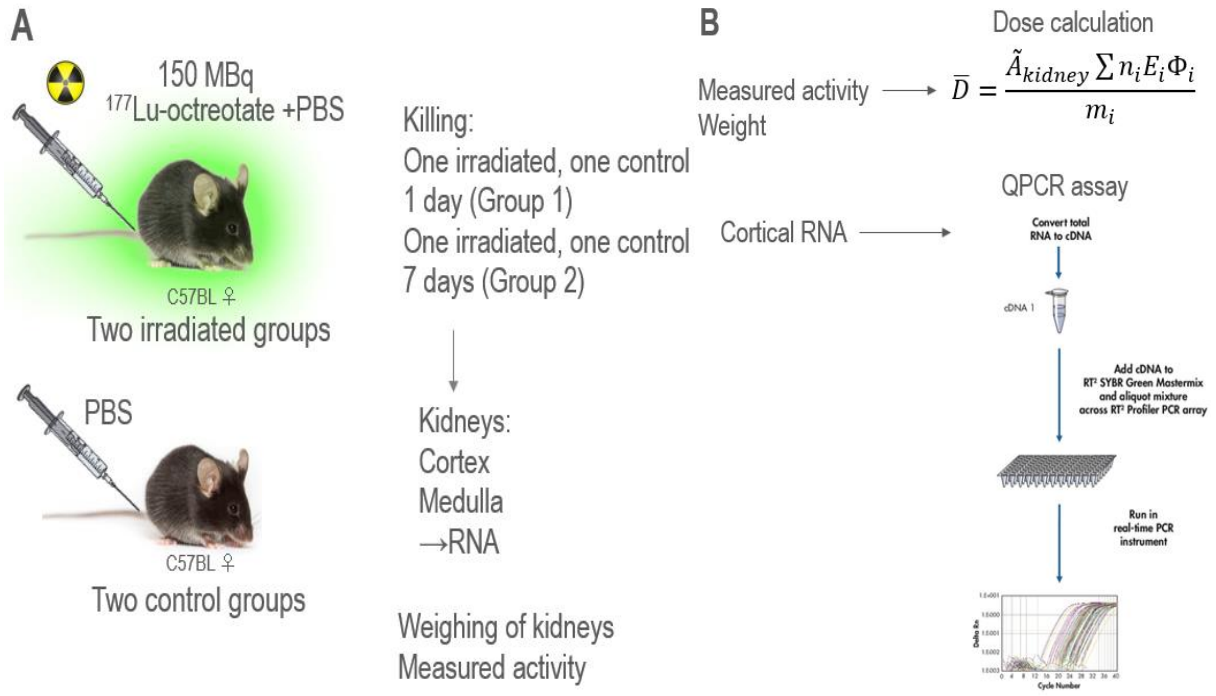


Figure 2: Schematic picture of the workflow prior to this project (panel A) and during the project (panel B). Image of the workflow for the QPCR assay was adapted from QIAGEN [17].

Dose calculation

In order to calculate the absorbed dose, the MIRD-formalism was used according to the equation [18]:

$$\overline{D} = \frac{\tilde{A}_{kidney} \sum n_i E_i \Phi_i}{m_i} \quad (1)$$

Where $\tilde{A}_{kidney}$ was the activity in the kidney integrated over time, $\sum n_i E_i$ the mean beta energy per decay, ignoring the photon energies, Φ_i the absorbed fracion and m_i the mass of the kidney.

The time-integrated activity was calculated with a trapezoid function in MATLAB, with the initial activity $A(0) = 0\ Bq$.

The activity was measured with a gamma counter as counts per minute (CPM). In order to obtain the measurement results in Bq, a calibration of the gamma counter was performed. A sample with ^{177}Lu was measured with a well-type ionization chamber (CRC-15R, Capintec, Ramsey, New Jersey, USA) and solutions with eight different activities varying from 6.25 kBq up to 800 kBq were prepared. Six samples were prepared with the same activity and measured in the gamma counter. The mean CPM per activity was calculated and plotted, where a linear fit was made where the plot was linear. Dead time and background corrections were made before plotting the activities. The slope from the linear fit gave the calibration factor. The CPM obtained for each kidney was divided by the calibration factor, giving the activity in each kidney.

Data for the activity concentration in the kidney up to the point of killing was taken from a previous study [19] investigating the biodistribution in different organs at different times from injection. The activity concentration was multiplied by the injected activity and the mass of each kidney. A time correction between measurement of the activity and killing was made. This gave a set of discreet values for the total activity in the entire kidney for specific times up to the killing.

Data for the fraction of energy absorbed by the renal cortex was considered for the entire kidney as a source organ and taken from literature [20]. The mean beta energy emitted from ^{177}Lu is 147 keV per decay [1].

The value for the absorbed fraction was 0.58 in cortex for kidneys weighing 0.15 grams.

Gene expression analysis

Before the reverse transcription and the QPCR assay was performed, the concentration of the total RNA was analysed. The analysis was performed with Qubit (Qubit ® RNA BR Assay Kit, molecular probes, Life technologies) to ensure that the same amount of RNA was used for the different samples. For the cDNA synthesis, 110 ng of RNA was used for each replicate.

The mRNA was synthesised to cDNA using the RT^2 First Strand Kit from QIAGEN (RT^2 First Strand Kit, 330404, QIAGEN). A Genomic DNA elimination mix was prepared, containing 110 ng RNA, 2 µL Buffer Ge and RNase free water up to 11 µL. The mix was incubated for five minutes at 45 °C and placed in ice for one minute. Afterwards, it was combined with a Reverse transcription mix, consisting of 4 µL 5x Buffer BC3, 1 µL Control P2, 2 µL RE3 Reverse Transcription Mix and 3 µL RNase free water. The Genomic DNA elimination mix and Reverse transcription mix were incubated at 42 °C for 15 minutes, allowing the reverse transcription to take place, then five minutes at 95 °C to denaturate the mRNA. 91 µL of RNase free water was added and the aliquots frozen at -20 °C until used for the QPCR assay.

The QPCR assay was performed using an array investigating 84 different genes related to apoptosis, 5 control genes and technical controls (Appendix) from QIAGEN (RT^2 PCR Profiler Array, Mouse Apoptosis, PAMM-012ZC, QIAGEN), using the RT^2 SYBR Green ROX qPCR Mastermix (QIAGEN). The cDNA was pipetted as 102 µL aliquots into the reaction mastermix and was added with 20 µL per well. The plate was analysed using a 7500 Fast Real-Time PCR system (Applied Biosystems). For two biological samples (group 1 and control group 1, mouse no.1 and control 1), the analysis was performed with a 7500 Real-Time PCR system (Applied Biosystems) with a different plate format (RT^2 PCR Profiler Array, Mouse Apoptosis, PAMM-012ZA, QIAGEN). Three plates per sample were analysed to achieve technical replicates.

QPCR works through a sequence-specific primer that binds to the gene of interest through the annealing. In addition, nucleotide probes, carrying a fluorescent dye and a quencher, bind to a certain sequence of the amplicon (amplified sequence section of the cDNA (gene) of interest). The reaction buffer contains nucleotides, salts and DNA polymerase. The QPCR reaction takes place by changing the temperature through three cycles and repeating the cycles around 40 times. Prior, an initialization step is required for hot-start DNA polymerase to be activated, where the temperature is around 95°C for 10 minutes.

The QPCR cycles go through three different phases, the stationary baseline phase, the exponential phase and the plateau phase, see figure 3. The stationary baseline, also called the lag phase, is when the concentration of PCR products is slowly increasing, but there is not enough fluorescence produced to be detected, or the consistency of the reaction is not fully exponential yet. This is where the baseline is set. In the exponential phase, the doubling of the amplicon is consistent and ongoing, and there is a large surplus of reagents available for the exponential chain reaction to continue. In the linear phase, there are fewer reagents left due to consumption in previous cycles, which causes a decrease in the slope of the fluorescence curve. A plateau is reached when the reagents have been fully consumed and the reaction cannot continue.

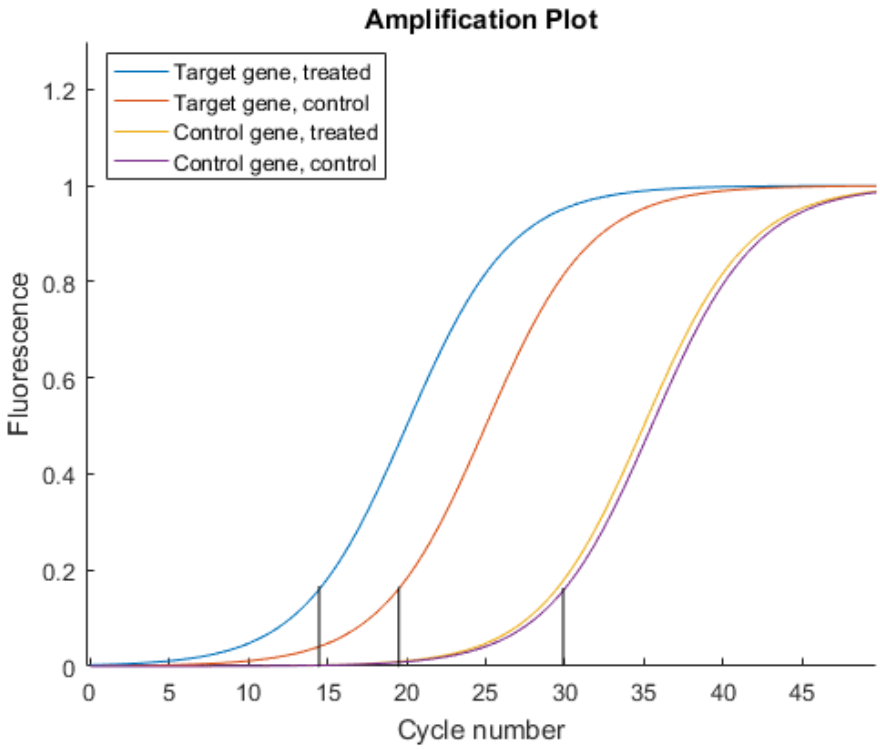


Figure 3: Representative illustration of the different phases of the fluorescence curve in QPCR. The threshold cycle (Ct-value) is marked for each gene with a straight line.

By measuring the cycle where the reaction becomes exponential, information about the expression level of the gene is obtained and quantification is possible [16]. This quantity is called the threshold cycle, C_t. The quantification of the expression level is obtained by normalising the C_t-value of the gene of interest to several control genes and by subtracting the normalised C_t-values of the irradiated group ($\Delta C_{t\ irr.}$), with that of the control group ($\Delta C_{t\ ctrl}$). This is used to calculate the fold change (FC), which gives the gene regulation by:

$$\Delta C_{t\ irr.} = C_{t\ irr.\ target\ gene} - C_{t\ irr.\ ref.gene} \quad (2)$$

$$\Delta C_{t\ ctrl} = C_{t\ ctrl.target\ gene} - C_{t\ ctrl.ref.gene} \quad (3)$$

$$\Delta\Delta C_t = \Delta C_{t\ irr.} - \Delta C_{t\ ctrl.} \quad (4)$$

$$FC = 2^{-\Delta\Delta C_t} \quad (5)$$

Significant gene regulation was accepted with FC ≥ 1.5 for upregulation or FC ≤ -1.5 for downregulation.

Results

Absorbed dose

The absorbed dose to the kidney cortex over one day (group 1) was 20 Gy (± 0.56 Gy SD) and over seven days (group 2) 51 Gy (± 0.11 Gy SD). The individual absorbed dose was calculated for each mouse and the mean value for all the mice in the group was used when analysing gene expression in correlation with the absorbed dose. The activity concentration at different time points up to the killing is shown in table 1 and figure 4.

Table 1: Activity concentration in the kidney up to the time of the killing [19].

Time after injection [h]	Activity concentration [% IA/g]
0	0
0.25	53
0.5	39
1	26
4	16
8	11
24	7.2 (from measurement in group 1)
72	2.8
168	0.3 (from measurement)

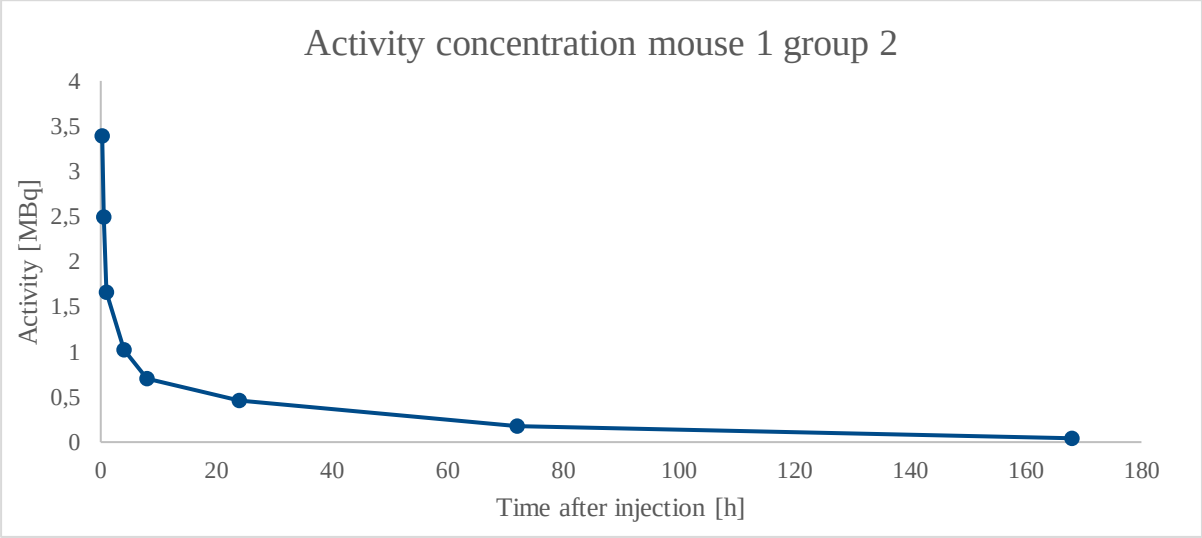


Figure 4: The activity concentration for mouse 1 in group 2 up to the time of killing. The data from table 1 was multiplied with the activity from the measurement.

Table 2: Absorbed doses to the kidney cortex for each mouse and mean absorbed dose in the two different irradiated groups.

Mouse	**Absorbed dose Group 1 [Gy]**	**Absorbed dose Group 2 [Gy]**
1	19	51
2	19	51
3	20	51
Mean (SD)	20 Gy (0.56)	51 Gy (0.11)

Gene expression

In total, 16 genes were significantly regulated in at least one of the irradiated groups, see figure 5 and 6 (see Appendix for regulation of all genes). Twelve genes were regulated in group 1 and eight genes in group 2. In each group, both pro-apoptotic and anti-apoptotic gene regulation was observed. For the majority of the array-based genes, no significant regulation was detected.

The majority of the pro-apoptotic genes that were significantly regulated were upregulated. However, two pro-apoptotic genes (*Cidea* and *Bok*), were downregulated in group 1 and group 2 (*Fasl* and *Casp1*). The anti-apoptotic genes were in majority downregulated with the exception for *il10*, which was significantly upregulated in group 2. In the group that was killed after one day (group 1), the regulation of seven genes indicated the initiation of apoptosis, two genes indicated inhibition and two genes were regulated that could lead to both regulations of apoptosis. In group 2, four genes indicated activation, three genes inhibition of apoptosis and one gene that could be both pro- and anti-apoptotic.

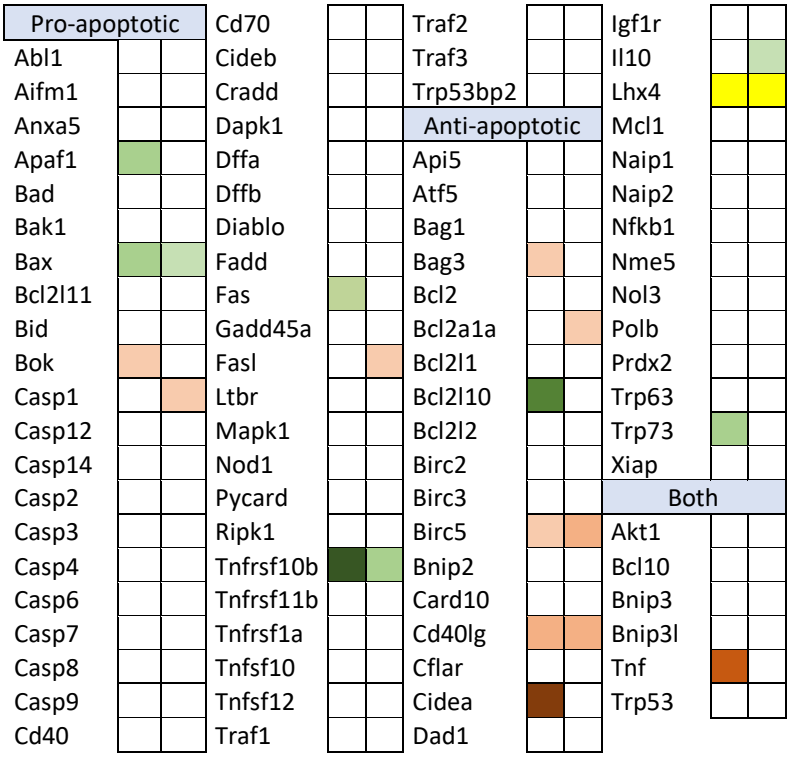
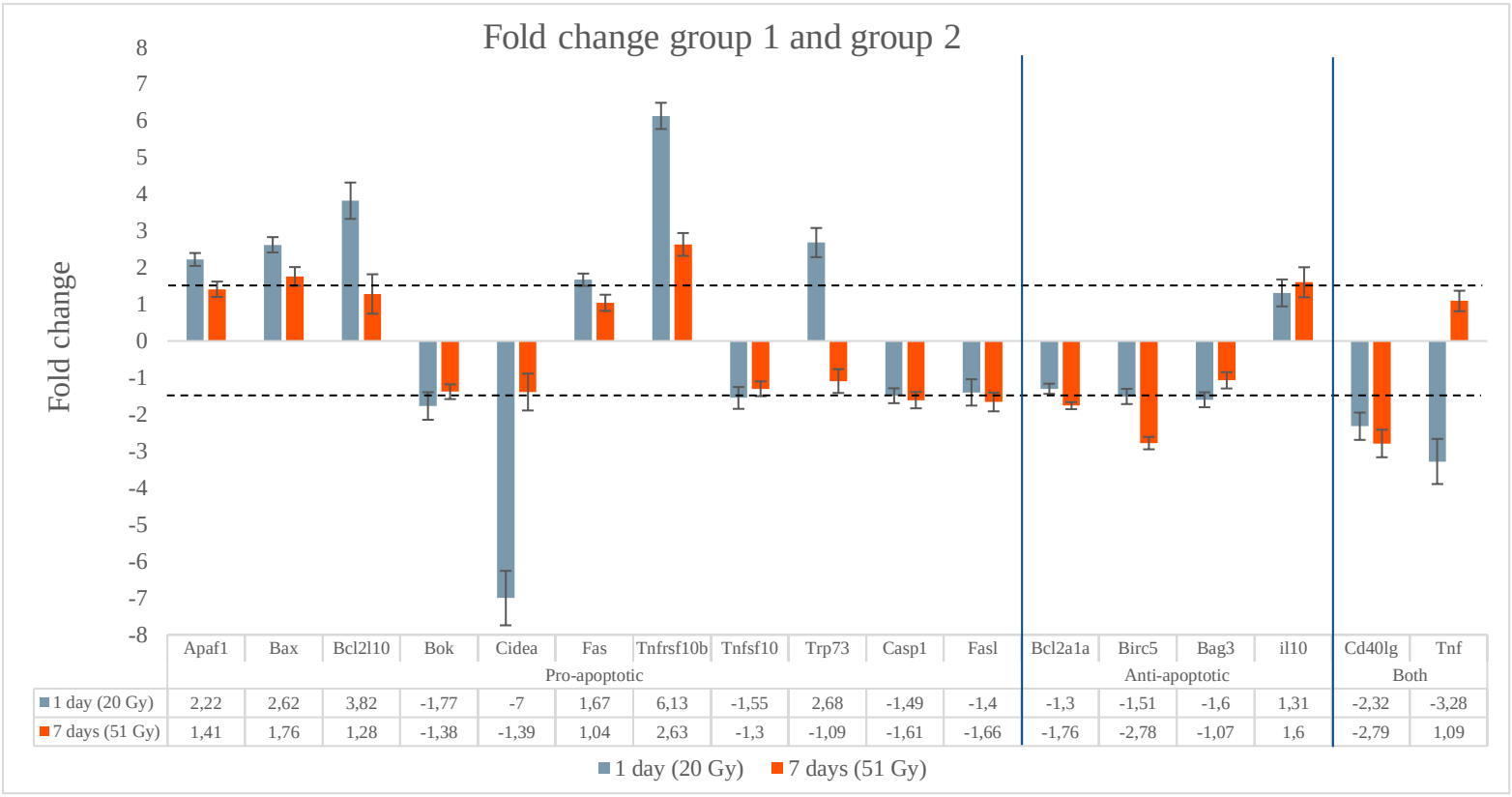


Figure 5: Overview of apoptosis-related gene regulation. First column shows results for group 1, second for group 2. Green indicates significant upregulation, orange significant downregulation, and white no significant regulation. Yellow for the gene Lhx4 indicates no results.



	Apaf1	Bax	Bcl2l10	Bok	Cidea	Fas	Tnfrsf10b	Tnfsf10	Trp73	Casp1	Fasl	Bcl2a1a	Birc5	Bag3	il10	Cd40lg	Tnf
	Pro-apoptotic											Anti-apoptotic				Both	
1 day (20 Gy)	2,22	2,62	3,82	-1,77	-7	1,67	6,13	-1,55	2,68	-1,49	-1,4	-1,3	-1,51	-1,6	1,31	-2,32	-3,28
7 days (51 Gy)	1,41	1,76	1,28	-1,38	-1,39	1,04	2,63	-1,3	-1,09	-1,61	-1,66	-1,76	-2,78	-1,07	1,6	-2,79	1,09

Figure 6: Significant gene regulation in group 1 and 2. Bars represent fold change values with error bars (SEM) for both biological and technical replicates. The horizontal lines represent a fold change of 1.5, indicating significance.

One of the control genes, *Gusb*, showed an increased expression relative to other control genes and was not used when normalizing the C_t-values. The reaction failed for *Lhx4*, so this gene was not considered in this study.

The error bars in figure 7 shows the uncertainties for both the biological and technical replicates, meaning that SEM was calculated for all of the analysed arrays for each gene. The difference between these uncertainties are illustrated for two genes, *Cd40lg* and *Tnfrsf10b*. In this figure, the individual gene regulation is presented with SEM as error bars.

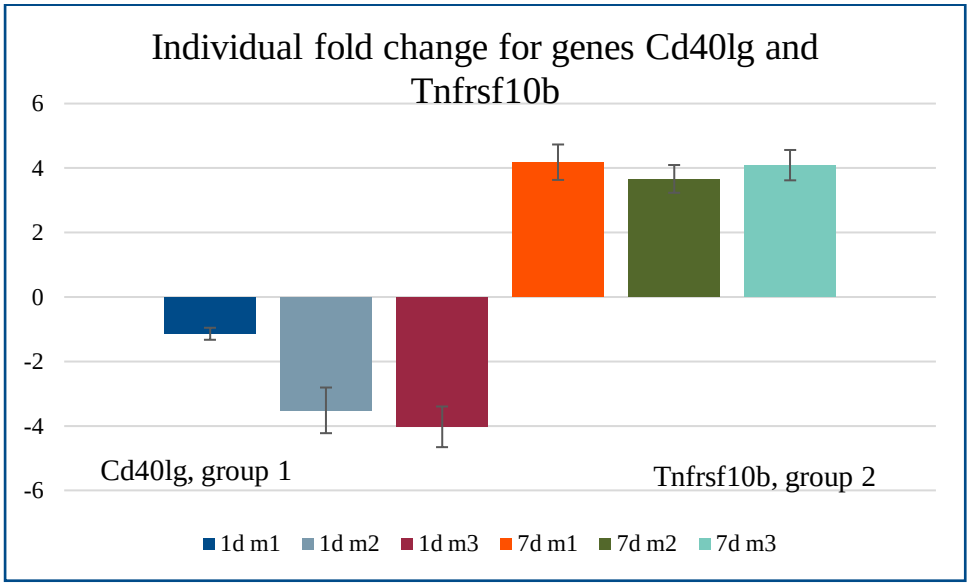


Figure 7: The gene regulation of Cd40lg and Tnfrsf10b for each mouse in group 1 and 2, respectively with error bars (SEM). The mice are represented as either group 1 that was killed after one day or group 2 that was killed after seven days, as 1d or 7d. The individual mice are represented as m1, m2 or m3.

Discussion

In this project, the regulation of 84 different genes related to apoptosis were investigated after one and seven days for different absorbed doses from ^{177}Lu. 46 of the genes in the QPCR array were pro-apoptotic, 32 anti-apoptotic and six genes can be considered both pro- and anti-apoptotic. This unequal ratio mean that an overrepresentation of pro-apoptotic genes was investigated. Only regarding statistics, this may give more information about whether apoptosis was initiated than inhibited. However, since anti-apoptotic genes represented almost 40 % of the panel, the results from the assay still give a clear image of the inhibition of apoptosis on a transcriptional level. Moreover, a direct interpretation by number of genes is not feasible in general, since gene products function in pathways where they can have differential impact on signal transduction and outcome. A direct comparison between treatment groups, however, is possible within the frame of the panel irrespective of these considerations. A clear trend of gene regulation indicating the occurrence of apoptosis appeared. An upregulation of pro-apoptotic genes and a downregulation of anti-apoptotic genes was found for both groups. Five of the 16 genes were significantly regulated in both groups. When studying a gene expression, it is important consider which genes interact in the same pathway in order to understand gene regulation in the context of cellular function and outcomes.

Overall, the group that was killed after one day showed an increased regulation in comparison to the group killed after seven days. The genes regulated in both groups indicate that the cells are signalling for apoptosis to be induced. When irradiating cells, there is often an early response in gene expression signalling for several processes that respond later, including signalling pathways for apoptosis. Apoptosis is a process that depends on the balance between proteins that inhibit apoptosis and proteins that activate it, and thus can be considered a late response to irradiation [14]. A late responding process is dependent of the synthesis of early responding genes.

In this study, there is a difference in the absorbed dose for the different groups, but also a difference in time between killing the mice. The results indicate that apoptosis-related regulation in the kidney cortex occurs as a response to an absorbed dose of 20 Gy protracted over 24 hours, mostly by an upregulation of pro-apoptotic genes, and after a prolonged absorbed dose of 51 Gy over seven days by a downregulation of anti-apoptotic genes. The slight downregulation of anti-apoptotic genes after seven days indicates a shift in the biological response over time. Since the former is not very pronounced considering the overall panel and possible pro-apoptotic regulation, a shift towards survival after seven days can be assumed. The results from this study do not show if the cells in the renal cortex undergo apoptosis, only indication towards a pro-apoptotic response. This means that if apoptosis occurs in the cells within a few days after irradiation, then the transcriptional regulation after seven days would indicate a shift towards pro-survival signalling.

These results are dependent on both absorbed dose and time of investigation. It is likely to assume that at higher dose would increase pro-apoptotic gene regulation, whereas a longer time period would reflect the tissue's response to the (initial) stressor. In addition, the time it takes for regulatory responses to lead to a cellular outcome also affects the interpretation of gene

expression results over time. This means that a change of transcriptional regulation may take hours until a change in protein expression may be observed. The change in protein expression in turn can take up to days until an effect on respective cellular processes can be observed. In addition, such changes may not manifest as acute responses but only after prolonged periods. In order to investigate the differential contribution of these factors independently, additional studies need to be designed in the future.

The gene *Apaf1* is a pro-apoptotic gene, encoding a protein that binds to cytochrome c, which activates procaspase 9, leading to the active form of the initiator caspase 9 and activation of the effector caspases. *Apaf1* is significantly upregulated after one day (group 1). *Bax* is a pro-apoptotic member of the Bcl-2 family and regulates the release of cytochrome c from the mitochondria. The upregulation of the *Bax* gene is an indicator of an increased level of cytochrome c in the cell [14].In mice, the protein encoded by *Bcl2l10* interacts with *Apaf1* and plays a role in apoptosis activated by *caspase 9* [21]. Hence, this upregulation is in agreement with the regulation of *Apaf1*.

Fas, Fasl (Fas Ligand), Tnfsf10 and *Tnfrsf10b* are members of the Tnf receptor superfamily and are pro-apoptotic. The activation of the FAS/FASL signalling pathway leads to apoptosis by forming signals activating a death domain, *caspase 8* and *caspase 3*, leading to a chain reaction that executes of apoptosis. The TNFRSF10B/TNFSF10 signalling pathway activates *caspase 3* and *caspase 8* and is pro-apoptotic. The upregulation of the receptor *Tnfrsf10b* in combination with the downregulation of the ligand *Tnfsf10l* is counterintuitive. This may indicate that the receptor and ligand do not interact in equal proportion (as more receptors may be recruited into the binding complex) or that it is a measure to fine-tune this signalling pathway. It may also mean that this signalling pathway is not (fully) activated in the cells; or that there is still enough Tnfsf10-protein in the cell for sufficient receptor binding to activate the signalling pathway. This pathway should be further investigated in future studies.

Bok is a pro-apoptotic member of the Bcl2 gene family, and even though it is downregulated in the kidney cortex, apoptosis can still be induced through other pathways controlled by other genes. *Cidea* is also pro-apoptotic and downregulated in group 1, interestingly, the baseline expression of the protein is very low in the kidneys, i.e. it is mainly expressed in soft tissue and breast tissue in humans [22].

Trp73 is a gene found in mice and is a member if the *p53* family and transactivates the p53-pathway which can activate apoptosis or cell cycle arrest (where the cell stops in its current state of the cell cycle, inhibiting mitosis and allowing reparations) [23]. The regulation of this gene is not a direct indication of apoptosis in the cell, meaning that in order to relate this expression to a biological response, investigation of other genes or proteins is necessary.

Tnf belongs to the Tnf superfamily and encodes a protein that can be a part of different processes in the cell depending on which receptor it binds to, including apoptosis. The translation of *Tnf* can either activate the signalling pathway NF-κB, an anti-apoptotic protein complex, or a signalling complex cleaving procaspase 8 into its active form (*caspase 8*) starting the process of apoptosis. Hence, the up- and down regulation of this gene in the mice can be both a pro-apoptotic and an anti-apoptotic indicator.

Cd40lg is also a member of the Tnf receptor superfamily and the protein regulates immune responses in the cell, either activating macrophages, B-cells or a step in a process that recruits leucocytes. When irradiated, the cells in the tissue can become inflamed, signalling to the bone marrow to initiate an immune response [24]. The immune response in the irradiated tissue in this study could also be caused by the cells undergoing apoptosis. Following the fragmentation of the cell, immune cells are recruited to phagocytize the apoptotic bodies [14]. The immune response by regulation of *Cd40lg* in the mice could be a direct effect of apoptosis or a response from inflammation caused by radiation.

When performing the QPCR assay, there were no reliable results for the gene *Lhx4*. This could be caused by different reasons. The primer could be located between two exons and be sensitive to different splicing variants; if the specific variant of the mRNA in this study does not match the primer, it cannot bind and start adding nucleotides. There is a possibility that the conditions for the amplicon were poorly adapted for this particular primer. The primer could also have difficulties binding to the cDNA due to suboptimal sequence homology, which prevented it from ever reaching the exponential phase, thus giving a false negative.

The results from the QPCR assay give an indication of the occurrence of apoptosis on the first stage of gene expression (transcription). It should be noted that the amount of mRNA in the cell is not in direct proportion to the protein level. The translation rate can differ between different genes and the same amount of mRNA of two different genes may not lead to the same amount of protein. There can also be an inhibition of translation by e.g. small interfering RNA molecules (siRNA) that degrade mRNA, or by so-called microRNA (miRNA) that bind to the mRNA and block translation by ribosomes (and thus building of a peptide chain).Another factor that may influence the apoptotic fate could be the occurrence of signals to degrade existing proteins in the cell, thus lowering the amount and the functional impact. Nevertheless, the gene expression assay shows that the cells are transcribing genes for pro-apoptotic proteins and suppressing anti-apoptotic ones.

The analysis used total RNA from cortical tissue samples and not individual cells, hence, the results do not reflect apoptotic regulation in individual cells. Another study analysing the tissue for apoptotic cells needs to be done in order to establish if the cells in the tissue undergo apoptosis after irradiation with ^{177}Lu-octreotate.

In a previous study by *Kristiansson et.al.* [25], the gene regulation was studied in the entire kidney for a different mouse strain, Balb/c nude mice. These mice have a genetic mutation making them unable to produce T-cells and therefore have an immunodeficiency. The same type of QPCR array and standard cycle protocol were used in this study rendering technical bias unlikely. However, *Kristiansson et.al.* only used one control gene to normalise the results, in contrast to our study where four control genes were used. In addition, *Kristiansson et.al.* pooled all samples within a group, meaning that information about biological variation and respective standard deviation was not obtained. In their study, the four genes that showed the greatest regulation, *Tnfrsf10b, Bax, Gadd45a* and *Bcl2l1,* were analysed with individual samples of each group, as in comparison to this study where all cortex samples were analysed individually with three technical replicates. In our study, the regulation of *Tnfrsf10b* and *Bax*

was observed as well, but no significant regulation for *Gadd45a* and *Bcl2l1*. The differences in the genes that were not regulated could be a result of the use of a different mouse strain, as well as the fact that the QPCR assay was performed with pooled groups and for the entire kidney, masking some gene expression as an effect of "watering down" potentially significant regulation.

In the study by *Schüler et.al*. [19] the kidney medulla showed to have an increased number of regulated genes compared to the kidney cortex, even though cortex received a higher absorbed dose compared to medulla [7, 8]. The genes analysed in that study were not only related to apoptosis, meaning that some of the genes that were regulated in medulla could be connected to different biological processes. In this project, we analysed the kidney cortex since it receives a higher absorbed dose than medulla and is therefore of great interest when studying apoptosis. However, future studies analysing kidney medulla are needed in order to compare the difference in apoptotic burden between the tissues. However, a previous study has shown that an administration of 150 MBq of ^{177}Lu-octreotate in mice leads to damages to the kidneys in mice after eight months [26].

When calculating the absorbed dose, several assumptions need to be made. The data that is obtained when performing the study is the weight of the entire kidney and the activity in the form of CPM. In order to calculate the absorbed dose, additional information is needed; the absorbed fraction in the kidney cortex, the biodistribution at different times after injection and the weight of the cortex in proportion to the kidney were obtained from previous projects [19, 20]. Assumptions are necessary when adapting these data, which creates uncertainties in the absorbed dose calculation, since the parameters in the previous studies do not precisely match the parameters in this project. In the work of *Svensson et.al*. [20], the data for absorbed fraction and the mass of the cortex in proportion to the kidney was taken for kidneys weighing 0.15 grams, and the kidneys in this project weighed between 0.09 grams and 0.13 grams. The importance of this difference is unclear, but in *Svensson*'s study, a noticeable difference between kidneys weighing 0.15 grams and 0.20 was observed, where a smaller kidney leads to a higher absorbed dose. Another assumption that might influence the results of the absorbed dose was the use of biodistribution data from *Schüler et.al.* [19]. The data used for biodistribution was taken from mice injected with 15 MBq ^{177}Lu-octreotate, while for our project, the mice were injected with 150 MBq ^{177}Lu-octreotate. That article clearly states that the biodistribution is dependent on the injected activity; nevertheless, the resulting differences for kidneys seem to be lesser than for other organs investigated.

Conclusions

This work demonstrates pro-apoptotic regulation in cortical tissue after ^{177}Lu-octreotate treatment and strongly advocates further analysis of the apoptotic fate in future studies. The results show a difference between the immediate response after one day and the prolonged response after seven days. The importance of the difference in absorbed dose and time after irradiation needs to be clarified in further studies, as well as a study of medullary tissue in order to assess the risk for the entire organ.

The potential induction of apoptosis after treatment emphasises the importance of finding protective treatments that minimise this side-effect.

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Reference list

1. Ljungberg, M., et al., *MIRD Pamphlet No. 26: Joint EANM/MIRD Guidelines for Quantitative 177Lu SPECT Applied for Dosimetry of Radiopharmaceutical Therapy.* J Nucl Med, 2016. **57**(1): p. 151-62.
2. Uusijarvi, H., et al., *Dosimetric characterization of radionuclides for systemic tumor therapy: influence of particle range, photon emission, and subcellular distribution.* Med Phys, 2006. **33**(9): p. 3260-9.
3. Bodei, L., et al., *Long-term evaluation of renal toxicity after peptide receptor radionuclide therapy with 90Y-DOTATOC and 177Lu-DOTATATE: the role of associated risk factors.* Eur J Nucl Med Mol Imaging, 2008. **35**(10): p. 1847-56.
4. Savill, J., *Apoptosis and the kidney.* J Am Soc Nephrol, 1994. **5**(1): p. 12-21.
5. Sanz, A.B., et al., *Mechanisms of Renal Apoptosis in Health and Disease.* Journal of the American Society of Nephrology, 2008. **19**(9): p. 1634-1642.
6. Vegt, E., et al., *Renal toxicity of radiolabeled peptides and antibody fragments: mechanisms, impact on radionuclide therapy, and strategies for prevention.* J Nucl Med, 2010. **51**(7): p. 1049-58.
7. De Jong, M., et al., *Inhomogeneous localization of radioactivity in the human kidney after injection of [(111)In-DTPA]octreotide.* J Nucl Med, 2004. **45**(7): p. 1168-71.
8. Melis, M., et al., *Localisation and mechanism of renal retention of radiolabelled somatostatin analogues.* Eur J Nucl Med Mol Imaging, 2005. **32**(10): p. 1136-43.
9. Eriksson, D. and T. Stigbrand, *Radiation-induced cell death mechanisms.* Tumour Biol, 2010. **31**(4): p. 363-72.
10. John C. Reed, D.R.G., *Apoptosis: Physiology and Pathology*. Vol. 1. 2011, New York, NY, USA: Cambridge University Press.
11. Kiraz, Y., et al., *Major apoptotic mechanisms and genes involved in apoptosis.* Tumour Biol, 2016. **37**(7): p. 8471-86.
12. Verheij, M. and H. Bartelink, *Radiation-induced apoptosis.* Cell Tissue Res, 2000. **301**(1): p. 133-42.
13. Egelberg. *Own work*. 13-November-12; Available from: https://commons.wikimedia.org/w/index.php?curid=22663211.
14. Eric J. Hall, A.J.G., *Radiobiology for the Radiologist*. 7th ed. 2012, Philadephia, PA, USA: Lippincott Williams and Wilkins.
15. Wikipedia. *Real-time polymerase chain reaction*. 190428 [cited 2019 05 01.
16. Biosystems, A., *Introduction to gene expression: getting started guide*. 2010: Life technologies™.
17. QIAGEN. *RT2 Profiler PCR arrays*. Available from: https://www.qiagen.com/~/media/nextq/image%20library/fc/00/25/fc_0025_pcrarray/1_5.
18. Bolch, W.E., et al., *MIRD pamphlet No. 21: a generalized schema for radiopharmaceutical dosimetry--standardization of nomenclature.* J Nucl Med, 2009. **50**(3): p. 477-84.
19. Schuler, E., A. Osterlund, and E. Forssell-Aronsson, *The amount of injected 177Lu-octreotate strongly influences biodistribution and dosimetry in C57BL/6N mice.* Acta Oncol, 2016. **55**(1): p. 68-76.
20. Svensson, J., et al., *Nephrotoxicity profiles and threshold dose values for [177Lu]-DOTATATE in nude mice.* Nucl Med Biol, 2012. **39**(6): p. 756-62.
21. Human protein atlas, p.o. *BCL2L10*. Available from: https://www.proteinatlas.org/ENSG00000137875-BCL2L10/tissue.
22. Human protein atlas, p.o. *CIDEA*. [cited 2019 05 10; Available from: https://www.proteinatlas.org/ENSG00000176194-CIDEA/tissue.
23. NCBI. *Trp73 transformation related protein 73 [Mus musculus (house mouse)]*. 7-May-19; Available from: https://www.ncbi.nlm.nih.gov/gene/22062.
24. Schaue, D., et al., *Radiation and inflammation.* Semin Radiat Oncol, 2015. **25**(1): p. 4-10.

25. Kristiansson, A., et al., *Protection of Kidney Function with Human Antioxidation Protein alpha1-Microglobulin in a Mouse (177)Lu-DOTATATE Radiation Therapy Model.* Antioxid Redox Signal, 2019. **30**(14): p. 1746-1759.
26. Schuler, E., et al., *Potential Biomarkers for Radiation-Induced Renal Toxicity following 177Lu-Octreotate Administration in Mice.* PLoS One, 2015. **10**(8): p. e0136204.
27. QIAGEN. *RT2 Profiler PCR arrays Gene List*. Available from: https://www.qiagen.com/se/products/discovery-and-translational-research/pcr-qpcr/qpcr-assays-and-instruments/mrna-incrna-qpcr-assays-panels/rt2-profiler-pcr-arrays/?catno=PAMM-012Z#geneglobe.

Appendix

Complete list of genes in RT2 profiler PCR array and type of gene [27]:

Gene	Function	Fold change group 1	Fold change group 2
Abl1	Positive regulation of apoptosis	-1.198	1.006
Aifm1	Caspase activation	1.002	1.114
Akt1	Anti-apoptotic, positive regulation of apoptosis	-1.197	1.111
Anxa5	Positive regulation of apoptosis	-1.129	-1.027
Apaf1	Caspase activation	2.216	1.407
Api5	Anti-apoptotic	-1.085	-1.033
Atf5	Anti-apoptotic	-1.266	-1.039
Bad	Pro-apoptotic	-1.044	1.081
Bag1	Negative regulation of apoptosis	-1.046	1.095
Bag3	Negative regulation of apoptosis	-1.597	-1.065
Bak1	Positive regulation of apoptosis	-1.111	1.030
Bax	Pro-apoptotic, caspase activation	2.620	1.762
Bcl10	Pro-apoptotic, negative regulation of apoptosis	1.003	1.051
Bcl2	Anti-apoptotic, negative regulation of apoptosis	-1.132	-1.555
Bcl2a1a	Negative regulation of apoptosis	-1.295	-1.758
Bcl2l1	Anti-apoptotic, negative regulation of apoptosis	1.464	1.156
Bcl2l10	Anti-apoptotic, negative regulation of apoptosis, caspase activation	3.821	1.283
Bcl2l11	Pro-apoptotic, positive regulation of apoptosis	1.373	1.192
Bcl2l2	Anti-apoptotic, negative regulation of apoptosis	-1.053	1.123
Bid	Pro-apoptotic, positive regulation of apoptosis	1.153	-1.044
Birc2	Negative regulation of apoptosis	-1.057	-1.129
Birc3	Anti-apoptotic, negative regulation of apoptosis	1.116	-2.776
Birc5	Anti-apoptotic, caspase inhibition	-1.513	-1.129
Bnip2	Anti-apoptotic, negative regulation of apoptosis	-1.201	1.203
Bnip3	Pro-apoptotic, anti-apoptotic, positive regulation of apoptosis, negative regulation of apoptosis	-1.103	1.070
Bnip3l	Pro-apoptotic, anti-apoptotic, positive regulation of apoptosis, negative regulation of apoptosis	-1.337	-1.378
Bok	Pro-apoptotic	-1.773	1.015
Card10	Negative regulation of apoptosis	1.139	-1.608
Casp1	Pro-apoptotic, caspase activation, initiator caspase	-1.487	-1.608

Casp12	Pro-apoptotic, caspase	1.066	-1.175
Casp14	Pro-apoptotic, positive regulation of apoptosis, caspase	-1.368	-1.338
Casp2	Pro-apoptotic, positive regulation of apoptosis, caspase	-1.196	-1.134
Casp3	Pro-apoptotic, positive regulation of apoptosis, caspase	1.309	-1.051
Casp4	Pro-apoptotic, positive regulation of apoptosis, caspase	1.344	1.038
Casp6	Pro-apoptotic, positive regulation of apoptosis, caspase	-1.051	1.079
Casp7	Pro-apoptotic, positive regulation of apoptosis, caspase	1.253	1.235
Casp8	Pro-apoptotic, positive regulation of apoptosis, caspase	1.015	1.100
Casp9	Caspase, caspase activation	-1.014	1.002
Cd40	Positive regulation of apoptosis	1.228	-1.060
Cd40lg	Anti-apoptotic, negative regulation of apoptosis	-2.325	-2.794
Cd70	Pro-apoptotic, positive regulation of apoptosis	1.190	1.413
Cflar	Extracellular apoptotic signals, anti-apoptotic, negative regulation of apoptosis	-1.128	1.081
Cidea	DNA damage and repair, negative regulation of apoptosis	-7.002	-1.392
Cideb	DNA damage and repair, positive regulation of apoptosis	-1.249	1.284
Cradd	Death domain receptor, positive regulation of apoptosis	-1.061	1.138
Dad1	Anti-apoptotic	1.093	1.229
Dapk1	Extracellular apoptotic signals, death domain receptor, positive regulation of apoptosis	1.038	1.022
Dffa	Pro-apoptotic, positive regulation of apoptosis	1.142	1.103
Dffb	Pro-apoptotic	1.081	-1.060
Diablo	Pro-apoptotic	1.139	1.132
Fadd	Death domain receptor, positive regulation of apoptosis	1.156	1.150
Fas	Pro-apoptotic, anti-apoptotic, negative regulation of apoptosis	1.670	1.039
Fasl	Pro-apoptotic, positive regulation of apoptosis	-1.401	-1.655
Gadd45a	Positive regulation of apoptosis	-1.314	-1.044
Igf1r	Anti-apoptotic	-1.192	1.063
il10	Anti-apoptotic	1.308	1.604
Lhx4	Anti-apoptotic	-	-
Ltbr	Positive regulation of apoptosis	1.086	1.119
Mapk1	Pro-apoptotic	-1.006	1.029

Mcl1	Anti-apoptotic, negative regulation of apoptosis	1.186	-1.134
Naip1	Anti-apoptotic	1.179	1.053
Naip2	Anti-apoptotic	-1.389	-1.642
Nfkb1	Death domain receptor, anti-apoptotic	-1.186	-1.058
Nme5	Anti-apoptotic	1.027	1.064
Nod1	Pro-apoptotic, positive regulation of apoptosis, caspase activation	-1.447	-1.036
Nol3	Anti-apoptotic, negative regulation of apoptosis	-1.037	-1.449
Polb	Anti-apoptotic	-1.074	-1.054
Prdx2	Anti-apoptotic	1.036	1.032
Pycard	Pro-apoptotic, positive regulation of apoptosis, caspase activation	1.113	-1.168
Ripk1	Death domain receptor	-1.083	-1.055
Tnf	Pro-apoptotic, death domain receptor, anti-apoptotic	-3.281	1.086
Tnfrsf10b	Death domain receptor, positive regulation of apoptosis, caspase activation	6.131	2.626
Tnfrsf11b	Death domain receptor	-1.272	1.287
Tnfrsf1a	Death domain receptor	-1.113	1.047
Tnfsf10	Pro-apoptotic, Positive regulation of apoptosis	-1.546	-1.301
Tnfsf12	Positive regulation of apoptosis	-1.247	1.184
Traf1	Positive regulation of apoptosis	1.050	-1.430
Traf2	Positive regulation of apoptosis	-1.063	-1.191
Traf3	Pro-apoptotic, positive regulation of apoptosis	-1.178	1.050
Trp53	DNA damage and reparation, negative regulation of apoptosis, positive regulation of apoptosis, caspase activation	1.060	1.079
Trp53bp2	Pro-apoptotic, positive regulation of apoptosis	-1.472	-1.556
Trp63	DNA damage and reparation, anti-apoptotic	-1.300	-1.276
Trp73	DNA damage and reparation, negative regulation of apoptosis	2.677	-1.087
Xiap	Anti-apoptotic, negative regulation of apoptosis, caspase inhibition	-1.223	1.150
Actb	Control gene	-	-
B2m	Control gene	-	-
Gapdh	Control gene	-	-
Gusb	Control gene	-	-
Hsp90ab1	Control gene	-	-