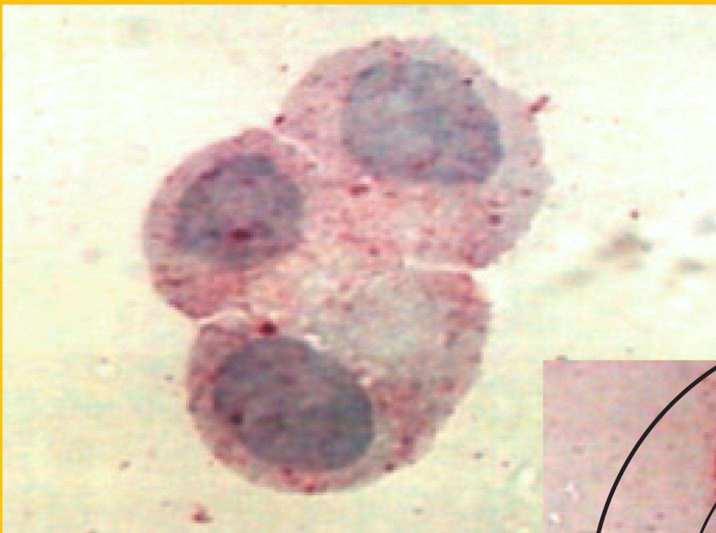
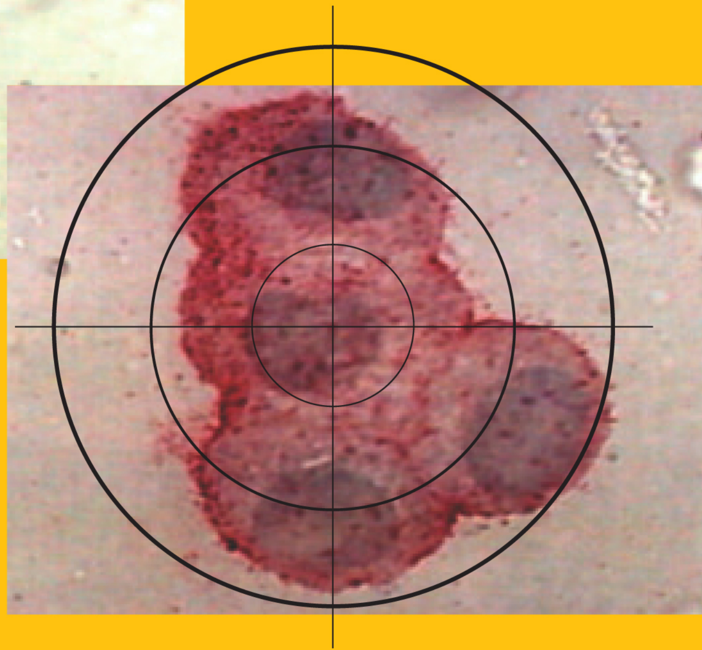
Induction of the Somatostatin Receptor on Colon Carcinoma Cells as a Target for Radiolabeled or Cytotoxic Somatostatin Analogs





A. Mearadji

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Amir Mearadji

“Ik zweer geen steenlijders te zullen snijden, doch bij die operatie voor deskundige plaats te zullen maken.”

Passage uit de Eed van Hippocrates

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INDUCTION OF THE SOMATOSTATIN RECEPTOR ON COLON CARCINOMA CELLS AS A TARGET FOR RADIOLABELED OR CYTOTOXIC SOMATOSTATIN ANALOGS

INDUCTIE VAN DE SOMATOSTATINE RECEPTOR OP COLON CARCINOOM CELLEN VOOR DOELGERICHTE RADIOGELABELDE OF CYTOTOXISCHE SOMATOSTATINE ANALOGA

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CONTENTS

Chapter 1	General Introduction	9
Chapter 2	Aims of the thesis	25
Chapter 3	Somatostatin receptor imaging, therapy and new strategies in patients with neuroendocrine tumors	29
Chapter 4	Induction of the somatostatin receptor on colon carcinoma cells as a target for radiolabeled octreotide	51
Chapter 5	Targeted therapy with [^{111}In-DTPA^{0}]octreotide on colon carcinoma cells transfected with a somatostatin receptor assessed in a rat liver metastasis model	65
Chapter 6	Anti-tumor effect and increased survival after treatment with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate in a rat liver micrometastases model	81
Chapter 7	Somatostatin receptor gene therapy combined with targeted therapy with radiolabeled octreotide: a new treatment for liver metastases	97
Chapter 8	AN-238, a cytotoxic somatostatin analog: an introduction	113
Chapter 9	The effect of targeted therapy with AN-238 on colon carcinoma cells transfected with a somatostatin receptor in a rat liver metastasis model	127
Chapter 10	Summary, Conclusions and Future Aspects	147
	Dankwoord en Curriculum vitae	161

Perfectie is niet haalbaar: daarom eisen we ze van anderen.

L. Tolstoi

Aan Ilona

en mijn ouders

CHAPTER 1

GENERAL INTRODUCTION

SOMATOSTATIN, SOMATOSTATIN RECEPTORS AND PRINCIPLES OF GENE THERAPY

SOMATOSTATIN

In 1973 Brazeau *et al.* isolated and identified a 14-amino acid peptide, named somatotropin-release inhibiting factor (SRIF), later changed to somatostatin (SS)(1). Subsequently, research showed that somatostatin had two forms of bioactive peptides, somatostatin 14 and somatostatin 28, and that SS was widely distributed throughout the body with a wide variety of effects on endocrine, gastrointestinal and central nervous systems. The diversity of effects mediated by SS led to the suggestion that the hormone could have a therapeutical purpose in a great number of indications. However, due to the short half-life of SS (approximately 2 minutes) there were some restrictions in the use of natural SS as a drug. Therefore, researchers began to synthesize more stable peptide analogs with a longer duration of action, such as octreotide (SMS 201-995), lanreotide (BIM 23014) and vapreotide(2). These analogs not only proved to be more stable, but had also different affinity for the five different somatostatin receptors (SSRs), making their mode of action also more selective. These new finding led to a broad use of SS and their analogs in a clinical setting.

SOMATOSTATIN RECEPTORS

At least five different human SSRs have been cloned, sst_1 to sst_5(2). The SSRs subtypes present a high degree of sequence identity. SS 14 and SS 28 bind with similar affinity for all SSRs, except sst_5, which shows a 10 fold higher affinity for SS 28. However, analogs show different affinities for the five receptor-subtypes. Analogs exhibit a high affinity for sst_2 and sst_5, whereas they bind with low affinity for sst_1 and sst_4 and bind with a moderate affinity for sst_3(2; 3). Using radiolabeled SS and analogs indicated that SSRs were expressed in a wide number of normal tissue such as hypothalamus, pituitary, gastro-intestinal tract, pancreas, endocrine glands and lymphoid tissue(4-6). Furthermore, binding studies showed also high expression of SSRs in various tumors, like gastroenteropanctreatic (GEP) tumors, thyroid carcinomas, breast tumors, ovarian cancers and pituitary adenomas. Interestingly, these binding studies suggested that SSRs were preferentially expressed in well-differentiated compared to less differentiated tumors(7; 8). These observations hypothesize that SSRs may play a role in the differentiation in some cancers and it can be used clinically as a marker in the progression of cancers. Thus, since SSRs play an important role in the physiological control of cell proliferation, loss of SSR expression in tumor cells would confer a proliferative advantage to those cells and

their progeny. In regard of this point genes of the SSRs can be regarded as tumor suppressor genes. This suggestion is supported by the observation that a point mutation in the sst_2 gene results in an increased proliferation of small cell lung cancer cells *in vitro*(9).

Each receptor subtype is coupled to multiple intracellular transduction pathways via GTP binding proteins. To date, a total of three signaling pathways are identified. The first signaling pathway is the inhibition of the adenylate cyclase system leading to reduction of intracellular cAMP(2; 3). A second key plasma membrane signaling pathway involved in SS action concerns K^+ and Ca^{2+} channels. Activation by SS causes hyperpolarization of the plasma membrane and leads to decreased Ca^{2+} influx and consequently to reduction of intracellular Ca^{2+} (10). SS can also decrease Ca^{2+} influx directly by inhibiting high voltage-dependent Ca^{2+} channels via $G_{0\alpha 2}$ (2). Both of these two signal transduction pathways, inhibition of Ca^{2+} and to a lesser extent inhibition of cAMP, mediate the negative regulation of hormone secretions induced by SS. A third important membrane signaling pathway involves an activation of a number of protein phosphatases induced by SS and analogs. Sst_1 to sst_4 have been reported to stimulate tyrosine phosphatase activity when expressed in NIH 3T3 fibroblast or CHO cells(11-13).

EFFECT OF SOMATOSTATIN ON CELL GROWTH

The effects of SS on tumor growth can be divided in indirect and direct effects.

Indirect effects

Indirectly, SS can inhibit the secretion of growth-stimulating hormones and factors which can stimulate the growth of various tumors. For example, octreotide has been demonstrated to negatively control the serum IGF-1 level by inhibiting GH secretion through sst_2 and sst_5(14).

Also a direct effect on IGF gene expression has been demonstrated(15). Clinical studies have shown a reduction of IGF-1 gene expression and serum levels of IGF-1 after treatment of breast cancers with octreotide(16).

In addition, an increased expression and secretion of IGF-binding protein-1, which specifically binds IGF-1 and negatively regulates plasma IGF-1, has been reported(17).

SS and its analogs can also indirectly control tumor and metastasis development by inhibition of angiogenesis(18). In various types of cancers (colorectal, breast, renal cell and lung cancers) an expression of peritumoral vascular SSRs has been demonstrated. Moreover, the expression of SSRs in tumor vessels was independent of receptor expression on the tumor(19). This suggests that SS and SSRs may play a role in angiogenesis and other hemodynamic aspects of the tumor.

Direct effects

SS can also directly inhibit cell growth in various cancer cell lines which express SSRs. However, this SS induced cell growth arrest is still poorly understood. To date, three different mechanism are described.

1. As a result of the blockade of mitogenic growth factor signal, SS may directly inhibit cell growth. This effect involves the stimulation of a tyrosine phosphatase(20; 21). The SS-sensitive tyrosine phosphatase has recently been identified as SHP-1. SHP-1 is associated with various activated tyrosylated growth factor tyrosine kinase receptors and cytokine receptors. It has been reported in several studies that SHP-1 may have a role in terminating growth factor and cytokine mitogenic signals by dephosphorylating critical molecules(22). Lopez *et al.* have shown that that SHP-1 is required for the antiproliferative signal initiated by sst_2(23).
2. The antiproliferative effect of SS can also result from apoptosis. This has been reported to be induced by sst_3 via a G protein-dependent signaling and to be associated with an intracellular acidification and activation of endonuclease and induction of p53 and Bax(24; 25).
3. Also SS can inhibit directly syntheses and secretion of autocrine growth factors, cytokines and hormones involved in the proliferation of tumor cells(26; 27). This is mediated through inhibition of Ca^{2+} and cAMP production. Furthermore, SS can directly interfere with the exocytotic machinery by inhibiting the protein phosphatase calcineurin(28).

PRINCIPLES OF GENE THERAPY

Gene therapy is defined as the transfer of nucleic acids (DNA) to cells which results in a therapeutic effect, by either correcting genetic defects or by overexpressing proteins that are therapeutically useful.

Gene delivery vectors

In order to transfer the DNA into cells a gene delivery vector is acquired. The gene delivery vectors can be divided in two groups: viral and non-viral vectors.

1. <u>Viral vectors:</u>

Viruses are able to effectively recognize and enter cells, traffic within the cytosol to the nucleus, translocate into the nucleus and express their genes in host cells. These properties made them among the first choices for gene delivery vectors.

The most frequently used viral vectors are retroviruses and adenoviruses (table 1). Although, recently other viral vectors are in preclinical development, including adeno-associated virus (AAV)(29), herpes simplex virus (HSV (30), pox and lentivirus (31) etc..

Retroviruses can lead to a stable integration of the transfected gene in the host genome, consequently leading to a ever-lasting gene transfer. Replication-deficient retroviruses are produced *in vitro* in specific packaging cells transfected previously with retroviral genes that have been deleted from the genome of the therapeutic retroviruses. Major limitations of the retroviral vectors are their low titres, their inability to infect non-dividing cells (this can be an advantage in the case of cancer gene therapy), and the potential risk of insertional mutagenesis(32).

Adenoviruses can be produced in high titres. As they do not integrate stable into the host genome, they lead to a transient transgene expression. An advantage is the effective gene transfer in both dividing as non-dividing cells, but an disadvantage is the produced immunological and inflammatory response by themselves(33) or via the proteins encoded in the transgene(34). Adenoviruses are human pathogens, and most patients have already been exposed to them during their lifetime resulting in the presence of various levels of circulating neutralizing anti-viral antibodies. This may decrease the effectiveness of their systemic application. Use of viral vectors which are not human pathogens (e.g., various serotypes of adenoviruses, AAV, or non-human

adenoviruses) can avoid this problem. Immune response evoked by the first application of the viral vector, however, may interfere with their repeated application.

2. Non-viral vectors

The synthetic vectors (naked DNA, cationic liposomes, etc.) are far from being ideal delivery systems. Although they are less pathogenic and may have reduced toxicity compared to some of the viral vectors, depending on the dose injected, liposomes may aggregate in blood and can cause severe toxic reactions(35). Plasmid and liposome complexes are easy to produce and are safe, but they have low gene-transfer efficiency. However, in the future, the "perfect" gene delivery vector may be synthetic, incorporating many of the advantages of viruses (e.g., cell recognition, cellular uptake, etc.), but avoiding the unwanted properties of viruses (e.g. pathogenicity, cell toxicity, immune response, etc.).

Table 1: Gene Therapy vehicles

Vector	Advantages	Disadvantages
Retrovirus	- stabile transduction - requires cell division for transduction (advantegeous for cancer gene therapy) - lower anti-viral immune response	- low transduction-efficacy - requires cell division for transduction - limited DNA-packaging capacity - insertional mutagenesis? - low production titres
Adenovirus	- high production titres - transduction in dividing and non-dividing cells - high transduction-efficacy - large DNA-packaging capacity	- transient transduction - higher anti-viral immune response - low cell-specificity of transduction

Gene delivery targeting

Targeting of gene therapy vehicles is not only important for the improvement of the effectiveness of expression of the transfected gene, but can also play a vital role in toxicity of this treatment. Especially, when cancer gene therapy is concerned, most gene therapy vehicles transfect both dividing and nondividing cells, as a result of

which normal cells can also be transfected, leading to toxicity of the treatment. For these reasons a lot of research has been done in targeting the gene therapy vehicles.

1. Physical targeting

A variety of physical gene delivery methods have been introduced to achieve better local tissue targeting of vectors. The most simple manner of physical targeting is intramuscular, -intratumoral, intradermal delivery of the gene therapy vehicles. Another example of the effective physical targeting is catheter-mediated gene transfer to various regions of the circulation (e.g., liver, heart, limbs, etc.)(36-38). These delivery methods offer certain advantages in specific disease targets, but when a disease is more widely spread in the whole human body (e.g. metastasized cancer), this targeting strategy becomes obsolete.

2. Transcriptional targeting

As many target tissues or diseases have a specific property, this property can be used in order to achieve a targeted vector. Incorporation of a tissue- or disease-specific promotor into the vector allows therapeutic gene expression only in cells which express transcription factor proteins binding to these specific promotor sites. Some of the tissue- or disease specific promotors are prostate-specific antigen (PSA)(39), carcinoembryonic antigen (CEA)(40) and hypoxia response element (HRE) activated by hypoxia inducible factors (HIF) in hypoxic/ischemic tissue (e.g. tumors)(41).

Cancer gene therapy

There are at least five different approaches presently developed for cancer gene therapy:

1. Gene replacement (tumor suppressor-genes)

Transfer of tumor suppressor genes, like wild-type p53, into p53 mutant tumor cells can induce apoptosis *in vitro* and in animal studies(42-44). Other cell cycle regulatory genes like p16 or p21 are also potential targets for gene replacement. Replacement of tumor suppressor genes can be combined with chemo- or radiotherapy to increase apoptosis of tumor cells. Treatment of lung cancer patients with wild-type p53 delivered locally with an adenovirus has shown promising result in a human clinical trial (45).

2. <u>Suicide gene therapy</u>

One of the most effective approaches of cancer gene therapy is suicide gene therapy, in which tumor cells are transduced with genes whose product convert a nontoxic prodrug into a toxic metabolite. Examples of such genes include the herpes simplex thymidine kinase (HSV-tk) gene and cytosine deaminase (CD) gene which convert the nontoxic drugs ganciclovir and 5-fluorocytosine to the toxic products ganciclovir-tryphosphate and 5-fluorouracil, respectively(46). A key property of this approach is the so called "bystander effect" consisting in the induction of killing of nontransduced cells by neighboring transduced cells(47; 48). Explanations for the bystander effect include the transfer of toxic metabolites through gap junctions of cells and the induction of immune responses against tumor antigens released from dying cells(49; 50). The bystander effect contributes to a significant increase of antitumor effect in cancer gene therapy

3. <u>Cytokine gene therapy</u>

Transduction of tumor or adjacent tissue with cytokine genes, generates an antitumor immunity by recruitment and stimulation of immune effector cells. Cytokine used to date include IL-2, IL-4, IL-6, IL-7, IL-12, GM-CSF, TNF-α and IFN-γ(51-58). Moreover, this strategy is often combined with suicide gene therapy, as the bystander effect induces a more intense antitumor effect (59).

4. <u>Drug resistance gene</u>

Transduction of the bone marrow with multidrug resistance (MDR-1) gene results protection of the bone marrow against chemotherapeutic agents, thus permitting the use of higher doses of chemotherapy (60; 61). MDR encodes for p-glycoprotein which functions as a cellular efflux pump extruding toxic drugs from out of transfected cells. Several clinical trials using this concept are currently under way.

5. <u>Anti-angiogenesis</u>

The growth of tumors is strongly dependent of angiogenesis to provide nutrients and oxygen to tumor cells. This process of neovascularization is modulated by tumor cells that produce angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Specific inhibition of these genes or gene transfer of angiogenesis inhibiting factors like endostatin or angiostatin, could have a significant antitumoral effect and is currently under investigation(62-64).

The therapeutic goal in any treatment modality for cancer is effective killing of most (if not all) cancer cells without serious damage to normal cells and tissues. Therefore,

gene therapy should also aim at effective and selective killing of cancer cells. So far, there has been a disappointing inability to reach cancer cells with sufficient efficacy to generate high enough levels of direct killing. Most successful results are in an experimental setting, although a few clinical trials are also under way and preliminary results are promising.

SSR gene therapy

As stated earlier the genes of the SSRs can be regarded as tumor suppressor genes. Zhang *et al.* observed that a point mutation in the sst_2 gene resulted in an increased proliferation of small cell cancer cells (10). Also Delesque *et al.* showed that loss of the sst_2 expression could lead to an increased tumorigenicity of pancreatic tumor cells (65). Through this observation a new approach was developed for the treatment of pancreatic cancer by means of sst_2 gene transfer (66). By inducing the SSR on the tumor cells, antitumor effects were obtained which might be attributed to several mechanisms. First, an autocrine negative feedback loop in which transfected tumor cells start to produce SS, which binds in an autocrine manner to the induced SSR, may provide an inhibitory effect on tumor cell growth. Second, binding of the SS to sst_2 may upregulate p27, a tumor suppressor gene, which leads to cell cycle arrest in the G0-G1 phase, and subsequently causes apoptosis. Local and distant bystander effects have also been noted (67). The local bystander effect might be attributed in part to apoptosis, these cells release apoptotic vesicles and enzymes, which in turn might kill neighboring cells. The distant bystander effect may be explained by a paracrine effect. SS can upregulate the expression of sst_1 on parental tumor cells, thereby rendering them sensitive to the antiproliferative effect of SS. All the abovementioned mechanisms may contribute to successful treatment of certain types of cancers with SSR gene therapy.

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CHAPTER 2

AIMS OF THE THESIS

AIMS OF THE THESIS

Somatostatin is a widely distributed peptide that negatively regulates a number of cellular processes, including growth of multiple epithelial cell types. Previous studies have shown that the somatostatin analog, octreotide, slightly suppress the growth of cancer cells expressing somatostatin receptors (SSRs). However, high radioactive doses with [^{111}In-DTPA^{0}]octreotide for peptide receptor radionuclide therapy (PRRT) could inhibit the growth of SSR positive liver metastases in a rat model strongly. The results of these experiments suggests that the antiproliferative effects were predominantly mediated by the specific SSR.

Among the five cloned SSR-subtypes (sst_{1-5}), subtype 2 was found to mediate the growth inhibitory effects of somatostatin analogs *in vitro* the strongest. A new targeted cytotoxic somatostatin analog, named AN-238, which could be more efficacious and less toxic than presently used systemic chemotherapeutic agents, also do have a high affinity for sst_2.

Moreover, PPRT with new radiolabelled somatostatin analogs, as [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, could have even stronger antitumor effects than [^{111}In-DTPA^{0}]octreotide .

In order to use PRRT and AN-238 in SSR-negative tumors, induction of the SSR (subtype 2) is necessary.

The aims of this thesis are:

1. Is sst_2 *in vitro* transfection of CC531 SSR-negative colon carcinoma cells possible and is the transfected SSR functional?
2. Is PRRT with [^{111}In-DTPA^{0}]octreotide effective for the sst_2-transfected CC531 cells in a rat liver metastases model?
3. Is PRRT with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate effective for sst_2-positive CA20948 pancreas carcinoma cells in a rat liver metastases model?
4. Is PRRT with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate effective for sst_2-transfected CC531 cells in a rat liver metastases model?
5. Is targeted therapy with the cytotoxic somatostatin analog, AN-238, effective for sst_2-transfected CC531 cells in a rat liver metastases model?

Alleen licht zie je beter in het donker.

S. Brussaard

CHAPTER 3

SOMATOSTATIN RECEPTOR IMAGING, THERAPY AND NEW STRATEGIES IN PATIENTS WITH NEUROENDOCRINE TUMORS

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ABSTRACT

Somatostatin receptors have been found on a variety of neuroendocrine tumors like carcinoids, paragangliomas, as well as on most pancreatic endocrine and breast tumors. Somatostatin receptor scintigraphy with a radionuclide labeled somatostatin analog, [^{111}In-DTPA^{0}]octreotide, is a sensitive and specific technique to visualise *in vivo* the presence of somatostatin receptors on various tumors.

Material was identified from previous review articles, references citated in original papers and a Medline search of the literature. Additional material was obtained from recently published abstracts of meetings.

Somatostatin receptor imaging of neuroendocrine tumors is essential in the diagnostic work up for most of these tumors. The expression of somatostatin receptors *in vivo* not only predicts the outcome of somatostatin analog treatment but also opens the possibility for new therapeutic strategies. Since better information about spread of the disease can be obtained, more justified therapy options can be proposed.

SOMATOSTATIN RECEPTOR EXPRESSION

Somatostatin (SS) is a small regulatory peptide, isolated in the ovine hypothalamic gland in 1973 as a growth hormone (GH) release inhibiting factor[1]. SS is widely distributed in the human body and not only in the hypothalamus, but also in various parts of the gastro-intestinal tract, indicating that inhibition of GH is not its only function[2]. Apart from its function as a neurotransmitter in the central nervous system (CNS), SS also has inhibitory effects on the secretion of hormones by the pancreatic islands (insulin, glucagon) and on the exocrine pancreatic function. Also, somatostatin inhibits normal gastrin production and consequently gastric acid and pepsin production[3]. A number of observations have also suggested an anti-proliferative effect of SS and its stable analogs[4-6]. Critical to all these actions is the expression of somatostatin receptors (SSRs) on the cell membrane. These SSRs subserve two functions: (1) to recognise the ligand and bind it with high affinity and specificity, and (2) to generate a transmembrane signal that evokes a biological response. Large number of SSRs were found on most tumors with amine precursor uptake and decarboxilation (APUD) characteristics and neuroendocrine properties, such as carcinoids, paragangliomas, pheochromocytomas, medullary thyroid cancers and endocrine pancreatic tumors. In addition, large numbers of binding sites with high affinity for SS were also found on breast and brain tumors as well as on various cells of the immune system[7-10]. At least five different human SSR subtypes have been cloned[11]. All subtypes bind SS with high affinity, while their affinity for the SS analog octreotide differs considerably. Octreotide binds with high affinity to somatostatin receptor subtype 2 (sst_2) and sst_5, to a lesser degree sst_3, while no binding to sst_1 and sst_4 occurs. Other somatostatin analogs, that are in clinical use like BIM 23014 (Lanreotide) and RC-160 (Vapreotide) as well as the hexapeptide MK678 bind to three of the five SSR subtypes displaying also high affinity for sst_2 and sst_5 and moderate affinity for sst_3[12].

Somatostatin receptor mRNA subtypes are widely expressed in neuroendocrine tumors, but their distribution is not necessarily correlated with SSR subtype expression. Furthermore SSR subtypes show a differential subcellular localisation in human SSR positive tumors[13]. The majority of human endocrine pancreatic tumors such as gastrinomas, glucoganomas, VIPomas and “non-functioning” islet cells tumors express sst_2. *In vitro* studies have shown that 72% of the insulinomas express SSRs, however, these receptors are mainly sst_3, which has low affinity for

octreotide[14,15]. Although SSRs have been demonstrated on exocrine pancreatic cells in experimental animals mainly on the acinar cells, neither SSRs nor neuroendocrine properties could be confirmed on human exocrine pancreatic adenocarcinomas[16]. Carcinoids, paragangliomas, pheochromocytomas and medullary thyroid cancers all have a mixed distribution of the SSR subtypes but sst_2 is most frequently expressed. The presence of different combinations of SSR subtypes may explain the variable clinical response to SS analogs and the difference in successful localisation by somatostatin receptor scintigraphy (SRS) of these tumors. Metastases of primary SSR positive tumors initially do express also the same SSR subtype, however loss of these receptors have been described after dedifferentiation of tumor cells or after chemotherapy[17]. In addition, SSR overexpression was identified in peritumoral veins of primary tumors and veins surrounding lymph node, bone and lung metastases[18].

SOMATOSTATIN RECEPTOR SCINTIGRAPHY

The optimal management of patients with neuroendocrine tumors requires accurate imaging and staging. For the visualisation of SSR positive tumors somatostatin receptor scintigraphy (SRS) with [^{111}In-DTPA^{0}]octreotide (Octreoscan®) (DTPA = diethylenetriaminopentaacetic-acid) has been used for more than 10 years[19,20]. The efficacy of SRS using [^{111}In-DTPA^{0}]octreotide in patients with histologically or biochemically proven endocrine pancreatic tumors or carcinoids was evaluated in a European multicenter trial[21]. The highest success rates of SRS were observed with glucagonomas (100%), vipomas (88%), gastrinomas (73%),"non-functioning" islet cells tumors (82%) and carcinoids (87%). Insulinomas were detected in only 46% due to the low incidence of sst_2 on insulinoma cells. The low sensitivity in this study found for some tumors may be related to important differences in scanning procedures such as the amount of radioligand administered, the duration of the acquisition and the use of single photon emission computed tomography (SPECT)[22]. With SPECT, in which a rotating camera is used, image reconstructions can be made similar to spiral computed tomography (CT). This technique renders additional visual information, especially on tumors in the liver and upper-abdomen[23]. With SPECT 25% more liver metastases are detected if compared to planar SRS.

In a prospective study comparing the sensitivity of SRS with that of CT-scanning, magnetic resonance imaging (MRI), ultrasonography (US) and selective angiography in the detection of primary and metastatic gastrinomas, SRS altered clinical

management in 47% and had a superior sensitivity, and specificity[24] (*Table 1*). Cadiot *et al* compared the results of SRS with those of conventional imaging techniques including endoscopic ultrasonography (EUS), and with surgical findings in 21 consecutive patients with Zollinger-Ellison syndrome[25]. SRS added complementary information to other imaging techniques including EUS and improved the preoperative detection of extrapancreatic gastrinomas. By combining SRS with EUS they were able to detect 90% of the tumors in the upper duodenopancreatic area. SRS identified metastatic disease in 20-30% of patients after all other imaging techniques had failed[23]. An other study concerning 160 patients with biologically and/or histologically proven gastroenteropancreatic (GEP) tumors including pancreatic islet cell tumors, SRS changed the surgical therapeutic strategy in 40 (25%) patients (*Table 2*)[26]. Unsuspected liver tumors were discovered only by SRS in 7 patients, contralateral liver tumors before hepatectomy in 2 and extrahepatic disease in 31 patients. In 48 patients with histologically proven hepatic metastases of neuroendocrine tumors including carcinoids we compared conventional imaging methods (CIM) with SRS for the detection of extrahepatic disease. CIM consisted of US, CT thorax and abdomen and, when indicated, bone scanning. All patients were scanned according to our multiple spot (MS) views protocol. A minimal dose of ^{111}In of 200 MBq and least 10 μg of peptide was administered as an iv. bolus and acquisition was performed 24 and 48 hours thereafter. Acquisition time was 15 min. and abdominal SPECT was used systematically with a triple head camera. SRS alone, according to this protocol, demonstrated 114 extra-hepatic lesions in 37 patients, whereas CIM visualised only 50 extra-hepatic lesions in 22 patients. (*Table 3*)(manuscript in preparation).

Table 1 **Imaging methods for the detection of liver metastases and extra-hepatic metastases in patients with Zollinger-Ellison syndrome**

Procedure	*Extra-hepatic tumor* n = 80	*Liver metastases* n = 24
	Percentage of patients positive	
Ultrasonography (US)	9%	46%
Computed tomography (CT)	31%	42%
Magnetic resonance imaging (MRI)	30%	71%
Angiography (Angio)	28%	62%
US + CT + MRI + Angio (CIM)	48%	83%
SRS	58%	92%
SRS + CIM	68%	96%
SRS Only*	20%	12%
CIM Only**	10%	4%

Results of tumor localisation for the identification of liver metastases and an extra-hepatic tumor in patients with Zollinger-Ellison syndrome. Results are expressed as the percentage of the 24 patients with proven liver metastases and of the 80 patients with extra-hepatic disease SRS: Somatostatine receptor imaging; CIM: Conventional imaging methods; SRS Only: tumor detected only with SRS; CIM Only**: tumor detected only with CIM*

Table 2 **Clinical impact of SRS in 160 patients with GEP tumors**

Initial Classification	N	*SRS Classification* I	II	III
I *No metastases*	90	*65*	*7*	*18*
II *Only liver metastases*	59		*46*	*13*
III *Extra-hepatic metastases*	11			*11*

Table 3 **Detection of extra-hepatic metastases; SRS *vs* CIM**

	CIM negative	*CIM positive*	*Total*
SRS negative	11	-	11
SRS positive	15	22	37
			48

Forty eight patients with histological proven hepatic metastases of neuroendocrine tumors. Table shows number of patients with extra-hepatic disease detected by [^{111}In-DTPA^{0}]octreotide (SRS) or by conventional imaging methods (CIM) consisting of computed tomography, ultrasound and bone scanning. In 15 patients extra-hepatic metastases were detected with SRS only. Eleven patients were found to have no extra-hepatic disease.

In patients with paragangliomas SRS provides optimal information on potential other tumor sites in the body, since paragangliomas are often multicentric and asymptomatic[27]. For imaging of adrenal pheochromocytomas, scintigraphy with radiolabeled metaiodobenzylguanidine (MIBG) is more accurate than SRS, since the majority of benign pheochromocytomas are localised in the adrenal, which localisation is difficult to distinct from the kidney with SRS. Furthermore the majority of these benign lesions do not express SSRs. Recently, in a large European multicenter study, sensitivity of ^{131}I-MIBG to detect benign pheochromocytomas was 81%[28], while the detection rate of SRS is only around 25%. The majority of malignant tumors (80%), in contrast, do express these receptors and therefore SRS could be used to characterise adrenal masses suspected to be malignant[29]. In the management of medullary thyroid cancer (MTC) patients SRS has a limited role. SRS could be useful for the detection of residual and recurrent disease after total thyroidectomy or in patients who present primarily with metastatic disease in order to study the feasibility to perform peptide receptor radionuclide therapy (PRRT)[30,31]. To optimalize the detection of metastases in patients with recurrent MTC the combination of SRS and technetium-99m dimercaptosuccinic acid (^{99}Tc-DMSA) should be considered, since together they have a sensitivity of 84% for the diagnosis of MTC, whereas these percentages for either technique alone were 29% (SRS) and 69% acid (^{99}Tc-DMSA)[32].

Finally, the difference in SSR expression between islet cell tumors, especially "non-functioning" tumors and pancreatic duct cancers offers the possibility to differentiate between these tumors preoperatively[33]. This is important, as palliative surgery in patients with islet cell tumors is not only of value to relieve clinical symptoms, but also because a decrease in tumor burden might enhance the effect of medical treatment, resulting in a better clinical condition and a longer survival.

CLINICAL USE OF SOMATOSTATIN ANALOGS

Most endocrine pancreatic tumors, with the exception of insulinomas, have a malignant potential and have already metastasesed at the time of diagnosis. These tumors are in general slow growing and most of the clinical distress is related to the hypersecretion of hormones, which often incapacitates the patient and causes long and repeated hospital stay. The clinical use of the somatostatin analog octreotide in this type of patients is of considerable help in controlling symptomatology. Debilitating

diarrhoea, dehydration and hypokalemia (VIPoma) and necrolytic skin lesions (glucagonoma) can be well controlled during chronic treatment with octreotide. There is no doubt that octreotide therapy is of great benefit for most of these patients and improves their quality of life dramatically[34]. In selected patients peptic ulceration with hyperplasia of fundic argyrophil cells (gastrinoma) and life-threatening attacks of hypoglycemia (metastatic insulinoma) octreotide could be of therapeutic benefit[35,36]. Clinical studies in patients with hormone producing islet cell tumors showed a close parallel between the presence of SSRs on the tumors and the *in vivo* and *in vitro* suppressive effects of octreotide on hormone release[37]. This indicates that SRS can predict a possible suppressive effect of octreotide on hormonal hypersecretion by endocrine pancreatic tumors. Although octreotide is able to inhibit gastrin release in Zollinger-Ellison syndrome patients, proton-pump inhibitors are currently the first choice, since more than 80% of patients are controlled by omeprazol, lansoprazole or pantoprazole. A major problem in the treatment of patients with islet-cell tumors and carcinoids with octreotide is that the inhibition of the secretion of tumor related hormones is transient. Most patients finally become insensitive to octreotide treatment, possibly by downregulation of SSR expression or outgrowth of SSR-negative clones[6,38,39]. The beneficial effects on clinical symptomatology in patients with metastatic endocrine pancreatic tumor and carcinoids is highly variable. Increasing the dose of octreotide or intermittent administration reverses these problems in most patients. The median duration of improvement of diarrhoea and flushing attacks by octreotide in patients with metastatic carcinoid disease was more than 12 months[40].

In some patients with (metastatic) medullary thyroid cancer, continuous treatment with very high doses of octreotide can be of temporary relief. Long-term therapy with somatostatin analogs of catecholamine-secreting (malignant) paragangliomas and pheochromocytomas has not shown clinical benefits[41].

The role of octreotide in the treatment of pancreatitis is controversial. A meta-analasys of 6 studies showed a significant decrease in mortality in patients with mild or severe pancreatitis (6.3% octreotide *vs* 14% placebo)[42]. However Uhl *et al* did not find a significant difference in mortality-rate or complications in a large series of 302 patients with acute pancreatitis[43]. The decrease of digestive enzyme secretion and the increase of intestinal water and electrolytes absorption by octrotide can be beneficial in the treatment of pancreatic and enterocutaneous fistula[44,45]. In elective surgery for

pancreatic carcinoma or chronic pancreatitis peri-operative administration of octreotide has demonstrated its value. In a study of 246 patients that underwent pancreatic surgery octreotide treatment reduced mortality and complication incidence compared to placebo (3.2% and 32% *vs.* 5.8% and 55% respectively)[46]. Finally somatostatin and octreotide infusion reduce portal pressure by decreasing splanchnic blood flow. Though the exact mechanisms of acute hemodynamic changes in patients with portal hypertension are unknown there is a rationale for the use of somatostatin and its derivatives in the control of acute oesophageal variceal bleeding[47]. Early infusion of somatostatin in cirrhotic patients with acute variceal haemorrhage decreases treatment failure and mortality and reduces active bleeding after sclerotherapy, thereby improving the efficacy of sclerotherapy for acute variceal bleeding episodes[48].

ONCOLOGIC APPLICATIONS OF SOMATOSTATIN ANALOGS

The observation that somatostatin inhibits the release of various peptide hormones has stimulated the interest in its use as an anti-proliferative agent. In pre-clinical studies SS analogs inhibit the growth of a wide variety of SSR positive as well as SSR negative tumors *in vivo* and *in vitro*[4,6,49]. An indirect tumor growth inhibition may be achieved via the inhibition of circulating tumor growth-promoting circulating hormones (GH, Insulin like growthfactor-1, insulin) and inhibition of circulating, paracrine- and/or autocrine-secreted stimulatory growth factors. SS and its analogs can also modulate the activity of immune cells[50] and potentially influence tumor blood supply, whereas Reubi *et al.* showed a high density of SSRs on veins in the peritumoral zone of several types of malignant tumors[51]. A potentiation of the anti-proliferative effect of octreotide has also been suggested[52], however no beneficial effect of octreotide combined with tamoxifen was found in patients with metastatic breast cancer[53]. Critical to the direct antiproliferative effects of SS analogs is the presence of SSRs[54]. Both cAMP-dependent and –independent effector mechanisms have been suggested[55-58], while stimulation of phosphotyrosine phosphatase activity may play an important role in the inhibition of growth factor-stimulated cell growth[59,60]. The results of clinical trials in patients with GEP tumors, using SS analogs alone or in combination with interferon-α are rather disappointing with a biochemical response in 77% of patients with a median duration of 15 months but without any reduction in tumor size[61,62]. Octreotide has been clinically the most

commonly applied SS analog, yielding a biochemical response rates between 30 – 70 % but objective tumor shrinkage in less than 10%-15% of the patients[5,63-66]. Treatment with standard doses of the SS analog lanreotide does not appear to be better than with standard doses of octreotide. However tumor biopsies before and during treatment with high doses of lanreotide indicated apoptosis in responding patients[67,68]. Side effects of long-term administration of SS analogs are rare. The most relevant side effect is the development of gallstones, which is believed to derive from the inhibition of gallbladder emptying due to the inhibition of cholecystokinin release[69]. The incidence of developing cholelithiasis and/or gallbladder sludge is reported to be between 30% to 60% and seems to be dose dependent[70,71]. Patients require cholecystectomy for either symptomatic disease or acute cholecystitis in 15% of cases, therefore prophylactic cholecystectomy is not indicated, unless it is performed during elective cytoreductive surgery[71]. Octreotide might also influence blood sugar levels in patients with diabetes mellitus.

RADIONUCLIDE THERAPY: PRE-CLINICAL

A new and fascinating application is the use of radiolabeled octreotide and other peptides for Peptide Receptor Radionuclide Therapy (PRRT), further referred to as radionuclide-therapy. After systemic injection of [^{111}In-DTPA^{0}]octreotide the radioligand is internalised and transported into the lysosomes by SSR specific and temperature dependent process starting with endocytosis[72,73]. The radionuclide as metabolite ^{111}In-DTPA-D-Phe is not capable of passing the lysosomal membrane resulting in a biological half-life in human tumor tissue of > 700 hours[74,75]. ^{111}In not only emits gamma-rays, which can be visualised during scintigraphy, but also emits internal conversion- and Auger electrons having a medium to short tissue penetration (200-550 µm, 0.02-10 µm, respectively). Therefore, an effect on tumor cell proliferation could be expected, as the radiotoxicity of the radionuclide is very high if the DNA of the cell is within the particle range[76,77]. Three experimental studies demonstrated tumor growth-inhibition of solid subcutaneous tumors by radiolabeled SS analogs in animal models[78-80]. We investigated the anti-proliferative effect of [^{111}In-DTPA^{0}]octreotide on, SSR positive, CA20948 pancreatic tumor cells in a rat liver metastases model with intra-portal tumor cell injections[81]. Treatment with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide was given on day 1 and/or day 8. After 21 days the number of tumor colonies was significantly decreased by all treatment regimens compared to control animals and

treatments on day 1 and 8 proved to be superior to single treatment on either day (*Table 4*). In repeated experiments, most treated animals showed none or a few tumor colonies, an outcome that was not observed earlier in experiments using high doses of non-radiolabeled octreotide[54].

Table 4 **Effect of PRRT with [^{111}In-DTPA^{0}]octreotide on** SSR **positive liver metastases**

	Number of animals with 0 - > 100 metastases			
Number of metastases	0	1 - 20	21 - 100	> 100
Treatment				
Controls	-	-	-	6
PRRT day 1	-	4	1	-
PRRT day 8	-	3	2	-
PRRT day 1 and 8	3	2	-	-

Slooter et al Int J Cancer 1999;767-771

Number of animals with given range of metastases (5 or 6 animals per group), 21 days after direct injection of SSR *positive CA20948 tumor cells into the portal vein. Peptide Receptor Radionuclide Therapy (PRRT) with 370 MBq (0.5 μg) [^{111}In-DTPA^{0}]octreotide was given on day 1 or 8 or on day 1 and 8. The effect of all treatment schedules was significantly different ($p < 0.01$) from the effect of 0.5 μg "cold" [DTPA^{0}]octreotide on day 1 and 8 (Controls). Treatment on day 1 and 8 was significantly different from treatment on day 8 alone ($p<0.05$). No significant difference was found between the effect of treatment on day 8 or day 1.*

We also demonstrated that this tumor growth inhibition is predominantly due to the specific binding of [^{111}In-DTPA^{0}]octreotide to the SSR and not to a systemic or a secondary mechanism since pre-treatment with 1 mg of octreotide prior to treatment, resulting in saturation of the SSRs, almost completely abolished the effect of [^{111}In-DTPA^{0}]octreotide on SSR positive cells. Furthermore, PRRT had no effect on the growth of SSR negative CC531 tumors in the same experiment[81]. During the experiments with PRRT there was no sign of toxicity in the animals and there was no histological damage to the kidneys, which is the first organ to be at risk[82]. We earlier demonstrated that liver regeneration after 70% partial hepatectomy (PHx) in this model accelerates tumor growth dramatically[83]. PRRT was found to completely annihilate this tumor growth stimulation of SSR positive tumor cells rendering only a few tumor colonies. In an experiment without tumor we found that [^{111}In-DTPA^{0}]octreotide does not influence liver regeneration or -function, suggesting that PRRT could also be a safe option for adjuvant treatment after liver resection or arterial embolisation. Although

PRRT with ^{111}In-labeled somatostatin analogs could be a promising treatment option for patients with locally irresectable or disseminated GEP tumors, more PRRT experiments, in more advanced stages of tumor development, with different doses of [^{111}In-DTPA^{0}]octreotide or other radioligands are necessary.

RADIONUCLIDE THERAPY: CLINICAL

A phase 1 study on the side effects and antiproliferative effect of high, multiple radiotherapeutic doses of [^{111}In-DTPA^{0}]octreotide started in 1995[88]. Thirty end-stage patients with mainly neuroendocrine tumors were included. After scoring tumor radioactivity uptake using scintigrams obtained 24 hr after the injection of a diagnostic dose (220 MBq) of [^{111}In-DTPA^{0}]octreotide treatment was initiated. All patients received doses of 6-7 GBq ^{111}In incorporated in 40-50 µg [DTPA^{0}]octreotide with at least 2 weeks intervals between administrations. A total of 8 injections was aimed at, with a possible extension to 12-14 administrations. Twenty-one patients received a total cumulative dose of at least 20 GBq [^{111}In-DTPA^{0}]octreotide with a maximum of 75 GBq. Of the 9 patients treated with a total dose lower than 20 GBq, 7 had to stop prematurely because of too progressive disease despite treatment and 2 did not conclude the first course of four administrations at the time of the interim analysis With a maximum follow up of 26 months no major clinical side effects were observed except for a transient decline in platelets count and the number of white blood cells. Kidney function was only influenced slightly and without clinical relevance. Although in a number of patients temporary because of end-stage disease, impressive effects on the clinical condition of the patient and hormone or tumor marker production were observed after the administration of high doses of [^{111}In-DTPA^{0}]octreotide. Prior to the start of treatment all 21 patients had progressive disease. In 8 patients treatment resulted in stable disease and in 6 patients in actual tumor shrinkage, monitored with CT or MRI. In these patients beneficial effects on hormone production and clinical symptoms were found. There was a tendency towards better result of PRRT in patients whose tumors had a higher accumulation of the radioligand on their scoring scintigram, from PRRT with [^{111}In-DTPA^{0}]octreotide that have described smaller groups of patients with end stage GEP tumors. *Table 5* summarises the results and characteristics of patients treated with PRRT with a minimal dose of 20 GBq [^{111}In-DTPA^{0}]octreotide that have been described in recent literature[88-93].

For radiotherapeutic applications, besides ^{111}In also other radionuclides like ^{90}Y (yttrium) and ^{177}Lu (lutetium) have been proposed for coupling to somatostatin analogs. ^{90}Y, with a half-life time of 2.7 days, is a pure high β-emitter with a tissue range up to 1 cm and ^{177}Lu, with a half-life time of 6.7 days, emits besides gamma radiation, suitable for visualisation also intermediate β-particles with approximately 1 mm tissue penetration. For tumors with a heterogeneous distribution of SSRs ^{90}Y and ^{177}Lu labeled somatostatin analogs might have additional extra beneficial characteristics, due to the effect of "cross-fire". Tumor cells lacking the SSR might then be hit by an electron coming from a neighbouring cell, which has internalised the radioligand. This may lead to a high and more homogeneous radiation dose in larger parts of the tumor. Studies with these radiolabeled somatostatin analogs are ongoing.

Table 5 **Results and characteristics of patients treated with PRRT with a minimal dose of 20 GBq [^{111}In-DTPA^{0}]octreotide**

			Tumor size	
	n	Reduction	Stable	Progression
Carcinoid	13	3	8	2
Neuroendocrine tumor	6	2	2	2
Gastrinoma	1	-	1	-
Vipoma	1	1	-	-
Glucogonoma	1	1	-	-
Med. Thyroid cancer	3	-	1	2
Pap. Thyroid cancer	1	-	1	-
Glomus tumor	2	1	1	-
Pheochromocytoma	2	-	1	1
Astrocytoma	1		1	
Inflammatory breast cancer	1		1	
Total	32	8	17	7

Data are from Krenning[88], Caplin[89], Fjalling[90] and Tiensuu[93], all phase 1 studies.

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CHAPTER 4

INDUCTION OF THE SOMATOSTATIN RECEPTOR ON COLON CARCINOMA CELLS AS A TARGET FOR RADIOLABELED OCTREOTIDE

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ABSTRACT

Somatostatin receptors (SSRs) are found in various human tumors, especially in neuroendocrine tumors, but also breast tumors and malignant lymphomas. By use of SSR-scintigraphy, Octreoscan, it is possible to visualize these tumors. Moreover, Octreoscan can be administered in high dosage schemes, so SSR-targeted therapy can be given for these kind of tumors. This kind of treatment is named Peptide Receptor Radionuclide Therapy (PRRT). In previous experimental, but also clinical studies, successful and promising results were shown with PRRT. Unfortunately, not all human tumors express SSRs on their cell surface, thus consequently PRRT is not successful for these tumors.

However, recent developments in molecular biology have made it possible to transfect the SSR-gene on SSR-negative tumor cells. In this study SSR-negative colon carcinoma cells (CC531) were transfected with a SSR-gene *in vitro* and named CC2B hereafter. Scatchard analysis showed high expression of SSRs and a high binding affinity of CC2B cells. Moreover, the transfected SSR was also functional as it could internalize radiolabeled octreotide. Growth speed and other growth characteristics of CC2B did not differ significantly *in vitro* compared with their parental tumor cell line CC531.

All the above mentioned features of the CC2B cell line fulfil future to the conditions to apply this SSR-transfected cell line in an *in vivo* model to perform experiments, in which PRRT will be tested.

INTRODUCTION

Somatostatin, a tetradecapeptide, is widely distributed in the body and functions in a variety of physiological processes. In the pituitary and pancreas, for example, somatostatin is a potent inhibitory regulator of various secretion processes[1], and in the central nervous system somatostatin exerts both excitatory and inhibitory actions (2,3). Until now, five subtypes of somatostatin receptors (SSRs) have been cloned (8-14) and is found in a wide variety of tissues. Also SSRs are found in different human tumors, especially in neuro-endocrine tumors[2]. By means of SSR scintigraphy (Octreoscan) it is possible to detect these SSR-positive tumors. Moreover, in several studies it is shown that high dosages of radiolabeled octreotide can be effective in the treatment of these SSR-positive tumors[1-9]. This kind of treatment is named Peptide Receptor Radionuclide Therapy (PRRT). After administration of radiolabeled octreotide the peptide adheres to the SSR on the tumor cell surface, after which it is internalized. As the radioligand is charged negatively, it is trapped in the tumor cell, resulting in a long residence time, which causes DNA damage and consequently can lead to cell death. Through this mechanism PRRT is not only highly effective, but also this kind of treatment is targeted. Unfortunately, not all tumors express SSRs, as a result of which PRRT can not be given. The aim of this study was to transfect a SSR-negative tumor cell line (CC531) with a SSR-type 2 gene *in vitro*, so *in vivo* experiments can be performed in the future when the transfection would be successful. In this study SSR-gene expression, functionality and growth characteristics were investigated after transfection.

MATERIAL AND METHODS

Tumor

CC531 is a SSR-negative, moderately differentiated rat colon carcinoma, induced by 1,2-dimethylhydrazine and is transplantable in syngeneic WAG/Rij rats [10]. The tumor is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany.

SSR Transfection and Expression

For expression of the SSR-subtype 2 ($sstr_2$) in CC531 cells, human $sstr_2$ cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute, Chicago, IL, USA) was excised form pBS and inserted into the Nhe-1/Sali cloning site of the retroviral expression vector pCi-neo. Selection was made by the geneticine resistance gene (G418). This vector was used to stable transfect (using lipofectin) CC531 cells. Transfectants were selected and cultured in RPMI 1640 supplemented with 5% FCS and geneticine (0.5 mg/mL) (Gibco, Paisley, UK). Geneticine-resistant clones were examined for their ability to bind with [^{125}I-Tyr^3]octreotide. The clone with the highest expression (Scatchard analysis) of SSR was isolated and named CC2B.

Immunocytochemical Staining of the SSR

Immunocytochemical staining was performed as described previously [11]. Briefly, CC531 cells and CC2B cells were fixed with paraformaldehyde (PF) and washed twice with PBS and incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% BSA). Thereafter, the cells were incubated overnight at 4 C with SSR_{2A} (R2-88) (a kind gift of Dr. A. Schonbrunn) antibodies in a dilution of 1:1000. Finally, a standard streptavidin-biotinylated-peroxidase complex (ABC) kit (Biogenix, San Ramon, CA, USA) was used according to the manufacturers to visualize the bound antibodies.

In vitro Functionality of the SSR

To test *in vitro* functionality of the SSR an internalization study was performed as described previously[14].

In short, on the day of the experiment, CC531 and CC2B cells were seeded at a density of 0.5 x 10^6 cells/well in 12-well multiwell plates (Costar, Cambridge, MA, USA) and grown in confluency for 2 days.

On the day of the experiment, the cells were washed twice with internalization medium. The internalization medium consisted of Dulbecco's Modified Eagle's Medium supplemented with HEPES (30 mM) L-glutamine (2 mM), sodium pyruvate (1 mM), penicilline (105 U/L), fungizone (0.5 mg/L) and 0.2% BSA (fraction V, Sigma Chemical Co., St. Louis, MO, USA). The cells were allowed to adjust to the medium for 1 h at 37 C. Thereafter, approximately 10^{-10} M [^{125}I-Tyr^3]octreotide

(home-made [15]) were added to the medium, and cells were incubated at 37 C for a period of 4 h in quadruplicate, without or in excess of non-radiolabeled octreotide (10^{-6} M) to determine non-specific internalization. After the incubation the cells were washed twice with ice-cold internalization medium. Thereafter 1 mL sodium acetate (20 mM) in Hanks' Balanced Salt Solution, pH 5.0 (HBSS-Ac), was added to the cells and the cells were incubated for 10 min at 37 C. After 10 min, the supernatant was collected. Finally, the cells were washed with HBSS-Ac, and the supernatant was pooled with the supernatant of the previous step. The pooled supernatant fraction, acid-extractable radioactivity, represented the membrane-bound ligand. At the end of the incubation, after the HBSS-Ac treatment, the cells were extracted in 1 N NaOH. The radioactivity in this fraction represents internalized radioligand.

Radiolabeling and Quality Control of the Radioligand

[DTPA^0]octreotide (Pentetreotide, DRN 4920) and $^{111}InCl_3$ (DRN 4901, 370 MBq/mL in HCl, pH 1.5 to 1.9) were obtained from Mallinckrodt (Petten, The Netherlands). Octreotide was a gift of Novartis (Basle, Switzerland). Labeling was performed by diluting freeze-dried [DTPA^0]octreotide in 1 mL saline and adding this to the $^{111}InCl_3$. Thirty minutes after the start of this procedure, quality control was performed by instant thin-layer chromotography with silica gel and 0.1 M sodium-citrate, pH 5, as eluent. The labeling efficiency of [^{111}In-DTPA^0]octreotide was over 98%.

In vitro Growth and Growth Characteristics

We determined *in vitro* growth and growth characteristics by DNA analysis described by Hofland et al. previously[14].

In brief, in 24-well culture plates (Costar, Badhoeverdorp, The Netherlands) 10.000 CC531 or CC2B cells were cultures in 1 mL 1640 RPMI enriched with 1% fetal calf serum (FCS) (Life Technologies, Breda, The Netherlands) and placed in an incubator with a humidified atmosphere of 95% / 5% CO_2 at 37 C. The next day all wells were washed twice with 0.9% NaCl in order to wash away superfluous non-adherent cells. Hereafter, the cells were incubated with the different growth factors in 1 mL medium with 1% FCS in an incubator. Concentrations of octreotide used were: 0.1, 1, 10, 100 and 1000 nM. Concentrations of IGF used were: 0.1, 1, 5,10 and 100 nM. After 72 or

144 hours, the plates were collected by washing away superfluous non-adherent cells and kept at –20 C for DNA analysis.

DNA analysis

The total DNA content of each well was measured using bisbenzimide dye (Bhoehringer Diagnostics, La Jolla, California, USA) as described previously by Hofland et al.[14]. The DNA measured represents the total amount of cells per well.

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance. When significant effects were obtained by analysis of variance, multiple comparison were made by the Newmann-Keuls test. Statistical significance was defined as $p < 0.05$. Data are expressed as mean ± SD.

RESULTS

Expression and Functional Capacity of the transfected SSR

Immunocytochemical staining with an antibody against the sst_{2A}, clearly visualized this receptor subtype on CC2B cells, while absent on CC531 (fig. 1).

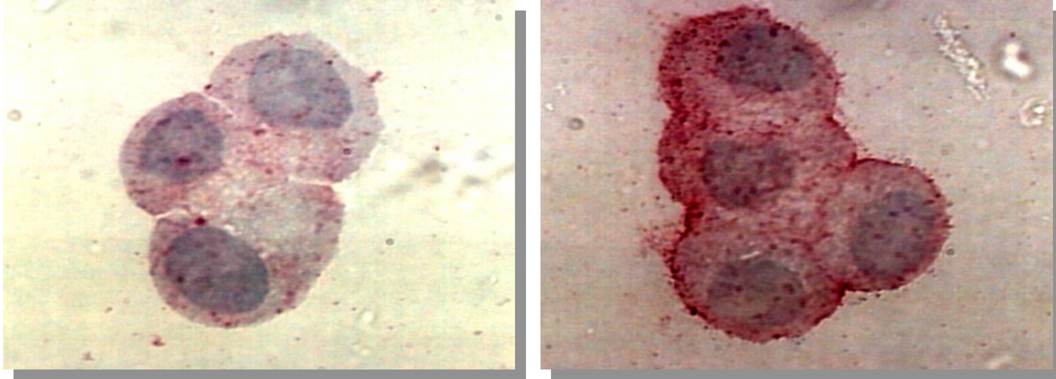

Fig.1: Immunocytochemical staining of the SSR (subtype 2A). Clearly staining on the membrane of the CC2B (right) is seen, in contrast to wild-type CC531 (left).

Scatchard analysis showed a maximum binding capacity (Bmax) of 760 fmol/mg per membrane protein with a dissociation constant (K_d) of 0.9 nM (fig. 2).

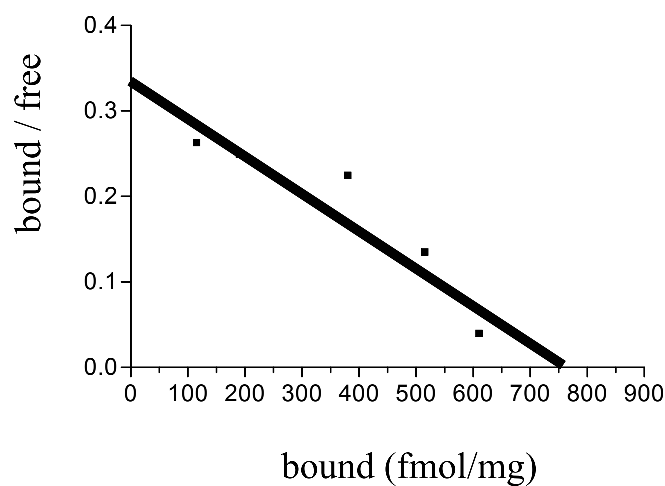

Fig. 2 : Scatchard analysis shows that CC2B has a Bmax of 760 fmol/mg membrane protein and a Kd of 0.9 nM.

Internalization studies showed a high uptake of [^{125}I-Tyr^{3}]octreotide in the CC2B cells (66 ± 4 kcpm *vs.* 1 ± 0.2 kcpm in controls ($p<0.001$) (fig. 3). Non-specific binding was low in the presence of 10^{-6} M octreotide for CC2B cells, but in CC531 cells this was equal for the specific. The ratio internalized/total radioactivity in CC2B cells was 0.85.

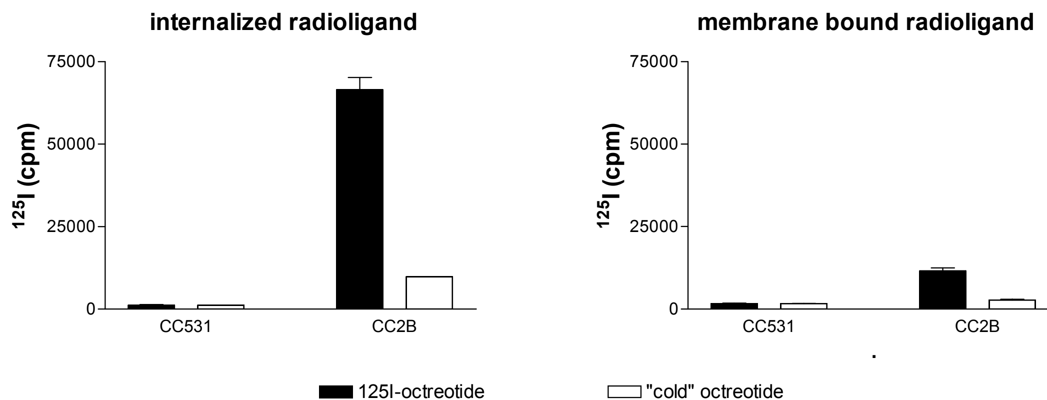

Fig.3: Amount of internalized (left) and mebrane bound (right) radioligand of CC2B and CC531 cells. (SD's in some bars are too small to observe.)

In vitro Growth and Growth Characteristics

In order to elucidate wether growth of the transfected cell line was changed after transfection, 10.000 CC531 or CC2B cells were cultured in a 24-well plate containing 1 mL 1640 RPMI enriched with 1% FCS. After 3 and 6 days the plates were collected for DNA analysis. No significant difference in growth was observed between the CC2B and CC531 cells (fig.4).

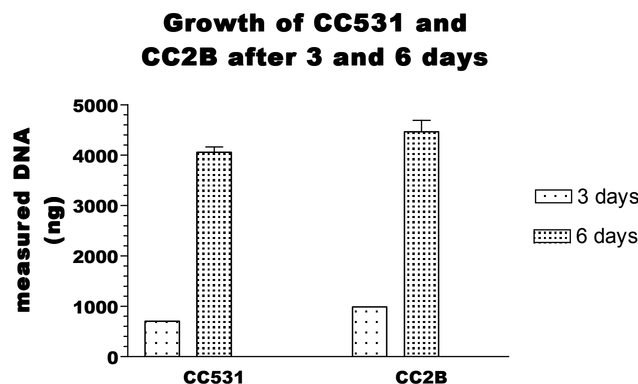

Fig. 4: No differences in growth were seen after SSR transfection between CC531 and CC2B cells.

Fig. 5 shows the effect of IGF on CC2B and CC531 cells. A comparable dose-dependent growth-stimulation with a maximum of 144% and 152%, respectively (not significant), was observed.

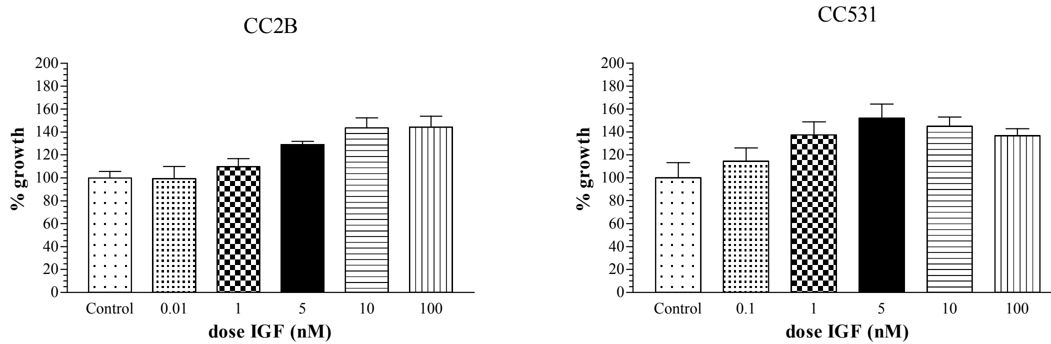

Fig. 5: A comparable dose-dependent growth stimulation was seen after addition of IGF on CC2B and CC531 cells.

In order to evaluate whether the transfected SSR could exert a growth inhibitory effect, 1000 nM octreotide was added to CC2B and CC531 cells. As expected no inhibition was seen in CC531 cells, however, also no inhibitory effect was seen in CC2B cells (fig. 6).

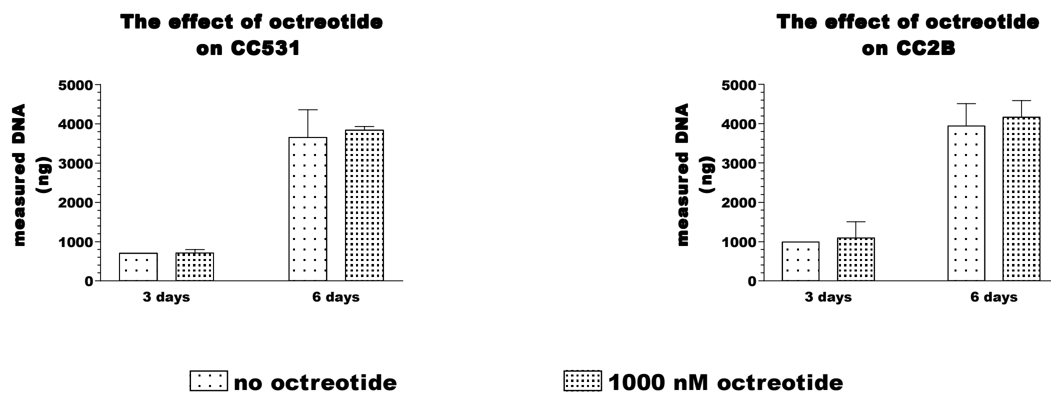

Fig. 6: No significant inhibitory effect of 1000 nM octreotide on CC531 and CC2B cells after 3 and 6 days was observed compared with controls.

Lastly, 10 nM IGF in combination with different doses octreotide had also no inhibitory effect on the growth-stimulation of IGF on CC2B or CC531 cells (fig. 7).

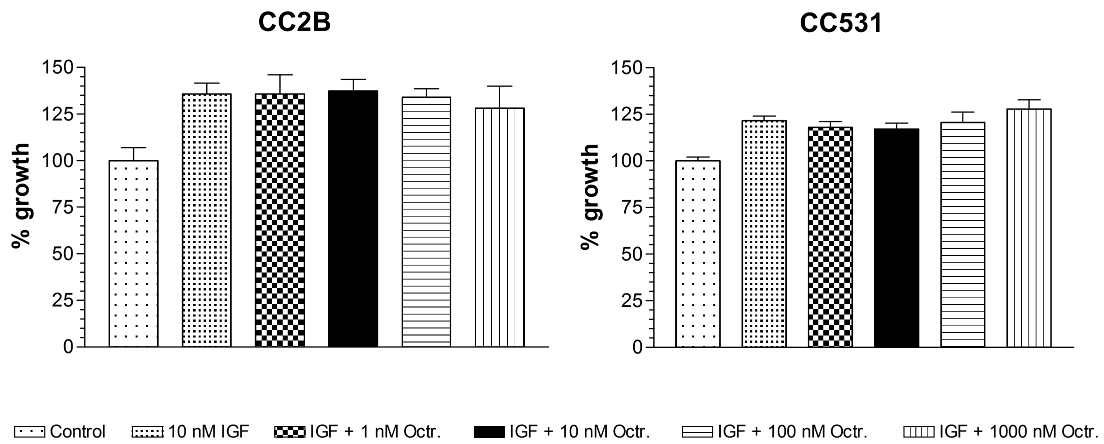

Fig. 7:Different doses octreotide did not have an inhibitory effect on the growth stimulation of 10 nM IGF on CC2B or CC531 cells.

DISCUSSION

SSRs are found in various human tumors, especially in neuroendocrine tumors, but also breast tumors and malignant lymphomas. By use of SSR-scintigraphy, Octreoscan, it is possible to visualize these tumors[2]. When Octreoscan is administered in high dosage schemes, these kind of tumors can also be treated. This kind of treatment is named Peptide Receptor Radionuclide Therapy (PRRT). In previous experimental, but also clinical studies, successful and promising results were shown with PRRT[2-4,9]. Unfortunately, not all human tumors express SSRs on their cell surface, thus consequently PRRT is not successful for these tumors.

By means of SSR-gene therapy, it is possible to make these SSR-negative tumor cells SSR-positive, so this kind of treatment can be given also to a larger population of patients with, for example, disseminated cancer disease, for which no ideal treatment is availible up to now.

We successfully transfected the SSR on colon carcinoma cells. After the transfection a high amount of SSRs (average 12.000 per cell, B_{max} = 760 fmol/mg per membrane protein) was expressed with high binding affinity (K_d = 0.9). Moreover, the transfected receptor was functional as it also could internalize (radiolabeled) octreotide. In order to elucidate whether SSR gene therapy in itself could exert an inhibitory growth effect, we performed an *in vitro* growth study. No inhibitory growth effect was observed in CC2B cells. This in contrast to Rochaix *et al*, who also used SSR gene therapy in human pancreatic adenocarcinoma, which had lost the ability to express SSRs[15]. By reintroducing the SSR into human pancreatic cancer cells these cells started to produce somatostatin again. The somatostatin released by SSR transfected cells could exert not only local but also a distant inhibitory effect on non-transfected tumor cells through an upregulation of the expression of SSR-subtype 1 on parental tumor cells, thereby rendering them sensitive to the antiproliferative effect of somatostatin. However, CC531 cells can not produce somatostatin after SSR gene therapy, as colon cells, in contrast to pancreatic cells, do not originally produce somatostatin. Moreover, also no inhibition of growth *in vitro* was seen after addition of 1000 nM octreotide. Probably, post-receptor cell signaling after internalization of octreotide does not function in a proper manner, so no antiproliferative effect is seen in CC2B cells.

However, our concept of SSR gene therapy is different than the group of Rochaix. The transfected SSR functions as a target for our therapy with radiolabeled octreotide.

As long as the SSR is functional (internalization and trapment of radiolabeled octreotide) this concept can be successful. In order to elucidate wheter the growth characteristics of CC2B were comparable with their parental tumor cell line CC531, we performed another study in which growth of CC2B cells was stimulated with IGF. A maximum of 144% growth stimulation was achieved after an addition of 10 nM IGF, which is comparable with CC531.

Lastly, different doses of octreotide did not have an inhibitory effect on the growth stimulation of 10 nM IGF, indicating that tyrosine phosphatase is not stimulated after internalization of octreotide and thus consequently dephosporylation and inactivation of tyrosine kinase such as those activated by IGF do not occur.

In conclusion, we transfected successfully the SSR on colon carcinoma cells with a high expression of SSRs and a high binding affinity. Moreover the transfected SSR was functional as it could internalize radiolabeled octreotide. Lastly, growth characteristics were comparable with their parental tumor cell line CC531.

All the above mentioned features makes this SSR-transfected cell line usefull to perform *in vivo* studies in the future: CC2B tumor cells will be used in a rat liver metastases model in order to investigate whether PRRT is feasible for the treatment of SSR-transfected liver metastases.

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CHAPTER 5

TARGETED THERAPY WITH [^{111}IN-DTPA^{0}]OCTREOTIDE ON COLON CARCINOMA CELLS TRANSFECTED WITH A SOMATOSTATIN RECEPTOR ASSESSED IN A RAT LIVER METASTASIS MODEL

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ABSTRACT

Previously, we have tested PRRT in a rat liver metastases model; successful treatment was only seen in SSR-positive tumors, while absent in SSR-negative tumors. Therefore the idea arose to transfect SSR-negative tumor cells with a SSR-subtype 2 gene to make PRRT applicable for these tumors. Using the same *in vivo* tumor model, here we describe the effect of PRRT with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide on CC531 (SSR-negative) rat colon carcinoma cells transfected *in vitro* with a SSR-gene further referred as CC2B.

No significant difference in tumorscore was seen in rats treated with PRRT *vs.* control animals with CC531 liver metastases. However, also no significant diffence in tumorscore was seen in CC2B liver metastases (PRRT *vs.* control). Autoradiography confirmed high SSR expression in CC2B liver metastases tissue, while absent in CC531 liver metastases. High uptake and retention of radioactivity in CC2B tumor was measured, indicating that the transfected receptor was functional. Moreover, the measured radioactivity and radiation dose was comparable with our positive control tumor CA20948, indicating that at our given dosage CC2B is not radiosensitive, which we could demonstrate in a clonogenic assay of our tumor cell lines treated with external radiation.

Although no antiproliferative effect of PRRT was seen on SSR transfected CC531 cells, we conclude that (1) SSR adherence, (2) internalization and (3) retention of radioactivity in the tumor cell did take place, indicating we transfected a functional receptor, but unfortunely the cell line was radioresistant to the given dosages.

INTRODUCTION

Somatostatin is a peptide hormone, which is widely distributed in the human body and can regulate many physiological processes. Somatostatin receptors (SSRs) are found in various tissues, like the hypothalamus and the intestines, but also in various tumors, like neuro-endocrine tumors, carcinoids and breast tumors[1]. The use of SSR scintigraphy with radiolabeled octreotide, [^{111}In-DTPA^{0}]octreotide (Octreoscan ®), is a sensitive and specific technique to visualize the presence of SSRs on various tumors. In addition, when radiolabeled octreotide is applied with high radioactive dosages it is also successful for peptide receptor radionuclide therapy (PRRT)[2]. Successful treatment of neuro-endocrine tumors with radiolabeled octreotide analogs with β-emitting radionuclides, such as ^{90}Y and ^{177}Lu, are described in several clinical trials [2-5].

Recently, we reported the antiproliferative effect of PRRT with [^{111}In-DTPA^{0}]octreotide in a rat liver metastasis model [6]. The presence and function of the SSR on the tumor was essential, since antiproliferative effects was only seen in SSR-positive tumor, while absent in SSR-negative tumor. Moreover, blocking of the SSR with an excess of octreotide, abolished this effect.

Therefore the idea arose to transfect SSR-negative tumor cells with a SSR-gene, opening the possibilities for application of SSR-targeted PRRT. Using the same *in vivo* tumor model, here we describe the effects of PRRT on SSR-negative colon carcinoma cells (CC531) after transfection with a SSR-gene.

MATERIAL AND METHODS

Animals

Male inbred Wag/Rij rats (Harlan-CPB, Horst, The Netherlands), 10 to 14 weeks old and 225 to 250 g, were kept under standard laboratory conditions (12 hr light/ 12 hr dark) and given a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to the rules of the Dutch Animal Experimental Act and was approved by the Committee on Animal Research of the Erasmus University.

Tumor

CC531 is a SSR-negative, moderately differentiated rat colon carcinoma, induced by 1,2-dimethylhydrazine and is transplantable in syngeneic WAG/Rij rats [7]. The tumor

is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany). A suspension of 0.5 x 10^6 living cells was used for direct injection into the portal vein [6].
The pancreatic tumor CA20948 was originally induced by azaserine. This SSR-positive tumor is of acinar origin and is transplantable in syngeinic Lewis rats. The tumor is maintained in tissue culture as a monolayer in RPMI 1640 (Gibco, Paisley, UK) supplemented with 5% FCS. Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany).

SSR Transfection and Expression

For expression of the SSR-subtype 2 ($sstr_2$) in CC531 cells, human $sstr_2$ cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute, Chicago, IL, USA) was excised form pBS and inserted into the Nhe-1/Sali cloning site of the retroviral expression vector pCi-neo. Selection was made by the geneticine resistance gene (G418). This vector was used to stable transfect (using lipofectin) CC531 cells. Transfectants were selected and cultured in RPMI 1640 supplemented with 5% FCS and geneticine (0.5 mg/mL) (Gibco, Paisley, UK). Geneticine-resistant clones were examined for their ability to bind with [^{125}I-Tyr^3]octreotide. The clone with the highest expression (Scatchard analysis) of SSR was isolated and named CC2B.

Immunohistochemical Staining of the SSR

Five µm sections of formalin fixed and paraffin-embedded CC531 or CC2B liver metastases tissue were deparafinized, dehydrated, exposed to microwave heating (in citric acid buffer, 10 min at 100 C), rinsed in tap water (1x) and PBS (1x), and processed further as described above for the immunocytochemical staining (SSR_{2A} antibody dilution 1:500). Negative controls for immunohistochemistry included omission of the primary antibody and preabsorption of the antibodies with the immunzing receptor peptide (at a concentration of 0.3 µg/mL = 100 nM)

In vitro and ex vivo SSR Autoradiography

In frozen tumor samples SSRs were measured by autoradiography on 10 µm cryostat sections. For *in vitro* SSR autoradiography 10^{-10} M [^{125}I-Tyr^3]octreotide (home made

[8]), as a radioligand, was used. Incubation and washing conditions were as described[9]. Non-specific binding was determined by adding an excess (10^{-6} M) of non-radiolabeled octreotide. For *in vitro* SSR autoradiography, radioactivity was measured for 24 hours in a Cyclone Storage Phosphor Screen (Packard Bioscience company, Meriden, USA), while for *ex vivo* SSR autoradiography (samples taken from the biodistribution study) radioactivity was measured for 72 hours. The screens were analysed using a Cyclone Phospor Imager and a computer-assisted OptiQuant 03.00 image processing system (Packard Instruments Co, Groningen, The Netherlands) [13].

Radiolabeling and Quality Control of the Radioligand

[$DTPA^0$]octreotide (Pentetreotide, DRN 4920) and $^{111}InCl_3$ (DRN 4901, 370 MBq/mL in HCl, pH 1.5 to 1.9) were obtained from Mallinckrodt (Petten, The Netherlands). Octreotide was a gift of Novartis (Basle, Switzerland). Labeling was performed by diluting freeze-dried [$DTPA^0$]octreotide in 1 mL saline and adding this to the $^{111}InCl_3$. Thirty minutes after the start of this procedure, quality control was performed by instant thin-layer chromotography with silica gel and 0.1 M sodium-citrate, pH 5, as eluent. The labeling efficiency of [^{111}In-$DTPA^0$]octreotide was over 98%. Each administration of the radioligand consisted of 370 MBq ^{111}In labeled with 0.5 μg [$DTPA^0$]octreotide, referred as 370 MBq (0.5 μg) [^{111}In-$DTPA^0$]octreotide.

Experimental Procedure

Under ether anesthesia, the abdomen was opened through a 2.5 cm midline incision. Then, 0.5 x 10^6 viable SSR-positive CC2B cells or SSR-negative CC531 cells in 0.5 mL RPMI 1640 were injected slowly into the portal vein through a 0.4 x 12 mm needle. The abdominal wall was closed in one layer by a continuous silk suture[6,86]. The day after the operation, rats were randomized in an experimental and control group. Each group consisted of 8 or 9 rats. Rats of the experimental group were treated with 370 MBq (0.5 μg) [^{111}In-$DTPA^0$]octreotide i.v. on days 1 and 8. Rats in the control group did not receive treatment.

All rats were sacrificed 28 days after inoculation of tumor cells. Livers were removed, washed and immersed in PBS. Tumor growth was determined by 2 independent investigators counting the number of metastases on the surface of the liver lobes (up to 100) while blinded for treatment modality. The number of metastases were subdivided in a semi-quantative tumorscore, with concordant ranking from 0 (no

metastases) up to 5 (>100 tumor colonies, with >50% of the liver affected as presented in table 1 and 2. Tumor tissue was snap frozen in liquid nitrogen for autoradiography to determine *in vivo* somatostatin receptor status of the tumor

Biodistribution Study

Under the same experimental procedure as described above, 12 rats were injected with 0.5×10^6 viable CC2B cells into the portal vein to produce liver metastasis. After 27 days 3 groups of 3 rats received a low dosage of 3 Mbq (0.5 µg) [^{111}In-DTPA^0]octreotide i.v.. These rats were sacrificed after 4, 24 and 48 hours after injection. Another group of 3 rats was coinjected i.v. with 0.3 mg octreotide, in order to saturate the SSR and to determine non-specific binding of radioactivity. These rats were sacrificed 24 hours after injection. After sacrification livers were removed and tumor and liver tissue were separated for biodistribution of radioactivity in a well-type LKB-1282-Compugammasystem and also samples were snap frozen in liquid nitrogen for ex vivo autoradiography [8].

Clonogenic Assay and Radiation

6-wells plates (Costar, Badhoevedorp, The Netherlands) were coated with 1 mL poly-l-lysine (Sigma, Zwijndrecht, the Netherlands) and were stored for 30 min at 4 C to harden. Hereafter, 200 CC531, CC2B or CA20948 tumor cells were seeded in six-plo for every measured radiation-dose in these coated plates with 3 mL medium and 5% FCS. The next day cells were irradiated with a Philips RT 250 x-ray machine at 1.5 Gy/min. The used radiation-doses were: 0, 1, 2, 4, 8 and 10 Gy. Irradiation was performed at room temperature. 10 days after culture, surviving fractions were fixed 10 min with 1mL methanol (LabScan Ltd., Dublin, Ireland) / glacial acetic acid (Merck, Darmstadt, Germany) (3:1) and stained with 1 mL Haematoxylin (Dako, Glostrup, Denmark). Surviving fractions were determined by clonogenic assay.

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance. When significant effects were obtained by analysis of variance, multiple comparison were made by the Newmann-Keuls test. Statistical significance was defined as $p< 0.05$. Data are expressed as mean ± SD.

RESULTS

Expression and Functional Capacity of the transfected SSR

Immunohistochemical staining with an antibody against the sst_{2A}, clearly visualized this receptor subtype on CC2B paraffin-embedded sections, while absent on CC531 paraffin-embedded sections (fig. 1).

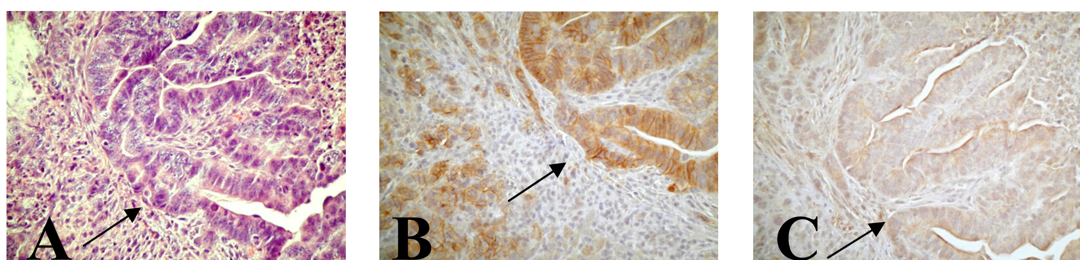

Fig. 1: Immunohistochemical staining of CC2B liver metastases (arrows) surrounded by normal liver parenchyma. Staining with: A. Heamtoxylin-eosin. B. SSR antibody. C. Non-specific binding of the SSR in the presence of an excess of octreotide.

Experimental Procedure

PRRT with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide on day 1 and 8 did not have an effect on the number of CC531 liver metastases in comparison with the controls. Mean tumor scores were 4.0 and 3.3, respectively (not significant) (table 1). Also no effect on the number of liver metastases was seen in the CC2B group in comparison with controls. Mean tumor scores were 2.5 and 2.6, respectively (not significant) (table 2).

Number of animals with 0 to >100 CC531 metastases						
	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**-**	**1**	**5**	**2**	**1**
PRRT	**-**	**-**	**1**	**1**	**3**	**3**

Table 1: Number of animals with given range of metastases, 28 days after direct injection of CC531 tumor cells into the portal vein. The effect of PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide is not significantly different (p>0.05) from that of controls.

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

Number of animals with 0 to >100 CC2B metastases						
	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**-**	**5**	**1**	**2**	**-**
PRRT	**-**	**1**	**4**	**2**	**-**	**1**

Table 2: Number of animals with given range of metastases, 28 days after direct injection of CC2B tumor cells into the portal vein. The effect of PRRT on days 1 and 8 with 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide is not significantly different (p>0.05) from that of controls.

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

In vitro and ex vivo SSR Autoradiography

Autoradiography of tumor tissue showed high expression of SSRs on CC2B tumors in contrast to CC531 tumors (fig. 2). The mean percentage uptake of radioactivity was approximately two decades higher in the CC2B group than in the CC531 control group (p<0.01). Also in the ex vivo autoradiography CC2B tumor tissue showed high uptake of radioactivity, in contrast to surrounding liver parenchyma tissue (data not shown).

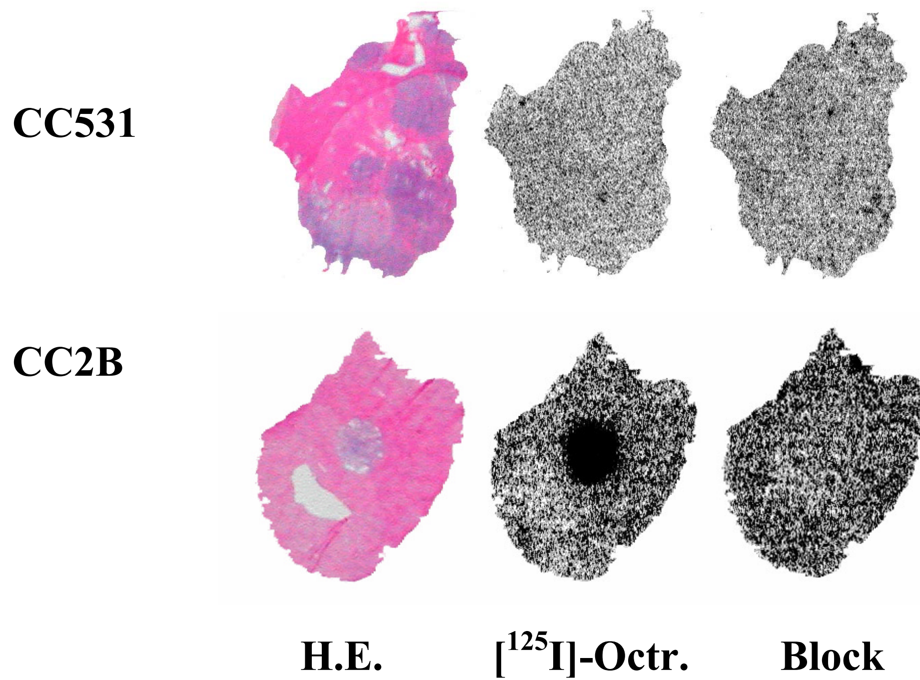

Fig. 2. In vitro autoradiography of CC2B and CC531 liver metastases tissue. H.E. = haematoxylin-eosin staining, [^{125}I]-Octr. = staining of the SSR with [^{125}I, Tyr^{3}]octreotide, Block = blocking of SSR with non-radiolabeled octreotide, before addition of [^{125}I, Tyr^{3}]octreotide

Biodistribution Study

Fig. 3, clearly shows the high uptake of radioactivity in the tumor after 4 hours, while uptake in liver tissue was significantly lower ($p<0.01$). After 24 and 48 hours the uptake of radioactivity was also higher compared to liver tissue ($p<0.01$). After 24 hours after injection of the radioligand the total binding *vs.* non-specific binding was 0.33% Injected Dose (ID)/g *vs.* 0.04% ID/g ($p<0.01$).

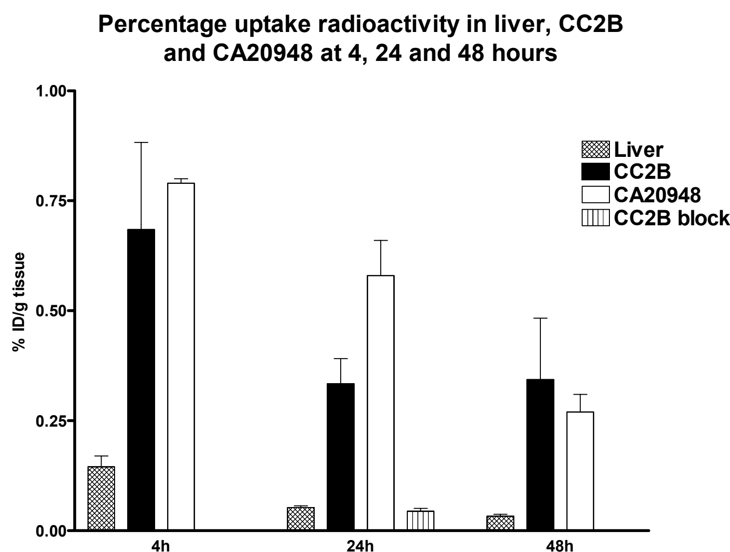

Fig. 3. The uptake of radioactivity after injection of [^{111}In-DTPA^{0}]octreotide expressed as percentage injected doses per gram tissue. Data of de Jong et al.[18] (CA20948) are taken over and added in the graph, in order to make a comparison with a positive-control tumor.

Clonogenic Assay and Radiation

The clonogenic assay of the tumor cell lines with external radiation shows that CC2B and CC531 are clearly less radiosensitive then our positive control tumor, CA20948. The surviving fraction at 2 Gy (SF2) was 0.78 and 0.76 for CC531 and CC2B respectively, while 0.48 for CA20948 ($p < 0.01$) (fig. 4)

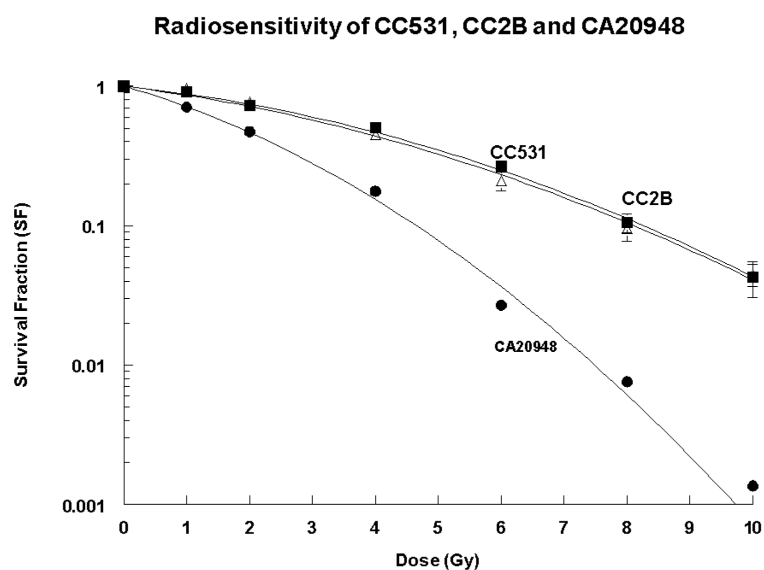

Fig. 4. Clonogenic assay of CC531, CC2B and CA20948 after external radiation. Clearly CC531 and CC2B are less radiosensitive than CA20948 (p < 0.01).

DISCUSSION

In previous studies we showed that PRRT was very effective in SSR-positive tumors, namely CA20948 rat pancreas carcinoma (B_{max} = 110 fmol/mg per membrane protein, K_d = 0.6 nM), in a rat liver metastasis model[6]. In another study Slooter *et al.* showed that tumor growth was stimulated by performing a partial hepatectomy[11]. Even under these tumor growth-stimulating conditions PRRT could provide a strong antiproliferative effect [8]. The SSR plays a crucial role in the mechanism of PRRT, as antiproliferative effects were only seen in SSR-positive liver metastases. When the SSR was blocked by giving an excess of non-radiolabeled octreotide before PRRT treatment, the antiproliferative effect was abolished. In this study SSR-positive CA20948 pancreas carcinoma cells versus SSR-negative CC531 colon carcinoma cells were used as tumors. By using two different tumors (CA20948 and CC531) in our PRRT model, we had two major drawbacks: 1. The difference in effects might be attributed to the fact that two different tumors were used, which can have differences in degrees of radiosensitivity [12,13]. 2. The effects could be also influenced by using two different strains of rats, namely the Lewis rats (CA20948) and Wag/Rij rats (CC531). The aim of this study was to transfect SSR-negative tumor cells with a SSR-gene, so SSR-targeted PRRT could also be applicable for these tumors. Also the two above mentioned drawbacks could be overcome by the transfection, as the transfected and the parental tumor cell have the same features (i.e. radiosensitivity) and can be tested in the same rat strain.

In a previous study, we successfully transfected the SSR on colon carcinoma cells *in vitro* (hereafter named CC2B), with a high expression of SSRs and a comparable growth and growth features as the parental tumor cell line CC531.

Through this result, in this study SSR-negative CC531 cells and SSR-positive CC2B cells can be compared in one tumor-model and in the same rat strain, in order to elucidate whether PRRT works or not in liver metastases model.

As stated before [^{111}In-DTPA^{0}]octreotide is widely used for visualization of SSR-positive tumors, like GEP tumors, with a gamma camera. However ^{111}In also emits internal conversion electrons and Auger electrons with a medium to short tissue penetration (200-550 µm, 0.02-10 µm, respectively) [14,15]. For *in vivo* PRRT therapy with [^{111}In-DTPA^{0}]octreotide, several successively steps are essential: the radiolabeled octreotide has to adhere to the SSR, after which it is internalized. Hereafter, the radioligand is trapped in the cell, since it is charged negatively,

resulting in a prolonged residence time of radioactivity in the tumor. The resultant DNA damage will consequently also be increased and can eventually lead to cell death [16]. For example, in a human tumor the biological half life of retention was >700 hours[16,17].

In our experiment PRRT did not have an antiproliferative effect on the CC2B cells. *In vitro* SSR autoradiography and a biodistribution study were performed to investigate whether adherence, internalization and (prolonged) residence of radiolabeled octreotide takes place. Immunohistochemical staining of the liver metastases showed SSR expression of CC2B liver metastases and no SSR expression in CC531 liver metastases. The *in vitro* SSR autoradiography confirmed high expression of SSR in CC2B cells, in contrast to normal CC531 cells, indicating that adherence of radiolabeled octreotide did occur.

In the biodistribution study a clear uptake of radiolabeled octreotide was seen in rats sacrificed after 4 hours, indicating that *in vivo* internalization also takes place. Rats sacrificed after 24 or 48 hours showed a stable measurement of % ID/g tumor tissue (0.34%), indicating high retention of radioactivity in the CC2B tumor cells. When the SSR was blocked the tumoral radioactivity uptake was reduced to surrounding liver level: underlining the involvement of SSR-mediated internalization. Compared to the CA20948 tumor almost the same amount of radioactivity was measured in the CC2B tumor after 4, 24 and 48 hours (see fig. 6) [10]. Despite of the same amount of radioactivity in the tumor cells and same radiation dose, no effect was seen on tumor growth, indicating that this tumor cell line apparently is less radiosensitive for our given dosage of radiolabeled octreotide. In order to elucidate the radiosensitivity of the cell lines, we performed clonogenic assays of the cell lines with external radiation. A poor radiosensitivity of the CC2B (and CC531) cells was seen in comparison with our positive control tumor CA20948, in which we previously had shown effiective therapy with radiolabeled octreotide. A possible explanation for this radioresistancy might be attributed to the p53 status of the tumor. Numerous *in vitro* and *in vivo* studies indicate that loss of p53 function increases post-irradiation clonogenic cell survival, which correlates with an abrogated G_1 checkpoint and changes in apoptosis[18]. Introduction of the wild type p53-gene by gene therapy resulted in increased tumoral radiosensitivity and a concordant decrease of radiation survival of these cells, both *in vitro* and *in vivo*. Spitz *et al* showed that introduction of wild-type p53 did sensitize colorectal cancer cells to ionizing radiation. This radiosensitization

correlates with restoration of the G_1 checkpoint and apoptosis [19]. In addition, reducing radiation survival *in vitro*, significantly increases apoptosis [20]. This combination therapy also resulted in delayed *in vivo* tumor growth in their xenograft mouse model [21]. Recently Geutskens *et al* showed p53-deletion of CC531 cells, so this may explain why PRRT is not effective in this tumor [22]. In theory, cotransfection of wild-type p53 and the SSR-gene might also sensitize the radiation caused by PRRT in CC531 cells.
Furthermore, a higher antiproliferative effects may be obtained by giving a higher and/or fractionated dosage of radiolabeled octreotide. However, toxic effects on kidney and bone marrow limit the height of the fractionated dosage [23].
Another method to increase antiproliferative effects is to replace the ^{111}In with a radionuclide with higher energy emitting particles, such as ^{90}Y or ^{177}Lu. Through the emission of β-particles not only a higher energy is produced (E_{β}max ^{90}Y = 2.3 MeV, ^{177}Lu = 0.5 MeV), but also a concordant higher tissue penetration is achieved (^{90}Y = 12 mm, ^{177}Lu = 2 mm), which might result in a more effective therapy. Currently clinical trials with [^{90}Y-DOTA^{0}]octreotide and [^{177}Lu-DOTA, Tyr^{3}]octreotate are ongoing with promising preliminary results [24,25 4,5,134,5], in which ^{90}Y is more successful for larger bulky tumors and ^{177}Lu is successfully used for therapy of micrometastases. Especially, when *in vivo* gene therapy is concerned, poor transduction rates results in poor efficacy of gene therapy. However, by using β-emitting radionuclides, like ^{90}Y or ^{177}Lu, transduced cells and neighboring non-transduced cells in a radius of a few mm are being radiated through the cross-fire of the β-particles, and can consequently result in a major cross-fire "bystander"-effect. Thus poor transduction efficacy might be overcome through strong antiproliferative effects with this strategy.
In conclusion: the expected antiproliferative effect of PRRT on SSR transfected colon carcinoma cells was absent, although (1) SSR-mediated adherence, (2) internalization and (3) retention of radioactivity in the tumor cell did take place. Furthermore clonogenic assays with external radiation showed a poor radiosensitivity of the CC2B cells. This means that we successfully transfected a functional receptor on radioresistent tumor cells. However, we still believe that SSR gene therapy followed by PRRT is promising. To prove the validity of this concept, we are currently transfecting a SSR-negative radiosensitive cell line and also are testing SSR-targeted PRRT with [^{177}Lu-DOTA, Tyr^{3}]octreotate in a rat liver metastasis model.

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CHAPTER 6

ANTI-TUMOR EFFECT AND INCREASED SURVIVAL AFTER TREATMENT WITH [^{177}LU-DOTA^{0},TYR^{3}]OCTREOTATE IN A RAT LIVER MICROMETASTASES MODEL

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ABSTRACT

Peptide receptor scintigraphy with [^{111}In-DTPA^{0}]octreotide (a stabilized radiolabeled somatostatin (SS) analog, OctreoScan®) is widely used for the visualisation and staging of somatostatin receptor-positive tumors. The application of likewise somatostatin analogs as vehicle for the deliverance of radionuclides to somatostatin receptor-positive targets are now in use for Peptide Receptor-targeted Radionuclide Therapy (PRRT). Currently preclinical and clinical investigation are ongoing trying to find the optimal combination of radionuclide and ligand. The anti-tumoral effects of such combinations, like [^{90}Y-DOTA^{0},Tyr^{3}]octreotide and [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, on SSR-positive solid tumors have been reported.

Aim: In this study we present the anti-tumor effects of ^{177}Lu-DOTA-tate on

a. a single SSR-positive cell model, and

b. on a SSR-positive tumor in a rat liver micrometastatic model, mimicing disseminated disease.

Results: ^{177}Lu-DOTA-tate showed anti-tumoral effects in **a**. and in **b**., and significant survival in the PRRT-treated rats.

In conclusion: ^{177}Lu-DOTA-tate is a very promising new treatment modality for SSR-positive tumors, including disseminated disease.

INTRODUCTION

Somatostatin receptors (SSRs) have been demonstrated on a variety of human tumors [1]. At least 5 different human subtypes (sst_{1-5}) have been cloned [2]. All subtypes bind somatostatin with high affinity, while octreotide, a somatostatin analog, binds with high affinity to sst_2, and with decreasing affinity for sst_5 and sst_3 [3]. The vast majority of human SSR-positive tumors express SSR subtype 2 [3, 4]. SSR-positive tumors, like carcinoids, breast cancer and various neuroendocrine tumors, can be visualised with [^{111}In-DTPA^0]octreotide (OctreoScan®). OctreoScan is nowadays recognised to be the imaging technique for the detection and staging of SSR-positive tumors. Peptide receptor radionuclide therapy (PRRT) with high dosages of [^{111}In-DTPA^0]octreotide showed anti-tumor effects in a rat liver micrometastases model [5] and in patients with neuroendocrine tumors [6-9]. The disadvantage of ^{111}In for PRRT is the short particle range and consequently small tissue penetration [10]. Therefore, other radionuclides, like ^{90}Y and ^{177}Lu, were coupled to DOTA-conjugated somatostatin analogs, such as [DOTA^0,Tyr^3]octreotide (DOTATOC), and [DOTA^0, Tyr^3]octreotate (see for a review [3]). These radionuclides emit high energy beta particles (2.27 and 0.5 MeV, resp.) with accordingly larger particle range of 10 and 2 mm, respectively [3].

Various studies showed favourable results of ^{90}Y-DOTATOC and ^{177}Lu-DOTA-tate in patients with neuroendocrine tumors [11-14]. PRRT with radionuclides emitting higher energy beta-particles (e.g. ^{90}Y) are in theory and in practice advantageous for larger tumors (>1g), but not for smaller tumors (<1g). For uniform activity distribution in these larger tumors, almost all energy of the emitted electrons will be deposited in the target. However, for the smaller tumors only a small energy fraction from the emitted electrons will be deposited within the tumor. [15-18]. Recently we presented successful PRRT with ^{177}Lu-DOTA-tate of SSR-positive solid tumors in rats [17] and also in patients[14]. However, still the question remains whether ^{177}Lu has anti-tumor effects on SSR-positive micrometastases. In the here presented study we describe a PRRT study with ^{177}Lu-DOTA-tate of SSR-positive tumor cells in an in vitro model (a) and in a rat liver micrometastatic model (b).

MATERIAL & METHODS

Animals

Male inbred Lewis rats, 10 to 14 weeks old with a body weight of 250 to 275 g, were obtained from Harlan-CPB (Horst, The Netherlands). Animals were kept under standard laboratory conditions (12 hours light/ 12 hours dark) and given a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to the rules of the Dutch Animal Experimental Act and was approved by the Committee on Animal Research of the Erasmus University.

Tumor

CC531 is a SSR-negative, moderately differentiated rat colon carcinoma, induced by 1,2-dimethylhydrazine, is transplantable in syngeneic WAG/Rij rats [5]. The tumor is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany).

CA20948 is a SSR-positive, pancreatic tumor of acinar origin, which was originally induced by azaserine and is transplantable in syngeneic Lewis rats [16]. The tumor is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% FCS. For the in vitro experiments, cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany). To produce artificial liver metastases for the *in vivo* experiments, tumors were excised from donor rat livers, cleaned from normal liver tissue and pressed through sieves with decreasing mesh size. The resulting suspension was washed twice in RPMI 1640. Viability was measured with trypan-blue exclusion (0.3% in a 0.9% NaCl solution). A suspension of 2.5 x 10^6 living cells per 5 mL was used for direct injection into the portal vein [5, 19].

Radiolabeling and Quality Control of the Radioligand

Reactor-produced ^{177}Lu from 70% enriched ^{176}Lu-target was obtained from IDB Holland (Baarle Nassau, The Netherlands). DOTA-tate was obtained from Mallinckrodt (St. Louis, Mo., USA). ^{177}Lu-labeling of DOTA-tate was performed as previously described [17, 18, 20]. Measurement of the incorporation of the

radionuclide was performed in the presence of 4 mM DTPA pH 4 on ITLC-SG with 0.1 M Na-citrate as eluens [17, 18]. The labeling yield always exceeded 99% and the radiochemical purity was higher than 90%. Post radiolabeling and prior to the administration of the radioligand-containing solution 4 mM DTPA pH 4 was added to a final mol/mol ratio (DTPA over DOTA-tate) of 30.

In vitro PRRT

One day before the experiment CC531 and CA20948 tumor cells were transferred to 6 well plates in a density of 200 and 400 cells per well. Cells were washed with PBS (Phosphate Buffered Saline) (37°C) and incubated for at least one hour in 1 mL incubation medium (RPMI-1640 medium without foetal calf serum (FCS) but with 1% bovine serum albumin and 20 mmol HEPES) containing ^{177}Lu-DOTA-tate. Control cells received only incubation medium for one hour. Thereafter, cells were thoroughly washed with PBS and allowed to form colonies during 2 days in growth medium. The medium was once refreshed after 3 days. At day 12 the cells were fixated with 1 mL methanol:glacial acid (3:1) for 15 minutes. Subsequently the cells were colored with haematoxylin (Dako, Glostrup, Denmark). Colonies that contained more than 50 cells per colony were scored as survivors.

In vivo PRRT

Under ether anaesthesia, the abdomen was opened through a 2.5 cm midline incision. 0.25 x 10^6 viable CA20948 cells in 0.5 mL RPMI 1640 were injected slowly into the portal vein through a 0.4 x 12 mm needle. The abdominal wall was closed in one layer by a continuous silk suture. The day after the operation, rats were randomised in an experimental and control group. Each group consisted of 12 rats. Rats of the experimental group were treated with 185 (1.9 µg) or 370 MBq (3.8 µg) [^{177}Lu-DOTA^0, Tyr^3]octreotate iv. on day 8. Rats in the control group did not receive treatment. Body weight and animal condition (i.e.: lethargy, scruffy coat and diarrhoea) were determined at regular intervals at least twice a week.

In order to evaluate anti-tumor effects, half of the rats were sacrificed 21 days after inoculation of tumor cells. Livers were removed, washed and immersed in PBS. Livers were weighed and 2 independent investigators counting the number of metastases on the surface of the liver lobes (up to 100) while blinded for treatment modality determined tumor growth. The number of metastases were subdivided in a

semi-quantitative tumor score, with concordant ranking from 0 (no metastases) up to 5 (> 100 tumor colonies, with > 50% of liver affected) as presented in table 1.

The remaining rats were used for a survival experiment. The experiment was ended 150 days post inoculation of the tumor, 150 days is the generally accepted equivalent of 5 years human survival. Body weight and animal condition was determined at regular intervals at least twice a week. When loss of body weight was more than 10% of original body weight, rats were sacrificed and livers examined.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance. When significant effects were obtained by analysis of variance, multiple comparison were made by the Newmann-Keuls test. Statistical significance was defined as $p < 0.05$. Data are expressed as mean ± SD. GraphPad Prism (GraphPad Prism Software, San Diego, CA) was used to plot survival curves for the different groups.

Results

In vitro PRRT

Fig 1 shows the % survival of CA20948 and CC531 cells after in vitro PRRT with various amounts of ^{177}Lu-DOTA-tate. No differences were found between the experiments with 200 and 400 cells (data not shown).

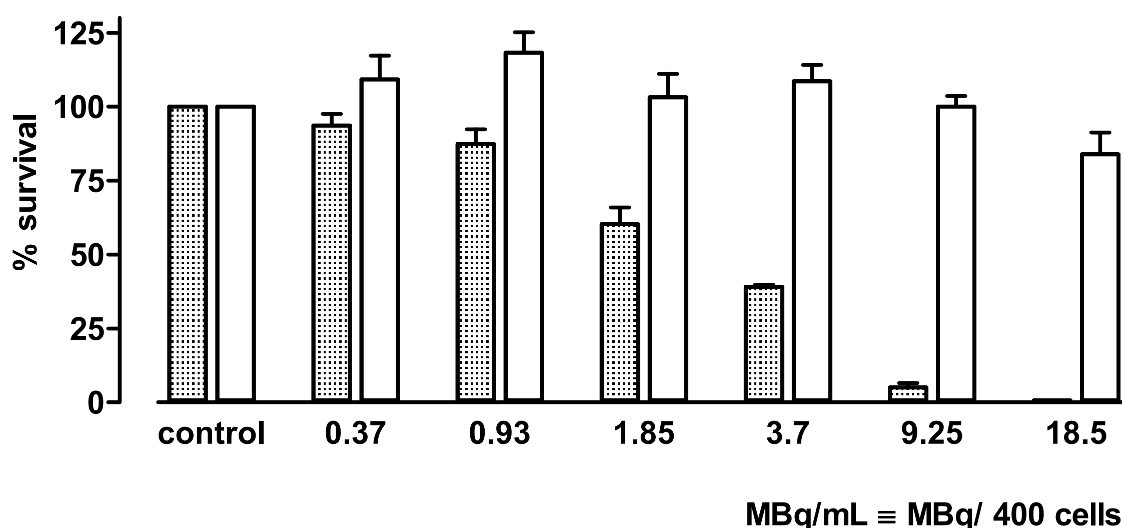

Fig 1. The percentage survival of CA20948 and CC531 cells (open bar) after in vitro PRRT with various amounts of ^{177}Lu-DOTA-tate

In vivo PRRT

The results of PRRT with 185 MBq (1.9 µg) or 370 MBq (3.8 µg) [^{177}Lu-DOTA^0, Tyr^3]octreotate on day 8 on the growth of CA20948 liver metastases compared with rats of the control group are summarised in table 1. Both 185 MBq and 370 MBq ^{177}Lu-DOTA-tate had an anti-tumor effect on CA20948 liver metastases compared with controls. Mean tumor scores were 2.0 ± 1.3, 1.3 ± 0.8 and 5.0 ± 0.0, respectively ($p < 0.001$ vs. controls). Liver weight was significant less in PRRT treated rats compared with controls: mean liver weights (in g) were 8.9 ± 0.9, 9.3 ± 0.7 and 19.2 ± 2.0, respectively ($p < 0.001$ vs. controls) (fig. 2).

Table 1. Effect of 185 or 370 MBq ^{177}Lu-DOTA-tate on CA20948 liver metastases

Number of animals with 0 to >100 CA20948 metastases						
	0	*1 - 20*	*21 - 50*	*51 - 100*	*>100[1]*	*>100[2]*
Rank	*0*	*1*	*2*	*3*	*4*	*5*
Controls	**-**	**-**	**-**	**-**	**-**	**6**
185 MBq	**1**	**1**	**1**	**3**	**-**	**-**
370 MBq	**2**	**-**	**4**	**-**	**-**	**-**

Number of animals with given range of metastases, 21 days after direct injection of CA20948 tumor cells into the portal vein. Treated rats (185 or 370 MBq ^{177}Lu-DOTA-tate) with CA20948 liver metastases had significant less liver metastases compared with control rats ($p < 0.001$).

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

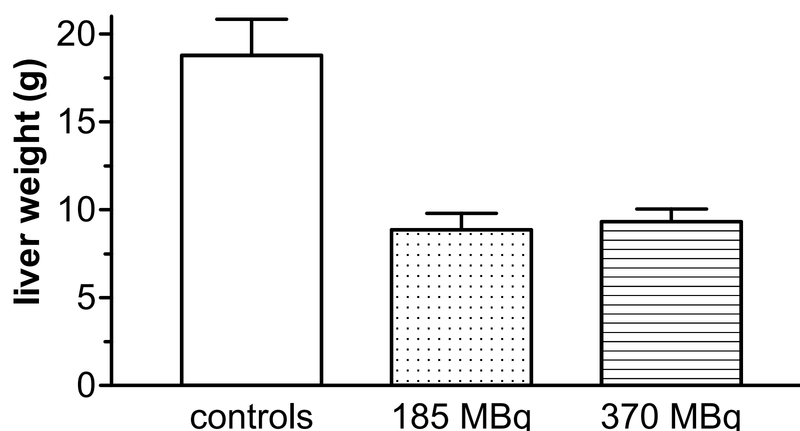

Figure 2. Liver weight of groups of rats with CA20948 liver metastases after indicated doses (expressed as mean ± SD, n=6). Mean liver weights of rats treated with 185 or 370 MBq ^{177}Lu-DOTA-tate are both significant less than control rats ($p < 0.001$)

Survival curves for the control and therapeutic groups are shown in figure 3. Tumors of the rats in the control grew excessively and no survival at 150 days was found. Treatment with a single dose of 185 MBq ^{177}Lu-DOTA-tate resulted in a significant increase of the survival of the rats of this group. In all cases of sacrification livers were full of metastases. At 150 days after tumor inoculation, 2 of the 6 rats (33%) in this group were still alive and autopsy after sacrification showed no visible liver metastases in these 2 rats ($p < 0.05$). Also animals that received a single injection of 370 MBq ^{177}Lu-DOTA-tate showed a significant increase in survival compared with controls, however, no animals survived 150 days ($p < 0.05$).
In the *in vivo* experiments no overt signs of toxicity as weight loss, scruffy coat, lethargy or diarrhoea were observed.

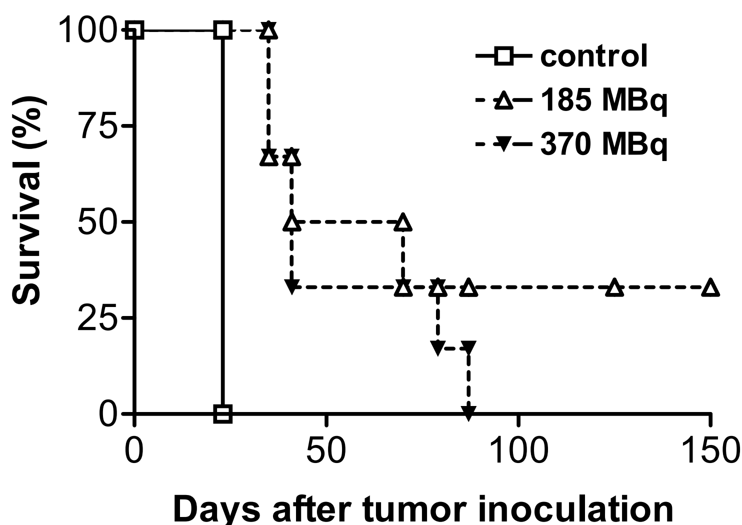

Figure 3. Survival curves of groups of rats with CA20948 liver metastases after indicated doses (n=6 per group). Both group of rats treated with 185 and 370 MBq ^{177}Lu-DOTA-tate showed a significant increase in survival compared with controls ($p < 0.05$). Two of the 6 rats treated with 185 MBq ^{177}Lu-DOTA-tate survived 150 days and had no visible liver metastases after sacrifice. 150 days survival equals 5 human years

DISCUSSION

Peptide receptor scintigraphy with [^{111}In-DTPA^{0}]octreotide is widely used for the visualisation and staging of SSR-positive tumors. The application of likewise ligands as vehicle for the deliverance of radionuclides to SSR-positive targets is now also in use for PRRT. Currently preclinical and clinical investigation are ongoing trying to find the optimal combination of radionuclide and ligand (with both their specific characteristics) for several parameters, including tumor volume. The anti-tumoral effects of such a combination, ^{177}Lu-DOTA-tate, on SSR-positive solid tumors have been reported [14, 17].

In this study we present the anti-tumor effects of ^{177}Lu-DOTA-tate:

1. in vitro in a single SSR-positive cell model, and
2. on a SSR-positive tumor in a rat liver micrometastatic model, mimicing disseminated disease.

As shown in figure 1, SSR-positive CA20948 tumor cell survival decreases with increasing doses of ^{177}Lu-DOTA-tate, and this in contrast to SSR-negative CC531 tumor cells in a parallel experiment. We reported earlier on experiments performed to study internalisation of radiolabeled somatostatin analogs such as [^{111}In-DTPA^{0}]octreotide and ^{90}Y-DOTATOC in rat pancreatic tumor cells [21, 22]. This process was found to be receptor specific and temperature dependent [22, 23]. Furthermore, the amount of radioligand taken up in the tumor depends on the total amount of unlabeled peptide present and thus on changes in specific activity of the radiolabeled peptide [24, 25]. Therefore, the success of the therapeutic strategy relies on the total amount of radioligand that accumulates within tumor cells, which among other things depends on the receptor affinity and rate of internalisation of ligand and receptor. The physical characteristics of radioactivity accumulated within tumor cells determines also the dosimetric dose on neighbouring cells. As stated above, ^{177}Lu emits β-particles with a maximum energy of 0.5 MeV and a high tissue penetration, with a maximum range of 2 mm. Assuming a cell diameter of 20 μm, this means, when internalized into the tumor cell, ^{177}Lu is able to potentially kill cells at approximately 12 cell diameters from a cell in which it is internalized, with a maximum cell-killing potential of approximately 50 cell diameters [26]. This extra cell killing potential is also called a radiological bystander effect [27] and can be an additional advantage in the PRRT of tumors with a heterogenous expression of receptors with affinity for the radiolabeled peptide.

In table 1 and in figure 2 the tumor growth control by ^{177}Lu-DOTA-tate is presented. Tumor growth in controls was excessive, 21 days post inoculation of the tumor cells, more than 100 tumor colonies were counted in the livers and, in addition, more than 50% of the liver was affected. The liver weight doubled ($p < 0.001$) vs. the 2 PRRT-treated groups of rats (Fig. 2). We recently reported that PRRT with [^{111}In-DTPA^{0}]octreotide had no effects on basic liver functions [28] in press). The effect of PRRT was also reflected in the survival curve: rats treated with 185 or 370 MBq ^{177}Lu-DOTA-tate had significantly lower tumor colonies present at day 21 (Fig. 2), and even 2 of 6 survived for 150 days, which is the generally accepted equivalent of 5 human years. In the PRRT-treated rats a higher survival was found in the rats treated with the lower dose. The amounts of injected radioactivity in these 2 groups were 185 and 370 MBq, and the concordant amount of injected ligand: 1.9 µg and 3.8 µg, resp. This increase in amount of injected ligand reduces the uptake in the SSR-positive targets: the uptake herein is a function of the injected mass [24, 25]. The uptake in SSR-positive tumor cells in rats injected with 1.9 µg is approximately 70% of the optimal uptake, and in the other group injected with 3.8 µg is 40%, resulting in no significant increase in radioactivity in the targets. So, although the radioactive dose was doubled, the uptake of radioactivity in these SSR-positive targets has not increased, and as a result, nor the dosimetric dose hereon. The latter in contrast to the SSR-negative kidney and bone marrow. These organs are critical organs for PRRT with radiolabeled somatostatin analogs [3], and by definition determine the maximal tolerated dose hereof. Since the radiation dose has doubled, the dosimetric dose for these organs has doubled. However, Lewis *et al*. recently reported no toxicity for ^{177}Lu-DOTA-tate, even up to doses of 4 GBq per kg, and up to 42 days post injection [29].

A major advantage of PRRT using radiolabeled peptides is that radiation can be delivered selectively, not only to (large) primary tumors, but also to tumors and metastases, which are too small to be imaged and thereby treated by, for example, surgery or external beam radiotherapy. Even tumors of less than 1 g are not able to absorb all electron energy emitted by ^{90}Y in their cells [10, 15, 18]. Therefore, patients bearing tumors of different sizes might be treated by a combination of radionuclides, e.g., the high-energy β-emitter ^{90}Y for large tumors (> 1 g) and low-energy ß-emitter, such as ^{177}Lu, or an Auger electron emitter, such as ^{111}In for smaller

(< 1 g) tumors and metastases. Preclinical and clinical PRRT experiments of SSR-positive tumors are ongoing.

In conclusion, ^{177}Lu-DOTA-tate showed anti-tumor effects in an isolated SSR-positive tumor cell model in vitro and in a rat liver SSR-positive micrometastic model setting, making it a very promising new treatment modality for SSR-positive disseminated disease.

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Arthur van Gameren and Erik de Blois are greatly acknowledged for technical assistance.

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CHAPTER 7

SOMATOSTATIN RECEPTOR GENE THERAPY COMBINED WITH TARGETED THERAPY WITH RADIOLABELED OCTREOTIDE: A NEW TREATMENT FOR LIVER METASTASES

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ABSTRACT

Objective: To evaluate the effect of Peptide Receptor Radionuclide Therapy (PRRT) on somatostatin receptor (SSR)-transfected colon carcinoma cells in a rat liver metastases model.

Summary Background Data: Previously, we showed highly effective therapy with PRRT of SSR-positive tumors. This treatment is SSR-mediated, while successful treatment is only seen in SSR-positive tumors and no effect is seen in SSR-negative tumors. As many tumors lack this receptor, the idea arose to transfect SSR-negative tumor cells with a SSR-gene, in order to apply PRRT on these SSR transfected tumor cells.

Methods: CC531 colon carcinoma cells (SSR-negative) were transfected *in vitro* with a SSR (subtype 2)-gene, further refered as CC2B. Liver metastases were developed after intraportal injection of these tumor cells in rats. On day 7, animals were treated with 185 or 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. After 21 days rats were sacrificed and liver metastases were counted.

Results: Treatment with 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate showed an impressive anti-tumor response in rats with CC2B liver metastases (SSR-positive) in comparison with controls ($p<0.001$), while no significant anti-tumor effect was seen in PRRT treated rats with CC531 liver metastases (SSR-negative). Also a (dose-dependent) tumor response was seen in rats with CC2B liver metastases treated with lower dose, 185 MBq, [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate compared with controls ($p<0.01$). In addition, rats with mixed liver metastases treated with 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate had significant less metastases compared with controls ($p<0.05$).

Conclusion: We showed an impressive anti-tumor effect of SSR (subtype 2) transfected colon carcinoma cells with PRRT in a rat liver metastases model. Moreover, rats with mixed liver metastases had also significant less liver metastases compared with control rats, which may be due to a radiological bystander effect of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. This phenomenon is beneficial in the concept of *in vivo* gene therapy.

INTRODUCTION

Somatostatin receptors (SSRs) have been demonstrated on a variety of human tumors (1). At least 5 different human subtypes (SSR subtype 1 – 5) have been cloned (2). All subtypes bind somatostatin with high affinity, while octreotide, a somatostatin analog, binds with high affinity to SSR subtype 2 and 5 and to a lesser degree to SSR subtype 3. Hofland *et al.* showed that the vast majority of human SSR-positive tumors express SSR subtype 2 (3).

SSR-positive tumors, like carcinoids, breast cancer and various neuroendocrine tumors, can be visualized with a low dose of [^{111}Indium-diethylenetriaminopenta-acetic acid (^{111}In-DTPA^0)]octreotide (Octreoscan®). Octreoscan has proven to be very useful in the detection and staging of SSR-positive tumors. High dosages of [^{111}In-DTPA^0]octreotide can also be given as a therapy to patients with these kind of tumors. Encouraging results have been reported in patients with progressive and metastasized neuroendocrine tumors, treated with high dosages of [^{111}In-DTPA^0]octreotide (4-7). This kind of treatment is called Peptide Receptor Radionuclide Therapy (PRRT). However, ^{111}In is not ideal for PRRT, because of the short particle range and consequently small tissue penetration. Therefore, other radionuclides, like ^{90}Yttrium (^{90}Y) and ^{177}Lutetium (^{177}Lu), were coupled to somatostatin analogs. These radionuclides emit high energy beta-particles with accordingly larger particle range of 10 and 2 mm, respectively. Various preliminary studies showed favorable results of [^{90}Yttrium-tetracyclododecanetetraacetic acid, Tyrosine (^{90}Y-DOTA^0, Tyr^3)]octreotide in patients with neuroendocrine tumors (8-10). Still the question remains whether ^{90}Y is the most ideal radionuclide for targeted radiotherapy, since a high maximum energy (E_{max} 2.27 MeV) can be an advantage for larger tumors, but this characteristic can also have drawbacks, while renal toxicity after PRRT was also observed (8; 11). Furthermore, other radionuclides with lower energies may be more useful for smaller tumors or even micrometastatatic disease. An alternative radionuclide for PRRT is ^{177}Lu (E_{max} 0.5 MeV). In a preliminary study by Kwekkeboom *et al.*, successful results were obtained in patients with progressive neuroendocrine tumors (12). Also in animal studies reported by de Jong *et al.*, convincing results were observed in rats bearing SSR-positive pancreatic carcinoma (13). However, since the uptake and internalization of this radioligand is SSR-mediated and many tumors lack this receptor, the idea arose to transfect SSR-negative tumor cells with a SSR-gene, in order to apply PRRT on these SSR-transfected tumor

cells. Using a rat liver metastases model, here we describe the effects of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on SSR-negative colon carcinoma cells (CC531) and SSR-transfected colon carcinoma cells (CC2B).

MATERIAL AND METHODS

Animals

Male inbred Wag/Rij rats, 10 to 14 weeks old with a body weight of 250 to 275 g, were obtained from Harlan-CPB (Horst, The Netherlands). Animals were kept under standard laboratory conditions (12 hours light/ 12 hours dark) and given a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to the rules of the Dutch Animal Experimental Act and was approved by the Committee on Animal Research of the Erasmus University.

Tumor

CC531 is a SSR-negative, moderately differentiated rat colon carcinoma, induced by 1,2-dimethylhydrazine and is transplantable in syngeneic WAG/Rij rats (14). The tumor is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany). A suspension of 0.5 x 10^{6} living cells was used for direct injection into the portal vein (15; 16).

SSR Transfection and Expression

For expression of the somatostatin receptor-subtype 2 in CC531 cells, human $sstr_2$ cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute, Chicago, IL, USA) was excised form pBS and inserted into the Nhe-1/Sali cloning site of the retroviral expression vector pCi-neo. Selection was made by the geneticine resistance gene (G418). This vector was used to stable transfect (using lipofectin) CC531 cells. Transfectants were selected and cultured in RPMI 1640 supplemented with 5% FCS and geneticine (0.5 mg/mL) (Gibco, Paisley, UK). Geneticine-resistant clones were examined for their ability to bind with [^{125}Iodine,Tyrosine (^{123}I-Tyr^{3})]octreotide. The clone with the highest expression (Scatchard analysis) of SSR was isolated and named CC2B (17).

Ex vivo and In vitro SSR Autoradiography

In frozen tumor samples SSR's were measured by autoradiography on 10 :m cryostat sections. The concentration of the radioligand, [^{125}I-Tyr^3]octreotide, was 10^{-10} M. Incubation and washing conditions were as described (18). Non-specific binding was determined by adding an excess (10^{-6} M) of non-radiolabeled octreotide (gift by Novartis, Basle, Switzerland). Radioactivity was measured 24 hours in a Cyclone Storage Phosphor Screen (Packard Bioscience company, Groningen, The Netherlands).

Radiolabeling and Quality Control of the Radioligand

Reactor-produced no carier added ^{177}Lu was obtained from IDB Holland (Baarle Nassau, The Netherlands).

[DOTA^0, Tyr^3]octreotate was obtained from Mallinckrodt (St. Louis, Mo., USA). ^{177}Lu-labeling of [DOTA^0, Tyr^3]octreotate was performed as previously described for [^{90}Y-DOTA^0, Tyr^3]octreotate (19). The labeling yield always exceeded 99% and the radiochemical purity was higher than 90%.

Experimental Procedure

Under ether anesthesia, the abdomen was opened through a 2.5 cm midline incision. Experiment 1: 0.5 x 10^6 viable SSR-negative CC531 cells or SSR-positive CC2B cells in 0.5 mL RPMI 1640 were injected slowly into the portal vein through a 0.4 x 12 mm needle. The abdominal wall was closed in one layer by a continuous silk suture (15; 16). The day after the operation, rats were randomized in an experimental and control group.

Each group consisted of 7 rats. Rats of the experimental group were treated with 370 MBq [^{177}Lu-DOTA^0, Tyr^3]octreotate i.v. on day 7. Rats in the control group did not receive treatment.

Experiment 2: in this experiment 0.5 x 10^6 viable SSR-positive CC2B cells in 0.5 mL RPMI 1640 were injected slowly in the portal vein. The day after the operation, rats were randomized in an experimental and control group. Each group consisted of 8 or 9 rats. Rats of the experimental group were treated with 185 MBq [^{177}Lu-DOTA^0, Tyr^3]octreotate i.v. on day 8. Rats in the control group did not receive treatment.

Experiment 3: in this experiment 0.25 x 10^6 viable SSR-negative CC531 cells mixed with 0.25 x 10^6 viable SSR-positive CC2B cells in 0.5 mL RPMI 1640 were injected

slowly in the portal vein. The day after the operation, rats were randomized in an experimental and control group. Each group consisted of 8 or 9 rats. Rats of the experimental group were treated with 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate i.v. on day 8. Rats in the control group did not receive treatment.

Body weight and animal condition (i.e.: lethargy, scruffy coat and diarrhea) were determined at regular intervals at least twice a week.

In the three experiments all rats were sacrificed 21 days after inoculation of tumor cells. Livers were removed, washed and immersed in PBS. Tumor growth was determined by 2 independent investigators counting the number of metastases on the surface of the liver lobes (up to 100) while blinded for treatment modality. The number of metastases were subdivided in a semi-quantitative tumor score, with concordant ranking from 0 (no metastases) up to 5 (>100 tumor colonies, with >50% of the liver affected as presented in table 1, 2 and 3. Tumor tissue was snap frozen in liquid nitrogen for autoradiography to determine *in vivo* somatostatin receptor status of the tumor.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance. When significant effects were obtained by analysis of variance, multiple comparison were made by the Newmann-Keuls test. Statistical significance was defined as $p< 0.05$. Data are expressed as mean ± Standard Deviation (SD).

RESULTS

In vivo PRRT

Experiment 1: The results of PRRT with 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 7 on the growth of CC531 and CC2B liver metastases are summarized in table 1. PRRT did not have a significant effect on the number of CC531 liver metastases in comparison with the controls. Mean tumor scores were 3.86 ± 0.38 and 4.14 ± 0.38 respectively. However, PRRT had a strong anti-tumor effect on CC2B liver metastases compared with controls. Mean tumor scores were 1.57 ± 0.79 and 4.43 ± 0.79 respectively ($p<0.001$). Examples of control and treated rats with CC2B liver metastases are shown in figure 1.

TABLE 1:

The effect of 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on CC531 or CC2B metastases

No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Control CC531	**-**	**-**	**-**	**-**	**6**	**1**
PRRT CC531	**-**	**-**	**-**	**1**	**6**	**-**
Control CC2B	**-**	**-**	**-**	**1**	**2**	**4**
PRRT CC2B	**-**	**4**	**2**	**1**	**-**	**-**

Table 1. Number of animals with given range of metastases, 21 days after direct injection of CC531 or CC2B tumor cells into the portal vein. Control received no treatmnent. PRRT received 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 7.

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

Experiment 2: The results of PRRT with 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 8 on the growth of CC2B are summarized in table 2. Again, PRRT had an anti-tumor effect on CC2B liver metastases compared with controls. Mean tumor scores were 2.63 ± 1.6 and 4.33 ± 0.71, respectively ($p<0.01$).

TABLE 2: The effect of 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on CC2B metastases

No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Control CC2B	**-**	**-**	**-**	**1**	**4**	**4**
PRRT CC2B	**-**	**3**	**1**	**1**	**2**	**1**

Table 2. Number of animals with given range of metastases, 21 days after direct injection of CC2B tumor cells into the portal vein. Control received no treatmnent. PRRT received 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 8.

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

Experiment 3: The results of PRRT with 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 8 on the growth of 50%/50% mixed CC2B/CC531 liver metastases are summarized in table 3. Also an anti-tumor effect, although less impressive, was found in PRRT treated rats with mixed (CC2B/CC531) liver metastases compared with controls. Mean tumor scores were 3.50 ± 1.20 and 4.78 ± 0.44, respectively ($p<0.05$). In all three experiment no overt signs of toxicity were observed after PRRT treatment (i.e.: weight loss, lethargy, scruffy coat and diarrhea).

TABLE 3: The effect of 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on mixed metastases

No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Control mixed	**-**	**-**	**-**	**-**	**2**	**7**
PRRT mixed	**-**	**-**	**2**	**2**	**2**	**2**

Table 3. Number of animals with given range of metastases, 21 days after direct injection of 50%/50% mixed (CC2B/CC531) tumor cells into the portal vein. Control received no treatmnent. PRRT received 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on day 8.

[1] >100 tumor colonies, but <50% of liver is affected

[2] >100 tumor colonies, and >50% of liver is affected

Ex vivo and in vitro SSR Autoradiography

The results of the ex vivo and *in vitro* autoradiography are summarized in table 4.

Ex vivo autoradiography did not show uptake of residual radioactivity in treated CC531, CC2B and mixed liver metastases. However, *in vitro* autoradiography of CC2B liver metastases tissue of control rats showed high expression of SSRs, in contrast to CC531 liver metastases tissue of control and treated rats (fig. 2). Moreover, when the SSR was blocked by an excess of non-radiolabeled octreotide non-specific binding was low. However, SSR expression of treated rats with CC2B liver metastases had a more heterogeneous uptake in the tumor colonies compared with control rats. This was also the case in control and treated rats with 50%/50% mixed liver metastases.

TABLE 4: Results ex vivo and *in vitro* autoradiography

		ex vivo	*in vitro*
CC2B	controls	n.r.r.a.	+
	PRRT	-	±
CC531/CC2B (mixed)	controls	n.r.r.a.	±
	PRRT	-	±
CC531	controls	n.r.r.a.	-
	PRRT	-	-

Table 4: Summarization of the results of the ex vivo and in vitro autoradiography. n.r.r.a. = no residual radioactivity, - = no SSR expression, ± = heterogeneous SSR expression, + = high SSR expression.

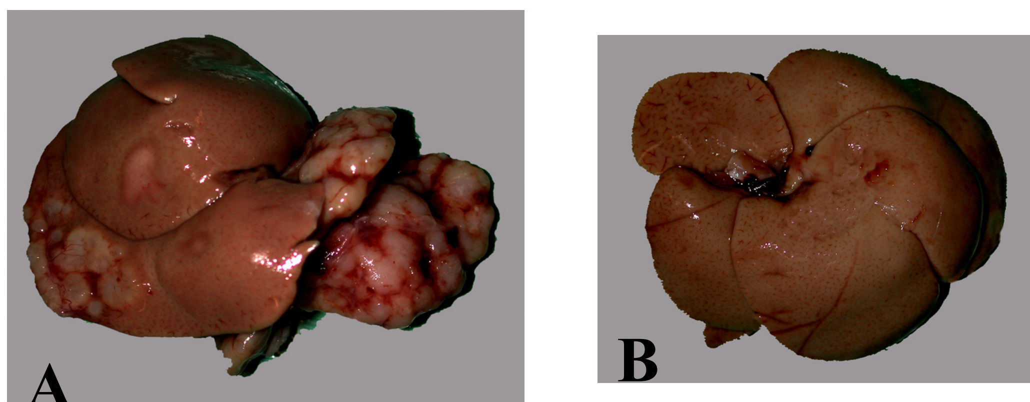

Figure 1: Examples of a liver of a control rat (A) and of a PRRT treated rat (B) with CC2B liver metastases.

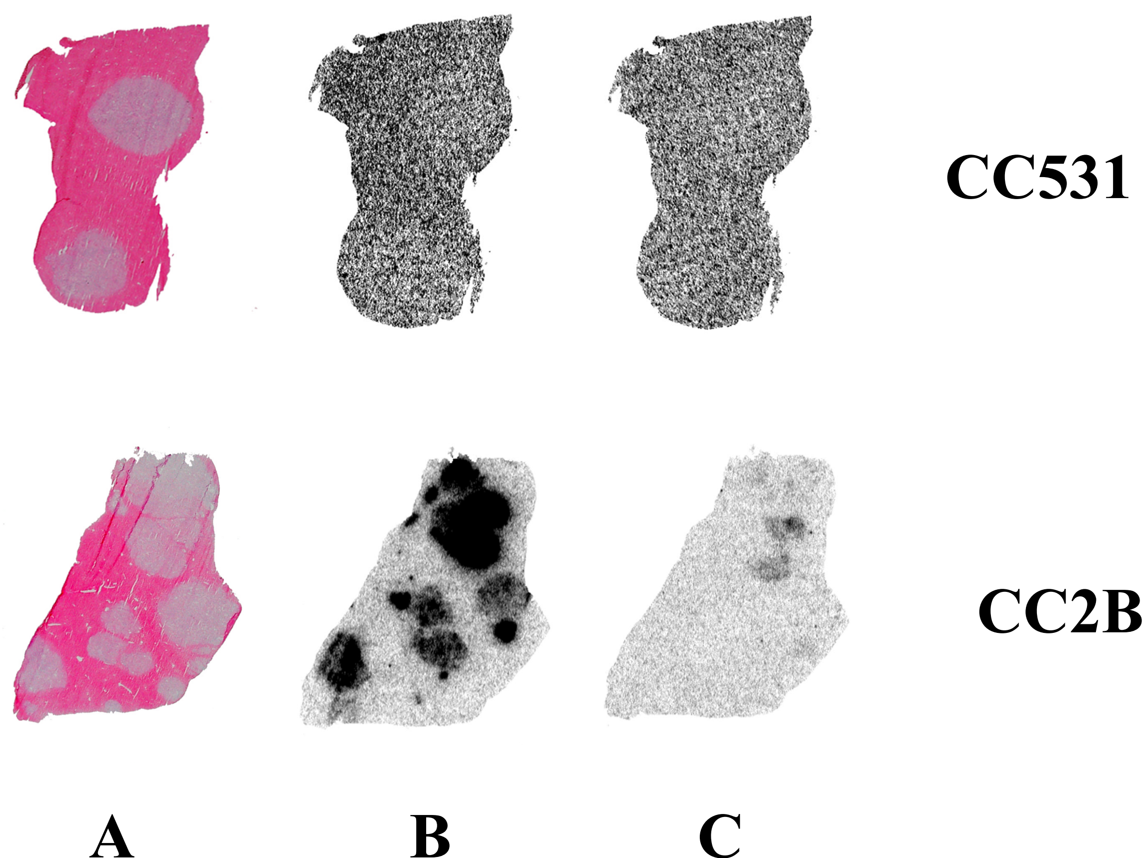

Figure 2: Examples of two frozen slides with liver metastases tissue of control rats with CC531 (up) and CC2B (down) liver metastases, respectively. (A). Hematoxylin-eosin staining shows the localization of the tumor colonies surrounded by normal liver parenchyma. (B). In vitro autoradiography clearly visualizes the SSRs on the CC2B tumor colonies, in contrast to CC531 liver metastases tissue. (C). When the SSR is blocked by an excess of non-radiolabeled octreotide non-specific binding is low.

DISCUSSION

In previous studies, Slooter *et al.* showed strong growth inhibition of SSR positive CA20948 pancreatic liver metastases in rats treated with 370 MBq [^{111}In-DTPA^{0}]octreotide (15; 16). This effect was abolished when the SSR was blocked by an excess of non-radiolabeled octretiode before PRRT treatment, indicating that radiolabeled octreotide was internalized specifically by the SSR. This SSR-mediated process was also underlined by the finding that no anti-tumor effects were seen in PRRT treated rats bearing SSR-negative CC531 colon carcinoma liver metastases. However, recent developments in molecular biology have made it possible to transfect SSR-negative CC531 cells with a SSR-gene, so SSR-mediated PRRT is also applicable in these tumors. We performed an retroviral *in vitro* transfection of tumor cells. The use of retrovirusses for *in vitro* transfection is highly efficient, although it has several drawbacks in its clinical use, like low production of its viral titres in the packaging lines, although currently improving, are still not sufficient high enough for many therapeutic applications, and insertional mutagenesis may also occur, inducing new tumors. However, the use of retroviral vectors has also an advantage in the transduction of liver metastases, while only active dividing cells (tumor cells) are transducted and non-dividing cells (hepatocytes) not, resulting in a highly specific transduction of liver metastases. This advantage can be useful in the future when liver metastases can be transduced, for example by isolated hepatic perfusion with a retroviral vector.

After retroviral *in vitro* transfection of the CC531 cells with a SSR (subtype 2)-gene, this cell line was named CC2B. This newly SSR transfected cell line had a high amount of SSR expression (B_{max} 760 fmol/mg membrane protein) and a high binding affinity (K_d = 0.9 nM) (17). Moreover, we showed that the transfected receptor was also functional as it could internalize radiolabeled octreotide. However, in the same rat liver metastases model we did not see anti-tumor effect after PRRT treatment with 370 MBq [^{111}In-DTPA^{0}]octreotide on CC2B liver metastases, despite the fact that we measured a high uptake of radioactivity inside these SSR-positive liver metastases after PRRT treatment (data not shown). In a previous study, we reported, as measured in a clonogenic assay with external radiation, that CC2B and CC531 are relatively radioresistant in comparison with our positive control tumor, CA20948, in which we previously shown highly effective therapy (17). Consequently, we decided to use another radionuclide than ^{111}In, which emits internal conversion and Auger electrons,

with smaller tissue penetrations (200- 550 μm and 0.02 – 10 μm, respectively) and lower energy (0.15 – 0.24 MeV and 0.025 MeV, respectively). In contrast, ^{177}Lu, a beta-emitting radionuclide, has a larger tissue penetration of a maximum of 2 mm and a E_{max} of 0.5 MeV. In addition, we used another somatostatin analog, namely [Tyr^{3}]octreotate instead of [DTPA^{0}]octreotide. This analog was found to have a very high affinity for the SSR(-type 2) (20), and high uptake of radioactivity in these SSR-expressing tumors were found to be 3 to 4 times higher than [DTPA^{0}]octreotide. De Jong *et al.* showed highly effective therapy with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate in a flank tumor model using the SSR-positive rat pancreatic CA20948 tumor cell line (13). All rats bearing small tumors showed complete regression of tumor mass after a total dose of 555 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. In this study, especially smaller tumors showed better results in comparison with larger tumors. In accordance with these findings, we were interested whether [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate was also feasible, using the same SSR-positive tumor cell line, CA20948, in a rat liver metastases model. A strong anti-tumor effects on CA20948 liver metastases bearing rats was found after treatment with 370 or 185 Mbq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate (manuscript in preparation), which confirmed our hypothesis that [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate is also feasible in this rat liver metastases model. Therefore, we were interested whether [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate could also exert an anti-tumor effect on our relatively radioresistant cell line, CC2B, in this liver metastases model. In our first *in vivo* experiment, we injected a dose of 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate i.v.. The results showed an impressive decrease in tumor score of CC2B liver metastases ($p < 0.001$). So, in contrast to our previous study, in which we injected 370 MBq [^{111}In-DTPA^{0}]octreotide, we were able to obtain an anti-tumor effect on SSR-transfected CC531 cells. The native cell line without SSR, CC531, did not show a decrease in tumor score after treatment with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, again underlining the importance of the SSR in this process. While a significant anti-tumor effect ($p<0.001$) was obtained in the first *in vivo* experiment, we repeated our experiment, but this time we injected half of the dosage [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, namely 185 MBq, in order to evaluate the tumor response with less radioactivity. Again, a (dose-dependent) significant anti-tumor effect was found ($p<0.01$), underlining the strength of the therapy even with lower dosages of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate.

As stated above, ^{177}Lu emits β-particles with a maximum energy of 0.5 MeV (average of 0.133 MeV) and a high tissue penetration, with a maximum range of 2 mm. Assuming a cell diameter of 20 µm, this means, when internalized into the tumor cell, ^{177}Lu is able to potentially kill cells at approximately 12 cell diameters from a cell in which it is internalized, with a maximum cell-killing potential of approximately 50 cell diameters (21). This extra cell killing potential is also called a radiological bystander effect and can be an additional advantage in the concept of gene therapy. As in many gene therapy trials a poor transduction rate *in vivo* results in a poor efficacy of the treatment. A bystander effect is vital to overcome this problem. The third *in vivo* experiment was performed, in order to evaluate the bystander effect of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate in 50% mixed CC2B and CC531, i.e. half SSR-positive and half SSR-negative cell population. Again a significant anti-tumor effect was found ($p<0.05$). Although these results were significant, it is not clear whether the obtained anti-tumor effect is due to the killing of only the SSR-positive cells (CC2B) or in addition a bystander effect also killed surrounding CC531 (SSR-negative) cells. Autoradiographical studies of excised livers were performed, in order to investigate SSR-status of the treated and control tumors. Ex vivo autoradiography, which shows residual radioactivity after PRRT, showed no macroscopically uptake of radioactivity in treated CC2B or 50/50% tumor colonies. However, *in vitro* autoradiography of these slides showed heterogeneous uptake of radioactivity, indicating that both SSR-positive and –negative cells are present in tumor colonies. In contrast, *in vitro* autoradiography of control rats with CC2B liver metastases showed high and uptake of radioactivity, indicating high (homogeneous) SSR expression. These findings suggests that mostly SSR-positive tumor cells are killed after PRRT and surviving tumor cells are mainly SSR-negative clones of the CC2B cell line. Since both *in vitro* autoradiograms of treated and control rats with 50%/50% mixed liver metastases showed macroscopically heterogeneous uptake of radioactivity in almost the same quantity, we conclude that SSR-positive cells were killed after PRRT, and in addition due to the cross-fire of beta-particles of internalized ^{177}Lu also a large quantity of SSR-negative tumor cells were killed, resulting in a radiological bystander effect. However, we can not quantify the amount of uptake exactly, therefore the exact amount of bystander effect can not be calculated.

In all three *in vivo* experiments no overt signs of toxicity were observed after treatment with 185 or 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. Comparable results

in toxicity were found in experiments reported by de Jong *et al.*, in which Lewis rats were injected with 277.5 or 555 MBq [^{177}Lu-DOTA^0, Tyr^3]octreotate (13). In addition, kidney and bone marrow are the critical organs for PRRT with radiolabeled somatostatin analogs and determined the maximal tolerated dose hereof in other studies (data not shown). Moreover, Lewis *et al.* recently reported also no toxicity for [^{177}Lu-DOTA^0, Tyr^3]octreotate, even up to doses of 4 GBq/kg in rats, and up to 42 days post injection (22). High dosages of [^{177}Lu-DOTA^0, Tyr^3]octreotate up to 130 mCi/kg did not give a significant change in platelet counts, hemoglobin, blood urea nitrogen, creatinine or alanine aminotransferase. In the gross necropsy and histopathological examinations of selected tissues, there were no apparent lesions, showing the lack of gross or histological signs of toxicity. The only sign of toxicity was a dose-dependent change of WBC in rats treated with 50 up to 130 mCi/kg. However, this change of WBC recovered in all cases within 35 days. Since our rats received a maximum dose of 370 MBq (= 1480 MBq/kg = 40 mCi/kg), we can conclude that our rats had a impressive tumor response with no overt signs of toxicity. We assume that higher or fractionated dosages may even give better results. Especially, when the bystander effect is concerned, higher and or fractionated dosages may result in more convincing bystander effect compared with our present results in experiment 3.

In conclusion, we found an impressive anti-tumor effect of SSR-transfected liver metastases after treatment with PRRT with both 185 and 370 MBq [^{177}Lu-DOTA^0, Tyr^3]octreotate, while no anti-tumor effect was seen in SSR-negative liver metastases, indicating that this therapy is targeted. Moreover, rats with mixed liver metastases had also significant less liver metastases compared with control rats, which may be caused by a radiological bystander effect of [^{177}Lu-DOTA^0, Tyr^3]octreotate. This proves that the combination of gene therapy followed by PRRT is an interesting concept, that might open possibilities in the treatment of micrometastases of the liver in the future.

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CHAPTER 8

AN-238, A CYTOTOXIC SOMATOSTATIN ANALOG: AN INTRODUCTION

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INTRODUCTION

Chemotherapy has been the most important modality for treatment of advanced or disseminated cancers in the past 50 years[1,2]. Also chemotherapy can be used in an adjuvantal setting after surgery and/or radiotherapy. However, treatment of cancer with conventional chemotherapy is limited by the development of drug resistance in cancer cells, a phenomena called multi-drug resistance (MDR), and toxicity of normal cells[1,2]. There are two options to increase the effectiveness of chemotherapy: modification of drug resistance or more selective delivery. Alterations in signal transduction involving cell differentiation, proliferation and death are involved in the pathogenesis of cancer. Increased understanding of the pathways by which chemotherapeutic drugs interact with mechanisms underlying signal transduction may lead to developments of drugs which may sensitize cancers to chemotherapy. Combinations of chemotherapeutic agents with signal transduction inhibitors or anti-signaling drugs are to be expected to combat MDR in a synergystic or additional manner.

A more selective delivery of the chemotherapy to the tumor cells allows a dose escalation and reduces the systemic toxicity. Methods such as isolated limb or isolated liver perfusion showed more effective treatment of cancer with low toxicity of normal cells[3-5]. However, this method needs invasive procedures before treatment can be started with also higher morbidity and mortality rates. A more preferable method is to target cytotoxic agents to their receptors on tumor cells. Almost 100 years ago Paul Ehrlich received the Nobel Prize in Medicine for his concept of the "magic bullets": based on the assumption that tumor cells possess specific antigens, specific antibodies could deliver toxic compounds to tumor cells and eradicate them[6]. In accordance with this concept the group of Schally *et al.* composed chemotherapy linked to various peptides which binds to their receptors on tumors, like cytotoxic LHRH analogs, cytotoxic analogs of bombesin/gastrin releasing peptide and cytotoxic somatostatin analogs. As with radiolabeled somatostatin analogs, the cytotoxic somatostatin analogs showed impressive anti-tumor effect with low peripheral toxicity in several tumor cell lines expressing SSRs[7-15]. A doxorubicin (DOX) derivate, 2-pyrrolino-DOX (AN-201), was coupled to the somatostatin analog, RC-121 and named AN-238[16]. In this small review the synthesis, mechanism of action and therapeutic application of AN-238 will be discussed.

SYNTHESIS

AN-201 can be formed by reacting DOX with a 5- to 10-fold excess of 4-iodobutyraldehyde in dimethylformamamide. The conversion of DOX takes place within minutes and is virtually 100%. AN-201 was shown to be 500-1000 times more potent than DOX *in vitro*[16].

AN-201 was linked through a glutaric acid spacer to the amino terminal D-Phe residues of the somatostatin carrier, RC-121 and named AN-238 (Fig.1).

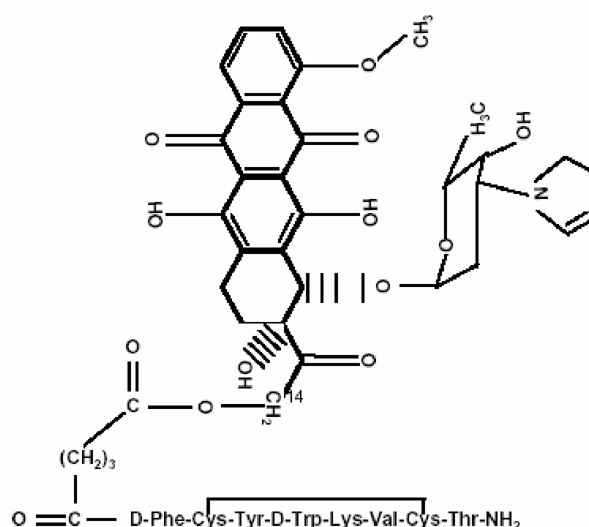

Fig. 1: Molecular structure of AN-238

MECHANISM OF ACTION

Although no tissue distribution test with AN-238 are published in literature, it is believed that these cytotoxic hybrids can accumulate in both normal and cancerous cells expressing SSRs, as demonstrated for radioactive somatostatin analogs[17-19]. After binding and internalization of AN-238[20], the cytotoxic radical may be released in the cells by carboxylesterase enzymes, which are ubiquitous[21]. It is also possible that the cytotoxic radical is released, in part, after binding but before internalization. This mechanism could still produce a higher concentration of the radical in SSR-positive tissue and, in addition, a "bystander effect", affecting SSR-negative neighbouring cells. As a result of the "bystander effect", it is very difficult to demonstrate targeting of these conjugates *in vitro*. Despite such difficulties, recently Schally *et al.* showed a more selective toxicity of AN-238 towards receptor-positive cells in mixed populations of SSR-negative and SSR-positive cells, using cell-line specific microsatellite markers for semiquantitive analysis[9,10].

Moreover, the esterase activity in plasma is an important factor in the efficacy of the treatment with AN-238, while release of radicals (AN-201) can occur in blood before the targeting is completed. A longer half-life of the ester bond allows more binding of AN-238 on the SSRs, leading to more efficient therapy and also leading to less toxic side-effects such as myelodepression. Schally *et al.* showed a half-life of the ester bond in rats of more than 60 minutes[12], while in mice the half life of ester bond was about 20 minutes[19]. Through this mechanism the difference in toxicity patterns between these two species can be explained. In addition, by means of an esterase inhibitor the plasma half-life of the ester bond was extended from 20 to 70 minutes in mice, resulting in a comparable tolerance pattern as in rats[9,22]. The half-life of the DOX-14-O-glutaryl ester bond in human plasma *in vitro* is approximately 2 hours, which allows a more complete targeting of the SSR-positive tumor cells, consequently resulting in a even more effecient treatment in a clinical setting compared to mice-models used by the group of Schally[22].

ONCOLOGICAL STUDIES WITH AN-238

SSRs are identified in various normal and neoplastic tissues, including brain tumors, pituitary tumors, gastro-intestinal tumors, breast cancers, colorectal cancers, prostate cancers and ovarian cancers[23-27]. Impressive results with radiolabeled somatostatin analogs were already shown in a experimental and clinical setting[18,28-32]. The group of Schally tested AN-238 on a wide variety of SSR-expressing tumor cell lines. In Table 1 a short summarization of the results are shown.

Prostate cancer

AN-238 and AN-201 was first evaluated on androgen-independent Dunning R-3327-AT-1 prostate carcinoma in Copenhagen rats. A dose of 300 nmol/kg AN-238 produced a 80% decrease in tumor weight 4 weeks after treatment. In contrast, AN-201 at a dose of 110 nmol/kg showed a weak effect and killed 9 of 10 rats at 115 nmol/kg[12].

Similarly results were shown in nude mice bearing PC-3 human prostate cancers. A single dose of 200 nmol/kg or two injections of 150 nmol/kg AN-238 reduced tumor weight and burden by more than 60%, mainly due to a significant increase of apoptosis in tumor cells. Again AN-201 was ineffective and more toxic[11].

Tumor model	Effect of AN-238 on tumor volume	Effect on AN-201 on tumor volume
Prostatic		
Dunning R-3327-AT-1	40% inhibition at 115 nmol/kg	35% inhibition at 110 nmol/kg
	80% inhibition at 300 nmol/kg	Lethal at 115 - 125 nmol/kg
PC-3		
- subcutaneous	62 and 74% inhibition at 2 x 150 nmol and 200 nmol/kg, resp.	No effect, 3 of 7 mice died at 200 nmol/kg
- orthotopic	77% inhibition at 2 x 150 nmol/kg	34% inhibition at 2 x 150 nmol/kg
- metastases	0 of 6 mice developed metastases	4 of 6 mice developed metastases
Renal		
SW-839	67% inhibition at 3 x 150 nmol/kg	27% inhibition at 3 x 150 nmol/kg
786-0		
- subcutaneous	78% inhibition at 3 x 150 nmol/kg	40% inhibition at 3 x 150 nmol/kg
- orthotopic	87% inhibition at 3 x 150 nmol/kg	35% inhibition at 3 x 150nmol/kg
- metastases	1 of 7 mice developed metastases	5 of 6 mice developed metastases
CAKI-1 (SSR-negative)	no effect at 2 x 150 nmol/kg	no effect at 2 x 150 nmol/kg
Breast		
MCF-7MIII	lasting regression at 250 nmol/kg	no effect at 250 nmol/kg
MDA-MB-231	initial regression at 250 nmol/kg	no effect at 250 nmol/kg
MX-1	5 of 10 mice cured, no deaths	1 of 10 mice cured, 1 death
Brain		
U-87-MG		
- subcutaneous	82% inhibition at 150 nmol/kg	35% inhibition at 150 nmol/kg
	30% shrinkage of very large tumor at 2 x 150 nmol/kg	32% shrinkage of very large tumors at 2 x 150 nmol/kg
- orthotopic	significantly prolonged survival	no significant effect
Lung		
H-69	55% inhibition at 200 nmol/kg	35% inhibition at 150 nmol/kg
	73% inhibition at 3 x 150 nmol/kg	33% inhibition at 3 x 150 nmol/kg
H-157 (SSR-negative)	91% inhibition at 200 nmol/kg	20% inhibition at 200 nmol/kg
	83% inhibition at 2 x 150 nmol/kg	2 x 150 nmol/kg not tested
Ovarian		
UCI-107	61% inhibition at 2 x 150 nmol/kg	34% inhibition at 2 x 150 nmol/kg
- with esterase inhibitor	70% inhibition at 2 x 400 nmol/kg	1 x 400 nmol/kg was lethal
Pancreas		
SW-1990	93% inhibition at 2 x 150 nmol/kg	68% inhibition at 2 x 150 nmol/kg
Colon		
HCT-116	74% inhibition at 2 x 150 nmol/kg	73% inhibition at 2 x 150 nmol/kg
HCT-15	78% inhibition at 2 x 150 nmol/kg	51% inhibition at 2 x 150 nmol/kg
HT-29	74% and 79% inhibition at 2 x 200 and 3 x 150 nmol/kg, resp.	49% and 43% inhibition at 2 x 200 and 3 x 150 nmol/kg, resp.
LoVo (SSR-negative)	38% inhibition at 4 x 150 nmol/kg	41% inhibition at 4 x 150 nmol/kg

Table 1: published results of the effect of AN-238 and AN-201 on various tumor cell lines.

Breast cancer

A number of studies have showed the expression of SSRs on primary breast cancers, especially invasive ductal carcinomas are positive[33]. To prove the effectiveness of AN-238 Kahan *et al.* performed experiments with nude mice bearing various estrogen dependent MCF-7-MIII and estrogen-independent MDA-MB-231 and MX-1 (DOX-resistant) breast cancers xenografts[15]. Single injection i.v. with AN-238 or AN-201 at 250 nmol/kg showed a tumor regression in 3 of 8 mices bearing MCF-7-MIII tumor xenografts treated with with AN-238, in contrast all tumors grew steadily in the group treated with AN-201. In the group with MDA-MB-231 tumors, 4 of 13 mice showed an initial regression after AN-238 treatment for about 2 weeks. Again AN-201 had no anti-tumor effect. In the mice with MX-1 tumors, both AN-238 and AN-201 had tumor inhibition. After 60 days 5 of 10 animals in the AN-238 group and only 1 of 9 animals in the AN-201 group were tumor free, indicating that AN-238 (and AN-201) is also active in DOX-resistant tumor cells.

Brain cancer

Kiaris *et al.* investigated AN-238 treatment in mice bearing U-87 MG human glioblastomas (SSR-positive)[14]. Animals with large subcutaneous xenografts were treated with AN-238 or AN-201 at 150 nmol/kg i.v. At day 19, the group treated with AN-238 showed an 82% tumor growth inhibition compared with controls. After blocking te SSRs with a high dose of RC-160 given prior to treatment this tumor inhibition could be reduced to 37%. AN-201 was not effective. In the same study, two injections of AN-238 at 150 nmol/mg gave also significant tumor inhibition in very large tumors, while AN-201 had no effect. Furthermore, orthopically grown U-87 MG tumors were treated succesfully after AN-238 i.v. injection, indicating that AN-238 can penetrate the blood-brain barrier.

Renal cell carcinoma (RCC)

About 70% of RCCs express SSRs[35]. AN-238 was tested on SW-839, 786-0 (both SSR-postive) and CAKI-1 (SSR-negative) human RCC[10]. As expected, no anti-tumor effect was observed in mice bearing CAKI-1 tumors treated with AN-238. In contrast, the growth of SW-839 and 786-0 was inhibited significantly. In a 786-0 metastastic tumor model, 3 of 7 mice treated with 3 i.v. injections of AN-238 at a dose of 150 nmol/kg biweekly were found to be tumor-free 6 weeks after initiation of treatment, and in 3 other animals the tumor mass was less than 30 mg. In one mouse, a mass of 320 mg was found which did not express SSRs. Moreover, no evidence of lymph nodes metastases were found in the AN-238 treated group,

except the mouse with SSR-negative tumor, while metastases were found in 5 of the 6 animals in the control and AN-201 treated group. Again in all studies, AN-201 was ineffective and more toxic than AN-238.

Pancreatic Cancer

Krenning *et al.* already showed that most endocrine pancreatic cancers can be shown by Octreoscan[18]. Also a tumor growth inhibition effect of CA20948, a SSR-postive pancreatic tumor cell line, is shown by treatment with "cold" octreotide and radiolabeled octreotide[29,30,36]. AN-238 and AN-201 were administered in a nuce mice bearing SW-1990 (SSR-positive) pancreatic cancers[8]. Two injections of AN-238 at a dose of 150 nmol/kg reduced tumor growth by 93% compared with controls, while the same treatment with AN-201 reduced tumor growth with 68%. Pretreatment with RC-160 (high dose) followed by AN-238 treatment reduced the efficacy of AN-238, resulting in an inhibition similar caused by AN-201 (69%).

Moreover, loss of the SSR-type 2 expression in pancreatic cancers could be associated with the proliferation of cells. In order test this theory, Benali *et al.* transfected PC-1.0 pancreatic cancer in Syrian golden hamsters with a SSR-type 2 gene to produce PC-1.0/SSR2 cells[37]. PC 1.0/SSR2 cells grew significantly slower than control PC-1.0 cells in an *in vivo* hamster model. In addition, when hamsters bearing SSR-expressing tumors received AN-238 at 100 nmol/kg i.v., the tumor growth was further slowed down in comparison with untreated PC-1.0/SSR2 tumors. Moreover, AN-238 had no effect on (SSR-negative) PC-1.0 tumors, demonstrating the importance of the SSR for the efficacy of the treatment of AN-238.

Lung Carcinomas

As small cell lung cancer (SCLC) is of neuroendocrine origin, most of the tumors and metastases have SSRs and thus can be visualized with Octreoscan[18]. Also non-SCLC can be visualized with Octreoscan, although the tumor cells itself do not express SSRs. The SSRs visualized by Octreoscan are mainly found in peritumoral tissue, like blood vessels and immune cells.

AN-238 was found to be very effective treatment in mice bearing H-69 SCLC tumors, resulting in a >50% tumor growth inhibition when a single dosage of 200 nmol/kg or two injections of 150 nmol/kg was given. AN-201 was less effective and more toxic[13]. Also mice bearing non-SCLC tumors (H-157), which is SSR-type 2, 3 and 5 negative, showed a 91% tumor grwoth inhibition after treatment with 200 nmol/kg of AN-238 compared with controls.

Again AN-201 was less effective and more toxic than AN-238. In this study it was also showed that AN-238 can also be a target for tumor vasculature, as control tumors showed high expression of SSR-type 2 and 5 after RNA extraction. So even when a tumor itself is SSR-negative it can be treated with AN-238 by targeting of the SSRs on the tumor vasculature.

Ovarian Cancer

Also ovarian cancer can express SSRs [38]. Plonowski *et al.* tested AN-238 in nude mice bearing UCI-107 ovarian cancer[9]. Two injections of 150 nmol/kg AN-238 gave a tumor weight reduction of 67% compared with controls, while AN-201 gave a nonsignificant effect on tumor weight. Moreover, in mice with inhibited serum carboxylesterase activity and very large tumors (>50% larger than the first study), AN-201 at 400 nmol/kg was lethal, whereas AN-238 at a total dose of 800 nmol/kg caused only 22% mortality and reduced tumor weight by 69%.

Colon Cancer

Various studies have indicated that SSRs are present on colon carcinomas[39-41]. AN-238 was tested in four tumor cell lines: SSR positive, HCT-116, HCT-15 and HT-29, and SSR-negative, LoVo. Moreover, HCT-116 and LoVo did express normal p53 tumor supressor gene, while HCT-15 and HT-29 had mutant p53 gene[7]. *In vivo* studies showed that AN-238, AN-201 and also DOX in equitoxic doses inhibited the growth of HCT-116 tumors that express wild-type p53. However, AN-238 also inhibited the growth of HCT-15 and HT-29 cancer that express mutant p53, whereas AN-201 and DOX showed no effect. None of the compounds could suppress the proliferation of LoVo tumors, as it lacks the SSR. This indicates that AN-238 may have a higher therapeutic potential on SSR positive colorectal cancers compared with the non-tageted compounds AN-201 and DOX independently of the p53 status.

CONCLUSIONS

As radiolabeled octreotide, AN-238, showed a impressive tumor growth inhibition in various SSR-positive tumor cell lines, whereas AN-201 was less effective and more toxic. Moreover, the group of Schally showed that AN-238 can be targeted to SSR-negative tumors by targeting of peritumoral vasculature. Although the authors claim that the treatment is targeted no clear blockage experiment was convincingly shown. After blocking the SSRs with a high

dosage of RC-160 almost 50% of the antitumor effect can still be obseved. In addition, no explanation was given by the authors when they observed that AN-238, AN-201 and DOX in equitoxic doses had the same growth inhibition in HCT-116 colon carcinoma tumor model despite the fact that the tumor cell line was SSR-positive.

Most of the preclinical studies, except the first study, were performed in nude mice, so human cancer lines could be tested. However, it would be also interesting to perform more studies in immune-competent animals in order to evaluate anti-tumor effect as a resultant of the immune system as well.

Despite these critics AN-238 seems an interesting option in the treatment of (SSR-positive) cancers.

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CHAPTER 9

THE EFFECT OF TARGETED THERAPY WITH AN-238 ON COLON CARCINOMA CELLS TRANSFECTED WITH A SOMATOSTATIN RECEPTOR IN A RAT LIVER METASTASIS MODEL

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ABSTRACT

In previous studies, the group of Schally *et al.* showed impressive results with the cytotoxic somatostatin analog, AN-238 in various tumors expressing somatostatin receptors (SSRs). AN-238, which consists of 2-pyrrolinodoxorubicin (AN-201) linked to the somatostatin carrier RC121, can be targeted to SSR-positive tumors.

We investigated whether AN-238 could be used for treatment of *in vitro* SSR-transfected colon carcinoma cells (CC2B) in a rat liver metastases model, in which we previously shown highly effective treatment with radiolabeled octreotide.

SSR-positive (transfected) CC2B and their parental SSR-negative CC531 cell line were tested *in vitro* with AN-238, AN-201 and doxorubicin (DOX) with and without addition of cyclosporin A (CsA). Hereafter two *in vivo* experiments were performed in which CC2B or CC531 cells were injected in the portal vein of rats, after which liver metastases were caused. A single injection of AN-238 at 200 nmol/kg or PBS (controls) on day 7 was administered i.v. in the first experiment. In the second experiment two injections of AN-238 at 250 nmol/kg or PBS (controls) on day 5 and 13 was administered i.v. WBC and body weight was measured twice a week to observe toxicity of the treatment.

In vitro exposure of CC531 or CC2B cells with AN-238 showed an equal IC50 for both cell lines, despite the presence of SSRs on CC2B cells. An attempt to make both cell lines more sensitive for AN-201/AN-238 with the p-glycoprotein pump inhibitor, CsA, was not effective. The use of AN-238 *in vivo* had no anti-tumor effect in both experiments. The severe (myelo)toxicity observed in AN-238 treated rats in the second *in vivo* experiment even resulted in a significant higher tumorscore compared with controls.

In conclusion, nor our *in vitro* either our *in vivo* data showed superior anti-tumor effect of AN-238 despite the presence of SSRs on CC2B cells compared with CC531 cells.

INTRODUCTION

Colon carcinoma is the third most common cancer and second leading cause of cancer deaths in the United States[1]. Despite improvements in early diagnosis, surgical techniques, and adjuvant chemotherapy, at least one-third of patients operated "for cure" die of recurrent local disease and metastases[2-4]. Cancer chemotherapy is limited by multidrug resistance (MDR) of tumor cells and toxicity of normal cells[5-7].

Several drugs have been reported to reverse MDR in vitro. One of the most effective reverters is the immunosuppressive drug cyclosporin A (CsA)[8]. Many in vitro studies have shown an increase in cytotoxicity to MDR cell lines when CsA is added to drugs that are affected by the MDR cross-resistance pattern, like doxorubicin, vincristine and colchicines[9-11]. Chemosensitization by CsA is mediated by p-glycoprotein. The cytotoxicity to parental cell lines that do not express p-glycoprotein is not influenced[12].

To increase the effectiveness of chemotherapy doses can be escalated, but toxicity limits the height of the dose. A more selective delivery of chemotherapy to primary tumors and their metastases would allow a dose escalation and reduce the peripheral toxicity[7].

A well established method to deliver selectively chemotherapy to tumor cells is to attach chemotherapeutic compounds covalently to hormones, for which receptors or antibodies are present on tumor cells[13,14] .

Somatostatin receptors (SSRs) are expressed in most neuro-endocrine tumors, but also in lymphomas and breast cancer. The group of Schally *et al.* recently developed a cytotoxic somatostatin analog (AN-238)[15]. AN-238 is composed of the doxorubicin derivate, 2-pyrrolinodoxorubicin (AN-201), and is linked to the somatostatin analog, RC-121. AN-201 is 500 – 1000 times more active *in vitro* than its parent compound[15]. In numerous *in vitro* and *in vivo* studies Schally *et al.* showed strong tumor growth inhibition in various human and rat SSR-postive tumors after treatment with AN-238 with low peripheral toxicity (transient fall of body weight and WBC)[16-26].

The aim of this study was to transfect SSR-negative tumor cells with a SSR-gene in order to make them feasible for therapy with AN-238 in rat liver metastases model.

MATERIAL AND METHODS

Animals

Male inbred Wag/Rij rats, 10 to 14 weeks old and 225 to 250 g, were obtained from Harlan-CPB (Horst, The Netherlands). Animals were kept under standard laboratory conditions (12 hr light/ 12 hr dark) and given a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experimental protocol adhered to the rules of the Dutch Animal Experimental Act and was approved by the Committee on Animal Research of the Erasmus University.

Tumor

CC531 is a SSR-negative, moderately differentiated rat colon carcinoma, induced by 1,2-dimethylhydrazine and is transplantable in syngeneic WAG/Rij rats[27]. The tumor is maintained in tissue culture as a monolayer in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were harvested from stationary cultures by gentle trypsinisation (Boehringer, Mannheim, Germany). A suspension of 0.5 x 10^6 living cells was used for direct injection into the portal vein [1,3].

SSR Transfection and Expression

For expression of the somatostatin receptor-subtype 2 ($sstr_2$) in CC531 cells, human $sstr_2$ cDNA in pBluescript (pBS) (a kind gift of G.I. Bell, Howard Hughes Medical Institute, Chicago, IL, USA) was excised form pBS and inserted into the Nhe-1/Sali cloning site of the retroviral expression vector pCi-neo. Selection was made by the geneticine resistance gene (G418). This vector was used to stable transfect (using lipofectin) CC531 cells. Transfectants were selected and cultured in RPMI 1640 supplemented with 5% FCS and geneticine (0.5 mg/mL) (Gibco, Paisley, UK). Geneticine-resistant clones were examined for their ability to bind with [^{125}I-Tyr^3]octreotide. The clone with the highest expression (Scatchard analysis) of SSR was isolated and named CC2B.

In vitro SSR Autoradiography

In frozen tumor samples SSRs were measured by autoradiography on 10 μm cryostat sections. 10^{-10} M [^{125}I-Tyr^3]octreotide, as a radioligand, was used. Incubation and washing conditions were as described[4]. Non-specific binding was determined by

adding an excess (10^{-6} M) of non-radiolabeled octreotide. Radioactivity was measured 72 hours in a Cyclone Storage Phosphor Screen (Packard Bioscience company, Meriden, USA.The screens were analysed using a Cyclone Phospor Imager (Packard Instruments Co, Groningen, The Netherlands)[28].

Chemicals

AN-238, AN-201, RC121 was a kind gift of Dr. A. Nagy (Tulane University, New Orleans, Louisiana, USA). Cyclosporin A was obtained from Sandoz.(Basel, Switzerland). Doxorubicin (Adriblastina (10 mg / 5 mL) was obtained from Pharmacia and Upjohn (Woerden, The Netherlands).

In vitro cytotoxicity

We determined chemosensitivity *in vitro* by DNA analysis described by Hofland *et al.*[29].

In brief, in 24-well culture plates (Costar, Badhoeverdorp, The Netherlands) 10.000 CC531 or CC2B cells were cultured in 1 mL 1640 RPMI enriched with 1% fetal calf serum (FCS) (Life Technologies, Breda, The Netherlands) and placed in an incubator with a humidified atmosphere of 95% / 5% CO_2 at 37 C. The next day all wells were washed twice with 0.9% NaCl in order to wash away superfluous non-adherent cells. Hereafter, the cells were incubated with the different chemicals in 1 mL medium with 1% FCS in an incubator. Concentration of AN-238 or AN-201 used were: 0.01, 0.1, 1, 10 and 100 nM. Concentration of doxorubicin used were: 0.034, 0.34, 3.4, 34 and 340 μM. Concentration of CsA used were: 0.5, 1 and 1.5 mM. After 72 hours , the plates were collected by washing away superfluous non-adherent cells and kept at –20 C for DNA analysis.

DNA analysis

The total DNA content of each well was measured using bisbenzimide dye (Boehringer Diagnostics, La Jolla, California, USA) as described previously by Hofland *et al.*[29]. The DNA measured represents the total amount of cells per well.

Experimental Procedure

Under ether anesthesia, the abdomen was opened through a 2.5 cm midline incision. Then, 0.5 x 10^6 viable SSR-positive CC2B cells or SSR-negative CC531 cells in 0.5

mL RPMI 1640 were injected slowly into the portal vein through a 0.4 x 12 mm needle. The abdominal wall was closed in one layer by a continuous silk suture[30-32]. The day after the operation, rats were randomized in an experimental and control group.

Experiment 1: Each group consisted of 7 or 8 rats. Rats of the experimental group were treated with 200 nmol/kg AN-238 i.v. on day 7. Rats in the control group were injected with PBS i.v.

Experiment 2: Each group consisted of 8 or 9 rats. Rats of the experimental group were treated with 250 nmol/kg AN-238 i.v. on days 5 and 13. Rats in the control group were injected with PBS i.v.

For both experiments, twice a week body weight were measured and blood samples of all rats were taken by tailcut for WBC.

All rats were sacrificed after 21 days after inoculation of tumor cells. Livers were removed, washed and immersed in PBS. Tumor growth was determined by 2 independent investigators counting the number of metastases on the surface of the liver lobes (up to 100) while blinded for treatment modality. The number of metastases were subdivided in a semi-quantative tumorscore as presented in table 1. Tumor tissue was snap frozen in liquid nitrogen for autoradiography to determine *in vivo* somatostatin receptor status of the tumor

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance. When significant effects were obtained by analysis of variance, multiple comparison were made by the Newmann-Keuls test. Statistical significance was defined as $p < 0.05$.

RESULTS

In vitro cytotoxicity of CC531 and CC2B cells

To evaluate the anti-tumor activity of AN-201 and AN-238 *in vitro* cytotoxicity experiments were performed. Exposure of CC531 or CC2B cells to these chemicals resulted in a dose response curve with an IC50 for AN-201 of 0.9 and 1.0 nM and an IC50 for AN-238 of 0.5 and 0.4 nM respectively (fig 1). Exposure of CC531 or CC2B to DOX resulted in an IC50 of approximately 0.2 µm (fig. 1b). As in previous studies van de Vrie *et al.*[33] showed that CC531 cells have p-glycoprotein pump, which could be inhibited by CsA, therefore we performed these experiments in combination with different doses of CsA, in an attempt to make these cells more sensitive to AN-201/AN-238. A dose depended growth reduction was observed when both CC531 and CC2B cells were exposed to CsA alone with a maximum reduction of 26% at 2 nM (Fig. 1b). However, addition of CsA to AN-201 or AN-238 did not influence the sensitivity of the cells to AN-201 or AN-238 significantly (Fig.1a)

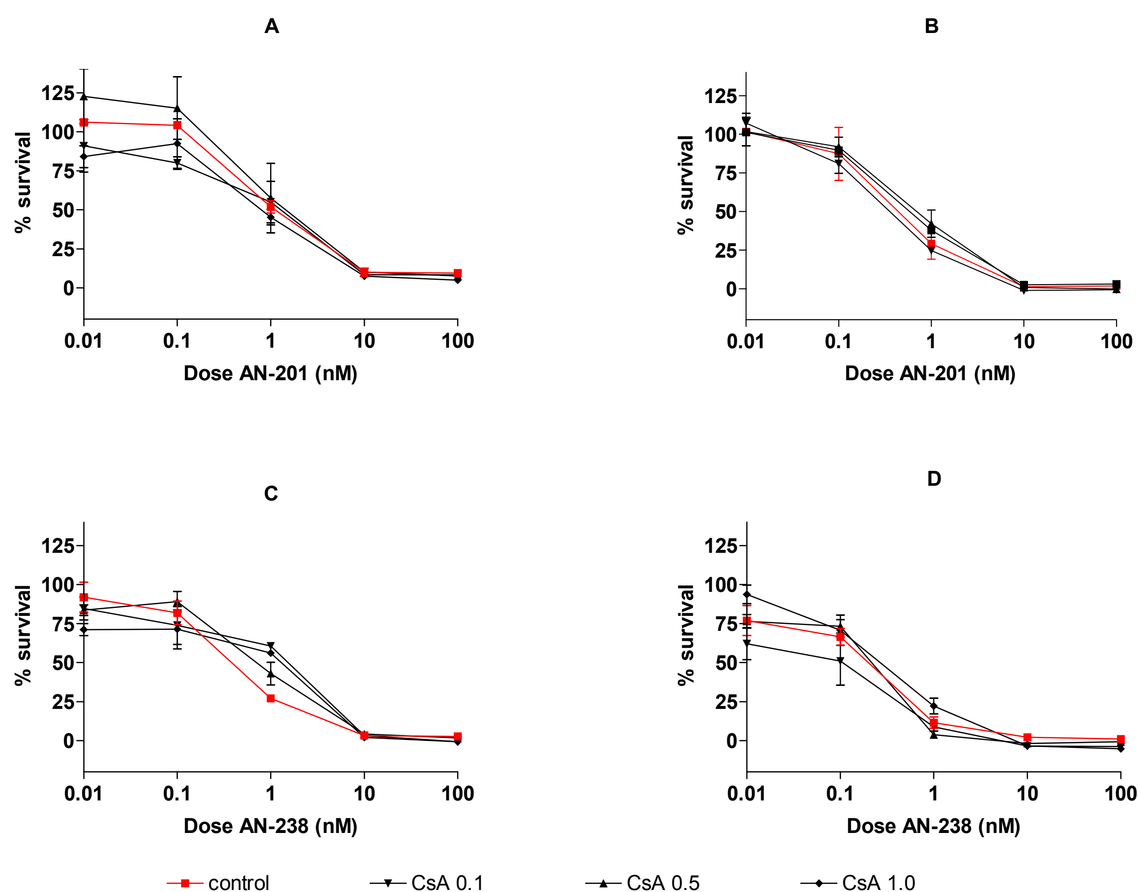

Fig 1a: The effect of AN-201 or AN-238 with or without CsA on CC531 (A + C) and CC2B (B + D)

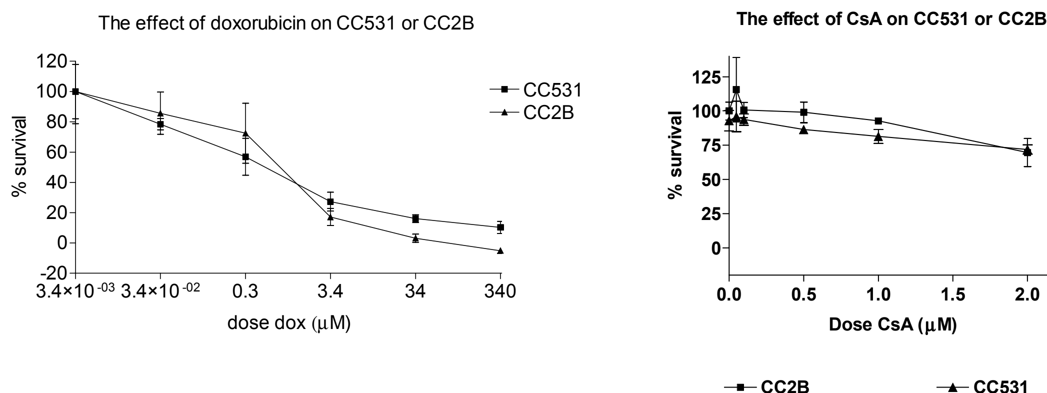

Fig 1b: The effect of DOX on CC531 or CC2B (left) and the effect of CsA on CC531 and CC2B cells (right).

In vivo experiments

The effects of cytotoxic SS analog AN-238 on tumorscore, bodyweight (BW) and WBC in rats bearing CC531 or CC2B liver metastases are shown in table 1 and 2 and figure 1a and b, respectively. In the first experiment, AN-238 was tested in a single dose (200 nmol/kg) i.v. on day 7. As expected, no significant differences in tumorscores were seen between treated rats compared with control rats with CC531 liver metastases (2.00 ± 0.82 *vs.* 1.78 ± 0.83). However, also no significant difference in tumorscores were seen between treated rats compared with control rats with CC2B liver metastases (3.00 ± 1.00 *vs.* 2.75 ± 0.71).

None of the rats died of the toxicity of the treatment. BW in the treated rats was slightly lower than control rats on day 11 (4%) (fig. 2a). On day 12 treated rats had an average decrease of 27% in WBC compared with control rats (fig 2b).

Number of animals with 0 to >100 CC2B metastases						
No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**-**	**3**	**4**	**1**	**-**
AN-238	**-**	**-**	**3**	**1**	**3**	**-**

Table 1: Number of animals with given range of metastases, 21 days after direct injection of CC2B tumor cells in the protal vein. The effect of AN-238 (200 nmol/kg) injected on day 7 is not significantly different (p>0.05) from that of controls.

[1] >100 liver metastases, but <50% of the liver affected

[2] >100 liver metastases, but >50% of the liver affected

Number of animals with 0 to >100 CC531 metastases						
No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**3**	**2**	**2**	**-**	**-**
AN-238	**-**	**2**	**3**	**2**	**-**	**-**

Table 2: Number of animals with given range of metastases, 21 days after direct injection of CC531 tumor cells in the protal vein. The effect of AN-238 (200 nmol/kg) injected on day 7 is not significantly different (p>0.05) from that of controls.

[1] >100 liver metastases, but <50% of the liver affected

[2] >100 liver metastases, but >50% of the liver affected

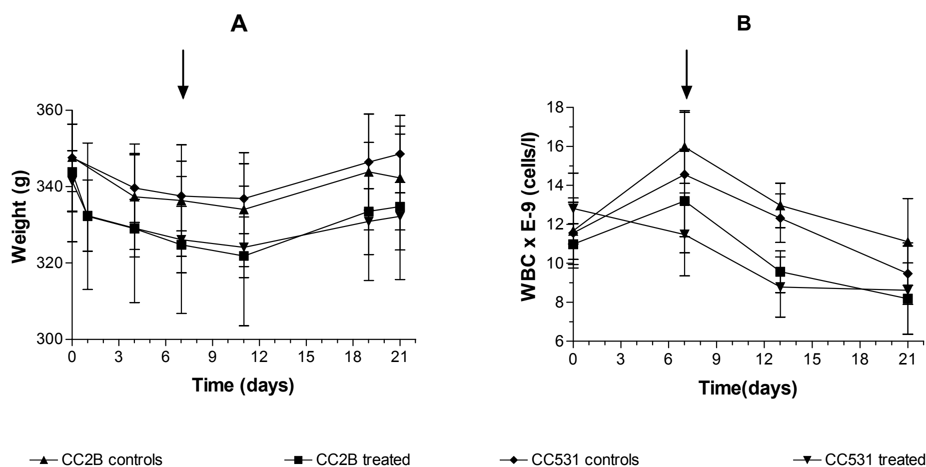

Fig 2: weight (table A) and WBC (table B) of the rats per group during the time of the experiment. ↓ Indicates the day(s) of treatment.

While no anti-tumor effects were seen in experiment 1, we did another experiment, in which we injected two dosages of AN-238 (250 nmol/kg) i.v. on day 5 and day 13 (Table 3 and 4). Again as expected, no significant differences in tumorscores were seen between treated rats compared with control rats with CC531 liver metastases (5.00 ± 0.00 *vs.* 4.33 ± 0.71). Surprisingely, in experiment 2 tumorscores of treated rats were significantly higher (p<0.05) compared with control rats with CC2B.
Two treated rats of the CC2B group died of the toxicity of the treatment on day 21 (just before sacrification). BW in the treated rats was 7% lower on day 9 and 11% lower on day 16 compared with control rats (fig 3a). This type of dosage scheme of AN-238 had severe myelotoxic effects, as shown by an average decrease in WBC of 38% on day 12 and a 78% decrease on day 21 compared with control rats (fig 3b).

Number of animals with 0 to >100 CC2B metastases						
No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**-**	**2**	**3**	**2**	**1**
AN-238	**-**	**-**	**-**	**1**	**1**	**6**

Table 3: Number of animals with given range of metastases, 21 days after direct injection of CC2B tumor cells in the protal vein. Control rats had significantly less liver metastases (P<0.05) compared with rats treated with AN-238 (250 nmol/kg) injected on days 5 and 13.

[1] >100 liver metastases, but <50% of the liver affected

[2] >100 liver metastases, but >50% of the liver affected

Number of animals with 0 to >100 CC531 metastases						
No. of metastases	0	1 – 20	21 – 50	51 – 100	>100[1]	>100[2]
Rank	0	1	2	3	4	5
Controls	**-**	**-**	**-**	**1**	**4**	**4**
AN-238	**-**	**-**	**-**	**-**	**-**	**8**

Table 4: Number of animals with given range of metastases, 21 days after direct injection of CC531 tumor cells in the protal vein. The effect of AN-238 (250 nmol/kg) injected on days 5 and 13 is not significantly different (p>0.05) from that of controls.

[1] >100 liver metastases, but <50% of the liver affected

[2] >100 liver metastases, but >50% of the liver affected

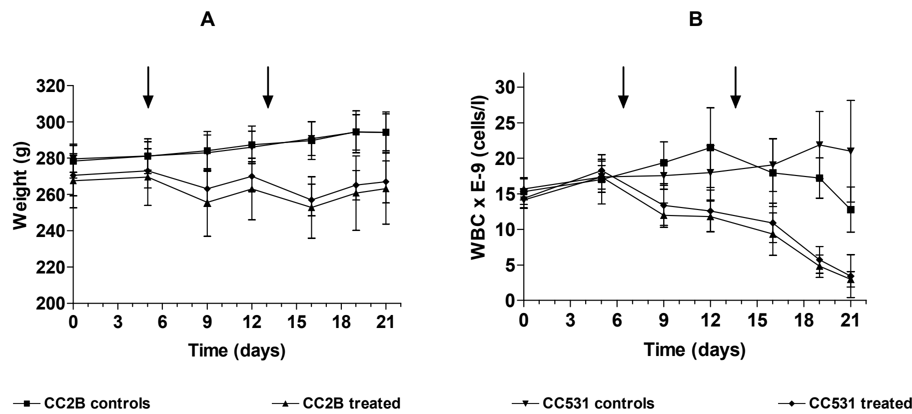

Figure 3: weight (table A) and WBC (table B) of the rats per group during the time of the experiment. ↓ Indicates the day(s) of treatment.

Autoradiography

As figure 4 shows CC2B tumor colonies stain black after addition of [^{125}I-DOTA,Tyr^{3}]octreotate, while no staining occurs on CC531 tumor colonies, indicating that the SSR is highly expressed on CC2B tumor colonies. When the SSR is blocked by an excess of non-radiolabeled octreotide and this experiment is repeated non-specific binding is low.

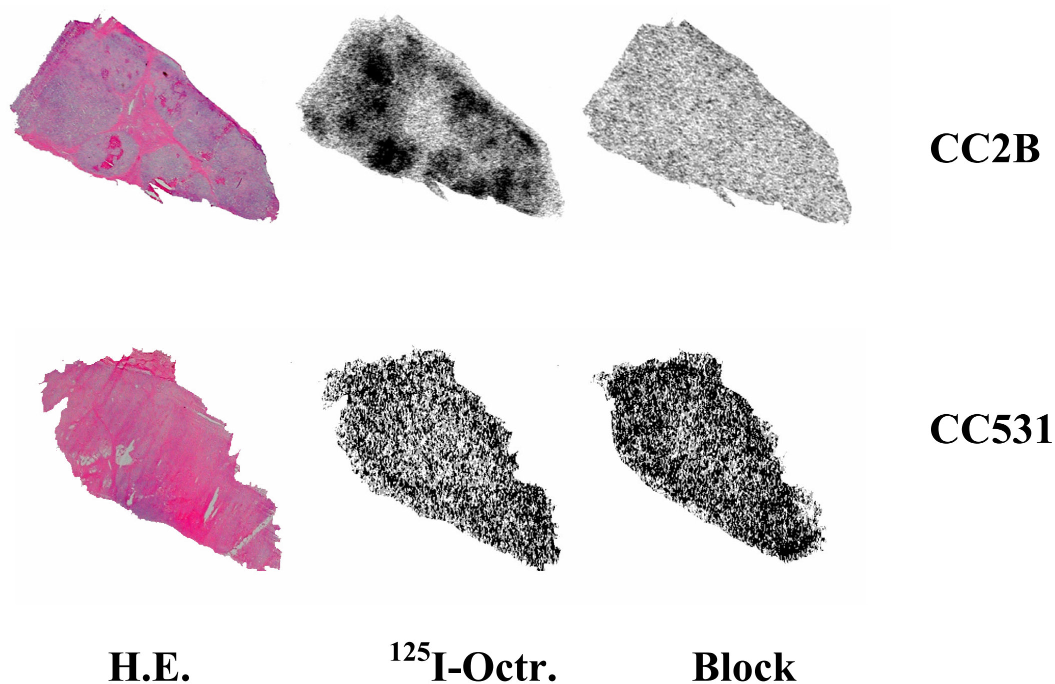

Figure 4: Examples of two frozen slides with liver metastases tissue of control rats with CC531 (down) and CC2B (up) liver metastases, respectively. H.E.: Hematoxylin-eosin staining shows the localization of the tumor colonies surrounded by normal liver parenchyma. ^{125}I-Octr. = In vitro autoradiography clearly visualizes the SSRs on the CC2B tumor colonies, in contrast to CC531 liver metastases tissue. Block = When the SSR is blocked by an excess of non-radiolabeled octreotide non-specific binding is low.

DISCUSSION

In previous studies we showed that therapy with radiolabeled octreotide and other somatostatin analogs could inhibit tumor growth strongly. This mechanism was SSR mediated, and succesfull treatment was only seen in tumors expressing SSRs.

In recent studies chemotherapy targeted to hormone receptors on tumors was also used to achieve a more effective and selective drug delivery to these tumors and thereby reducing peripheral toxicity. Since treatment with radiolabeled octreotide was highly effective in SSR transfected tumors, we wanted to investigate whether treatment with AN-238, a doxorubicin derivate linked to a SS analog, was also feasible in our tumor model. Moreover, in previous studies, performed by the group of Schally, AN-238 was used in two or more different tumor cell lines (SSR-positive or –negative). The authors concluded that the obtained anti-tumor effect of AN-238 was the result of the expression of the SSRs on the tumor cell lines, since the SSR-positive tumor cell lines showed a significant anti-tumor effect after treatment of AN-238, in contrast to the SSR-negative tumor cell lines. However, the differences in anti-tumor effect might also be attributed to the fact that two or more different tumors were used, which can have differences in degrees of chemosensitivity. In order to overcome this problem a SSR-negative cell line can be transfected with a SSR-gene, after which AN-238 can be tested in this SSR-negative and –positive cell line with the same chemosensitivity, resulting in a honest comparison.

Therefore, we transfected CC531 (SSR-negative) cells with a SSR-gene, so CC2B (SSR-positive) and CC531 cells could be compared in one tumor model.

First the *in vitro* cytotoxicity of AN-238 and AN-201 was tested. Both CC531 as CC2B showed a dose depended growth inhibition after addition of the chemotherapeutical drugs. Interestingly, AN-238 had a lower IC50 than AN-201 for both cell lines, despite the fact that CC531 is SSR-negative. In comparison to the cell lines used in a *in vitro* study by Nagy *et al.*[15], the chemosensitivity of these cell lines was slightly better compared to our cell lines (IC50 ranged from 0.18 and 0.5 nM for both AN-201 and AN-238). In our data no advantage of the SSR expression was seen, while AN-238 had no significant difference in IC50 for CC531 and CC2B. As our chemotherapeutics were incubated for three days, we hypothesised that maybe the chemotherapeutical compounds may diffuse direcly into the cell in such a long incubation period in stead of a receptor mediated internalisation. In order to investigate whether a shorter incubation period could show a SSR mediated process,

we repeated our experiment, but this time the incubation period of the chemotherapeutical drugs was just 15 or 30 minutes. In this condition again no significant differences in IC50 could be shown (data not shown). Incubation of our tumor cells with DOX resulted in an IC50 of 0.2 µm, which means that AN-201 is about 200 – 400 times more potent *in vitro* compared with DOX. In contradictory, Nagy *et al.* claimed that AN-201 was 500 – 1000 times more potent than DOX. One of the reasons why we found a less potent factor may be due to the p-glycoprotein pumps, that CC531 cells contain. In order to make our cell line more sensitive for AN-201 (and also AN-238), we performed another *in vitro* study, in which AN-201 and AN-238 was tested with and without addition of different doses CsA, a strong p-glycoprotein pump inhibitor[34]. However, no additional significant influence of CsA on the chemosensitivity of the tumor cells to AN-201 or AN-238 was found.

Despite the fact no specific receptor mediated therapy could be shown *in vitro*, we performed an *in vivo* experiment, in order to elucidate whether a SSR mediated process could be shown in this case. In our first *in vivo* experiment no anti-tumor effect was seen with a single injection of AN-238 at 200 nmol/kg on day 7. In most of the *in vivo* studies performed by the group of Schally *et al.* strong anti-tumor effects were obtained in comparable dosages schemes[16-26]. While no anti-tumor effects were seen and the *in vitro* data showed a relatively poor chemosensitivity, we decided to perform another *in vivo* experiment, in which we escalated the dosages scheme to two injections of AN-238 at 250 nmol/kg on day 5 and day 13. Again, no anti-tumor effect was seen. Interestingly, even higher tumorscores of treated rats with CC2B liver metastases were seen compared with control rats. This can be attributed to the fact that CC531 (as CC2B) is an immunogenic tumor and a severe myelotoxic effect was seen in this dosage-scheme, resulting in a an immunosupressive status of the treated rats. Similar results were reported by van de Vrie *et al.*[35], who showed enhancement of locoregional metastases after treatment with an immunosupressive drug, cyclosporin A. Also the treated rats with CC531 liver metastases in our second *in vivo* experiment had higher tumorscores, although these scores were not significant different, probably due to the fact that all rats in this group already had a maximum tumorscore of 5 and no higher tumorscore could be given to these treated rats. While no effect was seen in the treated rats, we evaluated the SSR-status of the SSR-transfected liver metastases by an autoradiographical study. Clearly, a high expression of SSR was shown in CC2B liver metastases, which also could be specifically

blocked by non-radiolabeled octreotide. As expected, no SSR were visualized in CC531 liver metastases. Moreover, we previously showed that the transfected SSR was also functional *in vitro* and *in vivo*, as it could internalize radiolabeled octreotide[36].

A possible explanation why the treatment with AN-238 did not work in our model, might be attributed to the serum levels of carboxylesterases (CE). AN-201 is a cytotoxic doxorubicin derivate, which is linked to the somatostatin analog, RC-121. In these conjugates, the glutaric acid spacer is linked to the cytotoxic radicals through an ester bond that is sensitive to hydrolysis by CE in blood. However, it is known that difference in enzymatic activity can be different in various animals and species. In a preliminary study Nagy *et al.* reported different t½ of AN-152, an LH-RH analog containing doxorubicin, in the serum of nude mice, Copenhagen rats, and men was about 10, 30 and 120 minutes, respectively[37]. These difference were based on differences in enzymatic activity of CE in the various animals. The enzymatic activity of CE in WAG/Rij rats, used in our experiments, is not known, but a high enzymatic activity can lead to hydrolysis of the ester bond, leading to a toxic dose of AN-201, which is not targeted anymore, consequently leading to severe myelotoxic effects and enhancement of tumor growth. This can also explain why reasonable antitumor effects were seen *in vitro*, comparable with *in vitro* results of the group of Schally *et al.*, which surprisingly were not observed *in vivo*. Diisopropyl fluorophosphate (DFP) can inhibit CE. Nagy *et al.* showed significantly prolonged t½ of AN-152 and less toxic effects of AN-238 in nude mice after addition of DFP[37]. In future experiments addition of DFP to AN-238 may also lead to a more effictive treatment in WAG/Rij rats.

Another reason why our results were not comparible with the group of Schally *et al.*, may be due to a difference in chemosensitivity of the used cell line. Although, *in vitro* data showed slightly less chemosensitivity, Geutskens *et al.* showed a large in-frame deletion, with junctions in exon 4 and 8, lacking the entire DNA-binding domain of the p53-gene[38]. Moreover, no wild-type p53 allele was retained and functional analysis showed that the mutated protein could repress the function of wild-type p53 protein. These findings may explain that our used cell line is relatively chemoresistant and consequently the obtained results of our experiments were poor.

In conclusion, our *in vitro* and *in vivo* results showed no clear SSR-mediated process of AN-238 on the CC2B cell line, despite the presence of SSRs. In contrast, therapy

with [^{177}Lu-DOTA^{0}-Tyr^{3}]octreotate resulted in highly effective anti-tumor activity in the same rat liver metastases model with the same CC2B cell line.

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CHAPTER 10

SUMMARY, CONCLUSIONS AND FUTURE ASPECTS

SUMMARY

Chapter 1 is the general introduction of this thesis. An overview of somatostatin, the effect of somatostatin on cell growth, somatostatin receptors (SSRs) and principles of gene therapy, concerning vectors, targeting and cancer gene therapy strategies is given.

In **Chapter 2** the aims of the thesis are summarized.

SSRs have been found on a variety of neuroendocrine tumors like carcinoids, paragangliomas, as well as on most pancreatic endocrine and breast tumors. SSR scintigraphy with a radionuclide labeled somatostatin analog, [^{111}In-DTPA^{0}]octreotide (Octreoscan®), is a sensitive and specific technique to visualise *in vivo* the presence of SSRs on various tumors. **Chapter 3** is an introduction of Octreoscan®. Both diagnostic, but also therapeutic purposes are discussed. Material was identified from previous review articles, references citated in original papers and a Medline search of the literature. Additional material was obtained from recently published abstracts of meetings.
SSR imaging of neuroendocrine tumors is essential in the diagnostic work up for most of these tumors. The expression of SSR *in vivo* not only predicts the outcome of somatostatin analog treatment but also opens the possibility for new therapeutic strategies. Since better information about spread of the disease can be obtained, more justified therapy options can be proposed, like Peptide Receptor Radionuclide Therapy (PRRT). PRRT results in a highly effective treatment for SSR-positive tumors in both experimental but also clinical setting.

Unfortunately, not all human tumors express SSRs on their cell surface, thus consequently PRRT is not applicable for these tumors.
However, recent developments in molecular biology have made it possible to transfect the SSR-gene on SSR-negative tumor cells. In **Chapter 4**, SSR-negative colon carcinoma cells (CC531) were transfected with a SSR-gene *in vitro* and named CC2B hereafter. Scatchard analysis showed high expression of SSRs and a high binding affinity of CC2B cells. Moreover, the transfected SSR was also functional as it could internalize radiolabeled octreotide. Growth speed and other growth characteristics of

CC2B did not differ significantly *in vitro* compared with their parental tumor cell line CC531.

All the above mentioned features of the CC2B cell line fulfil to the conditions to apply this SSR-transfected cell line in an *in vivo* model to perform future experiments in which PRRT will be tested.

Chapter 5 evaluates the effect of PRRT with 370 MBq (0.5 μg) [^{111}In-DTPA^{0}]octreotide on SSR-negative CC531 and SSR-transfected CC2B liver metastases in the rat.

Liver metastases were caused by injection of CC531 or CC2B cells in the portal vein in rats on day 0. On day 7 in half of the rats 370 MBq (0.5 μg) [^{111}In-DTPA^{0}]octreotide was administered, while the other half of the rats did not receive treatment. On day 28 all rats were sacrificed so tumorscores could be counted.

No significant difference in tumorscore was seen in rats treated with PRRT *vs.* control animals with CC531 liver metastases. However, also no significant diffence in tumorscore was seen in CC2B liver metastases (PRRT *vs.* control). Autoradiography confirmed high SSR expression in CC2B liver metastases tissue, while absent in CC531 liver metastases. High uptake and retention of radioactivity in CC2B tumor was measured, indicating that the transfected receptor was functional. Moreover, the measured radioactivity and radiation dose was comparable with our positive control tumor CA20948, indicating that at our given dosage CC2B is not radiosensitive, which we could demonstrate in a clonogenic assay of our tumor cell lines treated with external radiation.

Although no antiproliferative effect of PRRT was seen on SSR transfected CC531 cells, we conclude that (1) SSR adherence, (2) internalization and (3) retention of radioactivity in the tumor cell did take place, indicating we transfected a functional receptor, but unfortunely the cell line was radioresistant to the given dosages.

^{177}Lutetium is a β-emitting radionuclide, which emits β-particles in a range of approximately 2 mm with high energy (0.5 MeV). As a result of this higher energy and consequently larger tissue penetration, this radionuclide could be more advantegeous compared with ^{111}Indium for PRRT in the treatment of micrometastases. As most SSR-positive tumors are heterogeneous in expression the use of ^{177}Lu in PRRT results in radiation of these SSR-negative clones after the

radionuclide is internalized in neighbouring SSR-positive clones of the tumor. In addition, the use of octreotate in stead of octreotide results in a 3 to 4 times higher uptake in SSR-positive tumors, which consequently may lead to a more effective antitumor activity as well.

In **Chapter 6**, a study is presented in which the effect of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate on SSR-positive CA20948 rat pancreas carcinoma cells in an *in vitro* and *in vivo* experiment was evaluated.

CA20948 (SSR-positive) cells was injected in the portal vein of rats, and subsequently liver metastases developed. Treated rats (185 or 370 MBq [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate) had significant less liver metastases compared to control rats ($p<0.001$) 21 days after injection of tumor cells. Moreover, treated rats showed a significant increase in survival ($p<0.05$). Finally, an *in vitro* PRRT single cell model with [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate showed also a dose-dependent toxicity of CA20948 in contrast to SSR-negative CC531 tumor cells, underlining the importance of the SSR with this kind of therapy.

In conclusion, [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate was shown to be a very promising new treatment modality for disseminated SSR-positive tumors

As described in Chapter 5, PRRT with [^{111}In-DTPA^{0}]octreotide was not effective in SSR-transfected liver metastases, because the transfected cell line was relatively radioresistant to our given dosages. Chapter 6 clearly shows the advantages of [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate, a β-emitting radionuclide with higher energy compared to ^{111}Indium, for the treatment of SSR-positive liver metastases. The aim of the study, described in **Chapter 7**, was to evaluate the effect of [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate on SSR-transfected colon carcinoma cells in a rat liver metastases model. Liver metastases were developed after intraportal injection of these tumor cells (CC2B/CC2B) in rats. On day 7, animals were treated with 185 or 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, while control animals did not receive treatment. After 21 days rats were sacrificed and liver metastases were counted.

Treatment with 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate showed an impressive anti-tumor response in rats with CC2B liver metastases (SSR-positive) in comparison to controls ($p<0.001$), while no significant anti-tumor effect was seen in PRRT treated rats with CC531 liver metastases (SSR-negative). Also a (dose-dependent) tumor response was seen in rats with CC2B liver metastases treated with lower dose, 185

MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate compared with controls ($p<0.01$). In addition, rats with mixed liver metastases (half SSR-positive/half SSR-negative) treated with 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate had significant less metastases compared with controls ($p<0.05$), which may be due to a radiological bystander effect of [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. This phenomenon is beneficial in the concept of *in vivo* gene therapy.

AN-238, a cytotoxic somatostatin analog, is made of 2-pyrrolinodoxorubicin (AN-201) linked to the somatostatin analog, RC-121. AN-201 is claimed to be 500-1000 times more potent than doxorubicin *in vitro*. In **Chapter 8** an overview of the current literature about the cytotoxic somatostatin analog, AN-238, is given. Synthesis, mechanism of action and oncological studies with AN-238 performed by the Schally group are discussed.
Summarizing, just like PRRT, AN-238 also had impressive tumor growth inhibition on various SSR-positive tumor cell lines.

As impressive results were shown with AN-238 on various SSR-positive tumors, we were curious whether AN-238 was also effective in the treatment of SSR-transfected liver metastases, in which we previously shown highly effective treatment with PRRT. This was evaluated in **Chapter 9**.
SSR-positive (transfected) CC2B and their parental SSR-negative CC531 cell line were tested *in vitro* with AN-238, AN-201 and doxorubicin (DOX) with and without addition of cyclosporin A (CsA). Hereafter two *in vivo* experiments were performed in which CC2B or CC531 cells were injected in the portal vein of rats. A single injection of AN-238 at 200 nmol/kg or PBS (controls) on day 7 was administered i.v. in the first experiment. In the second experiment two injections of AN-238 at 250 nmol/kg or PBS (controls) on day 5 and 13 was administered i.v.. WBC and body weight was measured twice a week to observe toxicity of the treatment.
In vitro exposure of CC531 or CC2B cells with AN-238 showed an equal IC50 for both cell lines, despite the presence of SSRs on CC2B cells. An attempt to make both cell lines more sensitive for AN-201/AN-238 with the p-glycoprotein pump inhibitor, CsA, was not effective. The use of AN-238 *in vivo* had no anti-tumor effect in both experiments. The severe (myelo)toxicity observed in AN-238 treated rats in the

second *in vivo* experiment even resulted in a significant higher tumorscore compared with controls.

In conclusion, nor our *in vitro* either our *in vivo* data showed superior anti-tumor effect of AN-238 despite the presence of SSRs on CC2B cells compared with CC531 cells. These results are discrepant with the data of the group of Schally *et al.*.

CONCLUSIONS

- *In vitro* SSR-transfection of CC531 is possible. After transfection a high expression of functional SSRs with a high binding affinity is found.
- PRRT with [^{111}In-DTPA^{0}]octreotide is not effective for SSR-transfected CC531 cells in a rat liver metastases model, as the cell line is relatively radioresistant.
- PRRT with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate is highly effective for SSR-positive CA20948 cells in a rat liver metastases model. Treatment of animals resulted in an increased survival.
- PRRT with [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate is highly effective for SSR-transfected CC531 cells in a rat liver metastases model. Even in mixed (SSR-positive/SSR-negative) tumors PRRT with this agent is effective, probably due to a radiological bystander effect.
- Targeted therapy with the cytotoxic somatostatin analog, AN-238, is not effective for SSR-transfected CC531 cells in a rat liver metastases model. Nor *in vitro* nor *in vivo* any SSR-targeting of AN-238 could be shown.

FUTURE ASPECTS

As stated above, SSR gene therapy combined with PRRT seems to be a promising strategy for the treatment of liver metastases. In our studies the SSR-gene was transfected in vitro. However, if this method wants to have clinical relevance, SSR gene therapy has to be performed in vivo. In this case the hypotheses is applicable that, if higher transduction rates are achieved in vivo better response of the gene therapy is to be expected. This can be achieved through many factors, such as better vectors with the use of specific promotors. These promotors could enhance transduction rates in metastases tissue specifically. Also methods in which the vector is delivered locally, such as isolated hepatic perfusion or intrahepatic artery infusion,

could contribute to higher transduction rates, consequently leading to better results of the treatment. The developments in gene therapy are currently very rapid. With every month more than hunderds of newly published articles, in which new methods and strategies of gene therapy are described, improvements in this field are to be expected in the near future.

For PRRT, besides ^{111}In and ^{177}Lu, other radionuclides such as ^{90}Y (Yttrium) have also been proposed for coupling to somatostatin analogs. ^{90}Y, with a half-life of 2.7 days, is a pure high-energy β emitter with a tissue range up to 12 mm. ^{177}Lu works optimally in small tumors where ^{90}Y is better for larger tumors[1,2]. In a study combinations of these two radionuclides in PRRT resulted in a significant longer survival in rats bearing small and large tumors simultaneously, mimicing the clinical situation, in which small and large metastases are usually present in the same patient[3]. Moreover, also new stable somatostatin analogs with high affinity for different SSRs are currently being developed. An example is [DOTA, 1-NaI^3]octreotide, which has a high affinity for sst_1, sst_3 and sst_5[4]. Through this compound PRRT can also bind to tumors expressing sst_3 and sst_5, which receptors can not bind with such a high affinity on octreotide or octreotate.

A major problem in PRRT is caused by the uptake of radioactivity in the kidneys: small peptides in the blood plasma are filtered through the glomerular capillaries in the kidneys and subsequently reabsorbed by and retained in the proximal tubular cells. Renal uptake of the radioactivity can be reduced by 40 percent by intravenous administration of a combination of L-lysine and L-arginine. Clinically PRRT with somatostatin analogs should be carried out with co-infusion of these aminoacids[5]. Other methods to reduce toxicity are currently under investigation.

Also the concept of surgery in combination of PRRT is an interesting option in the treatment of SSR-positive tumors. The use of radiolabeled octreotide may be considered in an adjuvantal setting to eradicate occult metastases, possibly originating from tumor spill after surgery. Starting therapy, directly after surgery is favourable, because of the small tumor burden. However, the application of PRRT can be also given at a later stage. For example, in patients with non-resectable SSR-positive tumor(s), PRRT can be applied as a first step to reduce tumor size or number, followed by surgery.

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SAMENVATTING

Hoofdstuk 1 is de algemene introductie van dit proefschrift. Er wordt een overzicht over somatostatine, het effect van somatostatine op celgroei, somatostatinereceptoren (SSRs) en principes van gentherapie, betreffende vectoren, targeting en kanker gentherapie-strategieen gegeven.

In **Hoofdstuk 2** zijn de doelstellingen van dit proefschrift samengevat.

SSRs worden op verscheidene neuroendocriene tumoren, zoals carcinoiden, paragangliomen, maar ook op de meeste endocriene alvleesklier- en borsttumoren, gevonden. SSR scintigrafie met behulp van radionuclide gelabelde somatostatine analoog, [^{111}In-DTPA^{0}]octreotide (Octreoscan®), is een sensitieve en speciefieke techniek om de aanwezigheid van SSRs op vershillende tumoren te visualizeren. **Hoofdstuk 3** is een introductie over Octreoscan®. Zowel diagnostische maar ook therapeutische doeleinden worden besproken. Materiaal werd geidentificeerd uit voorgaande review-artikelen, geciteerde referenties van originele stukken en een Medline-zoekopdracht van literatuur. Additionele materiaal werd gebruikt uit recent gepubliceerde abstracts van congressen.
SSR imaging van neuroendocriene tumoren is essentieel in de diagnostische uitwerking van de meeste van deze tumoren. De expressie van SSRs *in vivo* voorspelt niet alleen de uitkomst van behandeling met een somatostatine analoog, maar geeft ook mogelijkheden om nieuwe therapeutische strategieen te gebruiken. Aangezien betere informatie over de uitgebreidheid van de ziekte verschaft kan worden, kunnen meer verantwoorde behandelingsmogelijkheden voorgesteld worden, zoals Peptide Receptor Radionuclide Therapie (PRRT). PRRT resulteert in een zeer effectieve behandeling van SSR-positieve tumoren in zowel experimentele maar ook klinische kaders.

Helaas brengen niet alle humane tumoren SSRs tot expressie op hun celoppervlak, waardoor behandeling middels PRRT voor deze tumoren ook niet toepasbaar is.
Echter is het tegenwoordig zeer goed mogelijk door recente ontwikkelingen in de moleculaire biologie om de SSR-gen op SSR-negatieve tumoren te transfecteren. In **Hoofdstuk 4** worden SSR-negatieve colon carcinoom cellen (CC531) *in vitro* getransfecteerd met een SSR-gen, waarna ze CC2B genoemd worden. Scatchard

analyse van CC2B cellen laat een zeer hoge expressie van SSRs en een hoge bindingsaffiniteit zien. Verder wordt aangetoond dat de getransfecteerde SSR ook functioneel is, aangezien het radiogelabeld octreotide kan internaliseren. Groei snelheid en groeikarakteristieken van CC2B cellen zijn *in vitro* niet significant verschillend ten opzichte van hun moeder tumor cellijn, CC531.
De bovengenoemde kenmerken van CC2B voldoen aan alle voorwaarden om deze SSR-getransfecteerde tumor cellijn te gebruiken in een *in vivo* model, waarbij toekomstige experimenten met PRRT uitgetest zullen worden.

Hoofdstuk 5 evalueert het effect van PRRT met 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide op SSR-negatieve CC531 en SSR-positieve CC2B levermetastasen. Levermetastasen werden veroorzaakt door injectie van CC531 of CC2B cellen in de poortader in ratten op dag 0. 370 MBq (0.5 µg) [^{111}In-DTPA^{0}]octreotide werd op dag 7 aan de helft van de ratten toegediend, terwijl de andere helft van de ratten geen behandeling kregen. Op dag 28 werden alle ratten opgeofferd, zodat de tumorscore vastgesteld kon worden.
Er werden geen significante verschillen in tumorscore gevonden in PRRT-behandelde ratten ten opzichte van de controle ratten met CC531 levermetastasen. Echter ook werden er geen significante verschillen in tumorscore gevonden in ratten met CC2B levermetastasen (PRRT *vs.* Controle). Autoradiografie bevestigde een hoge expressie van SSRs op CC2B levermetastasen, terwijl deze afwezig was in CC531 levermetastasen. Een hoge opname en retentie van radioactiviteit in CC2B tumoren werd gemeten, hetgeen aantoont dat de getransfecteerde receptor functioneel was. Tevens was de opgemeten radioactiviteit en toegediende doses radiatie vergelijkbaar met onze positieve controle tumor, CA20948, hetgeen aantoont dat CC2B niet radiosensitief is voor de door ons gegeven dosis. Dit werd tevens bevestigd in een clonogene assay van onze tumorcellen die door externe radiatie bestraald waren.
Alhoewel er geen antiproliferatief effect van PRRT gezien werd van SSR-getransfecteerde CC531 cellen, kunnen we concluderen dat (1) SSR aanhechting, (2) internalisatie en (3) retentie van radioactiviteit in de tumorcel wel plaats vond, hetgeen aantoont dat we een functionele receptor getransfecteerd hebben, maar onfortuinlijk de gebruikte cellijn radioresistent bleek te zijn voor de door ons gegeven dosis.

In **Hoofdstuk 6** wordt een studie gepresenteerd, waarbij het effect van [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate op SSR-positieve CA20948 rat pancreas carcinoom cellen in zowel een *in vitro* als *in vivo* model geevalueerd werden.
^{177}Lutetium is een β-emitterende radionuclide, die β-partikels in een straal van ongeveer 2 mm met hoge energie (0.5 MeV) uitstraalt. Als gevolg van deze hogere energie en tevens grotere weefsel penetratie kan dit radionuclide voordeliger zijn dan ^{111}Indium voor PRRT bij de behandeling van micrometastasen. Aangezien de meeste SSR-positieve tumoren heterogeen in expressie zijn, resulteert het gebruik van ^{177}Lu bij PRRT in bestraling van deze SSR-negatieve klonen, nadat het radionuclide geinternaliseerd is door naburige SSR-positieve klonen van de tumor. Tevens zorgt het gebruik van octreotate in plaats van octreotide voor een 3 tot 4 maal hogere opname in SSR-positieve tumoren, wat ook tot een meer effectievere antitumor activiteit kan leiden.
CA20948 (SSR-positief) tumor cellen werden in de poortader van ratten geinjecteerd, zodat levermetastasen veroorzaakt werden. 21 dagen na inductie van de levermetastasen hadden behandelde ratten (185 of 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate) significant minder levermetastasen in vergelijking met de controle ratten ($p<0.001$). Tevens hadden behandelde ratten een significante langere overleving ($p<0.05$). Tenslotte liet een *in vitro* PRRT één-cel model met [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate een dosis-afhankelijke toxiciteit van CA20948 zien in tegenstelling tot SSR-negatieve CC531 tumorcellen. Dit onderstreept het belang van de SSR bij deze vorm van therapie.
Concluderend kunnen we stellen dat [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate een zeer veelbelovende nieuwe behandelingsmodaliteit is voor SSR-positieve tumoren, inclusief de behandeling van gedissemineerde ziekte.

Zoals in Hoofdstuk 5 beschreven wordt is PRRT met [^{111}In-DTPA^{0}]octreotide niet effectief voor SSR-getransfecteerde levermetastasen, aangezien de getransfecteerde cellijn relatief radioresistent was voor de door ons gegeven dosis. Hoofdstuk 6 laat duidelijk de voordelen van [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, een β-emmiterende radionuclide met hogere energie in vergelijking met ^{111}Indium, zien voor de behandeling van SSR-positieve levermetastasen. De doel van de studie, beschreven in **Hoofdstuk 7**, was het evalueren van het effect van [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate op SSR-getransfecteerde colon carcinoom cellen in een rat levermetastase model.

Levermetastasen ontstonden door intraportale injectie van tumorcellen (CC2B en CC531) in ratten. Op dag 7 werden de dieren behandeld met 185 of 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate, terwijl controle dieren geen behandeling kregen. Na 21 dagen werden de ratten opgeofferd en de levermetastasen geteld.
Behandeling met 370 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate liet een duidelijke antitumor effect zien in ratten met CC2B levermetastasen in vergelijking met de controle dieren ($p<0.001$), terwijl er geen antitumor effect waar werd genomen in PRRT behandelde ratten met CC531 levermetastasen. Ook werd er een (dosis-afhankelijke) tumorrespons gezien in ratten met CC2B levermetastasen, die behandeled waren met een lagere (185 MBq) dosis [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate in vergelijking met de controle dieren ($p<0.01$). Tevens hadden ratten met gemixte levermetastasen (half SSR-positief/half SSR-negatief) behandeld met 185 MBq [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate significant minder levermetastasen vergeleken met de controle dieren ($p<0.05$), hetgeen verklaard zou kunnen worden door een radiologisch “bystander” effect van [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate. Dit fenomeen is zeer voordelig in het concept van *in vivo* gentherapie.

AN-238, een cytotoxisch somatostatine analoog, bestaat uit 2-pyrrolinodoxorubicin (AN-201) gekoppeld aan het somatostatine analoog, RC-121. Over AN-201 wordt beweerd dat het 500-1000 maal potenter is *in vitro* dan doxorubicin. In **Hoofdstuk 8** wordt een overzicht over de huidige literatuur over het cytotoxisch somatostatine analoog, AN-238, gegeven. Synthese, werkingsmechanismen en oncologische studies met AN-238 uitgevoerd door de groep van Schally worden besproken.
Samenvattend kan worden gesteld dat AN-238, net als PRRT, ook een zeer indrukwekkende tumorgroei inhibitie op verscheidene SSR-positieve tumor cellijnen had.

Aangezien indrukwekkende resultaten met AN-238 op verschillende SSR-positieve tumoren aangetoond waren, waren wij benieuwd of AN-238 ook effectief zou kunnen zijn voor de behandeling van SSR-getransfecteerde levermetastasen, alwaar wij eerder zeer effectieve behandeling met behulp van PRRT hadden aangetoond. Dit werd geevalueerd in **Hoofdstuk 9**.
SSR-positieve (getransfecteerde) CC2B en zijn moeder SSR-negatieve CC531 cellijn werden getest *in vitro* met AN-238, AN-201 en doxorubicin (DOX) met en zonder

toevoeging van cyclosporine A (CsA). Hierna werden twee *in vivo* experimenten uitgevoerd, waarbij levermetastasen werden veroorzaakt door intraportale injectie van CC2B of CC531 cellen. Een enkele intraveneuze toediening van AN-238 bij 200 nmol/kg of PBS (controle) op dag 7, werd bij het eerste experiment gegeven. In het tweede experiment werden twee intraveneuze injecties van AN-238 bij 250 nmol/kg of PBS (controle) gegeven op dag 5 en 13. WBC en lichaamsgewicht werd twee keer per week gemeten om toxiciteit van de behandeling te observeren.

In vitro blootstelling van CC531 of CC2B cellen aan AN-238 liet een gelijke IC50 voor beide cellijnen zien, ondanks de aanwezigheid van SSRs op CC2B cellen. Een poging om de beide cellijnen chemosensitiever te maken voor AN-238/AN-201 met behulp van de p-glycoproteine pomp remmer, CsA, was niet effectief. Het gebruik van AN-238 *in vivo* had in beide experimenten geen antitumor effect. De ernstige (beenmerg)toxiciteit, dat geobserveerd werd bij AN-238 behandelde ratten in het tweede *in vivo* experiment, resulteerde zelfs in significant hogere tumorscore vergeleken met de controle dieren.

Concluderend kunnen we stellen dat zowel onze *in vitro* data als onze *in vivo* geen superieure antitumor effect van AN-238 heeft kunnen aantonen, ondanks de aanwezigheid van SSRs op CC2B cellen vergeleken met CC531 cellen. Deze resultaten zijn tegenstrijdig met de resultaten van de groep van Schally en anderen.

CONCLUSIES

- *In vitro* transfectie van CC531 is mogelijk. Na transfectie werd een hoge expressie van SSRs met een hoge bindingsaffiniteit gevonden. Tevens is de getransfecteerde receptor functioneel en zijn de groeisnelheid en karakteristieken niet veranderd.
- PRRT met [^{111}In-DTPA^{0}]octreotide is niet effectief voor SSR-getransfecteerde CC531 cellen in een rat levermetastase model, aangezien de cellijn relatief radioresistent is.
- PRRT met [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate is erg effectief voor SSR-positieve CA20948 cellen in een rat levermetastase model. Bovendien hadden behandelde ratten een significant langere overleving.
- PRRT met [^{177}Lu-DOTA^{0}, Tyr^{3}]octreotate is erg effectief voor SSR-getransfecteerde CC531 cellen in een rat levermetastase model. Zelfs als

transfectie-percentages lager zijn, is PRRT met dit middel effectief, waarschijnlijk door een radiologische bystander effect.

- Doelgerichte behandeling met het cytotoxische somatostatine analoog, AN-238, is niet effectief voor SSR-getransfecteerde CC531 cellen in een rat levermetastase model. Noch *in vitro* noch *in vivo* werd een SSR-targeting van AN-238 waargenomen.

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Toen ik in februari 1999 begon aan dit onderzoek, kreeg ik met een enthousiaste groep onderzoekers en begeleiders te maken, die elk op hun manier wat hebben bijgedragen tot de realisatie van dit proefschrift. Een aantal van hen wil ik in het bijzonder noemen.

Mijn co-promotor, Dr. C.H.J. van Eijck, Casper, jij was de grote initiator van het gehele onderzoeksproject. Jouw ongekend groot enthousiasme en optimisme, ook als het een keer wat tegenzat, hebben mij zeer gestimuleerd om tot een goed einde te komen. Ook jouw aanwezigheid op de vele congressen samen met de "van Eijck-onderzoeksgroep" waren niet alleen leerzaam maar bovenal gezellig. Ik verheug mij zeer op mijn komende Dijkzigt-jaren, alwaar ik nog veel van je hoop te mogen leren.

Mijn promotor, Prof. dr. J. Jeekel: Al velen zijn mij voorgegaan als promovendus. Uw enthousiasme voor onderzoek is ongekend en zeer aanstekelijk. Na de vele discussies over mijn onderzoek, die wij gehouden hadden, wilde ik het liefst zo snel mogelijk terug naar het lab, om nieuwe experimenten uit te voeren. Ook de samenwerking met betrekking tot andere activiteiten, zoals de wintersport en Chirurgencup, stelde ik zeer op prijs.

Mijn tweede promotor, Prof. dr. E.P. Krenning: U bent de "vader" van de Octreoscan en alle nieuw ontwikkelde radiogelabelde somatostatine analoga. De waarde van dit middel laat fantastische resultaten zien en heeft nog veel meer potentie. De samenwerkingsverband tussen het chirurgische en uw nucleaire lab was zeer vruchtbaar en heeft al meerdere promoties opgeleverd.

Prof. dr. H.W. Tilanus en Prof. dr. S.W.J. Lamberts wil ik danken voor het kritisch doornemen van het manuscript. Tevens wil ik jullie en de overige leden danken voor de bereidheid om deel uit te maken van de promotiecommissie.

Dr. R.L. Marquet: Richard, jouw ervaring in het uitvoeren van experimenten, maar ook op andere dagelijkse zaken, is van onschatbare waarde geweest voor de totstandkoming van dit proefschrift. Ik heb als zeer bijzonder ervaren om in het lab onder jouw leiding gewerkt te mogen hebben.

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Je gaat het pas zien als je het door hebt.

J. Cruyff

CURRICULUM VITAE

Amir Mearadji werd op 25 april 1973 geboren te Rotterdam. In 1991 behaalde hij zijn VWO diploma aan het Erasmiaans Gymnasium te Rotterdam. Aansluitend studeerde hij aan de Rijksuniversiteit Leiden Geneeskunde. Tijdens zijn studie en als keuze co-schap deed hij onderzoek bij de afdeling Kindergeneeskunde in het Leids Universitair Medisch Centrum (Prof. dr. J.M. Wit). In 1996 werd het doctoraal examen behaald. Tijdens zijn co-schappen verrichtte hij onderzoek naar “het acute scrotum” in het Rode Kruis Ziekenhuis te Den Haag (Dr. H. Boutkan). In 1998 werd het artsexamen behaald.

In 1998 startte hij als AGNIO Chirurgie in het Leids Universitair Medisch Centrum (Prof. dr. O.T. Terpstra), waarna hij in 1999 een aanstelling als arts-onderzoeker kreeg op de afdeling Heelkunde in het Erasmus Medisch Centrum Rotterdam (hoofd: Prof. dr. J. Jeekel, begeleider: Dr. C.H.J. van Eijck). In deze periode werd het proefschrift geschreven. In 2002 begon hij met zijn opleiding tot algemeen chirurg in het Sint Franciscus Gasthuis (opleider: Dr. C.H.A. Wittens), welke voortgezet zal worden van 2005 tot 2007 in het Erasmus Medisch Centrum Rotterdam (opleider: Prof. dr. H.J. Bonjer). Tenslotte zal hij zijn laatste jaar van zijn opleiding weer volgen in Sint Franciscus Gasthuis.