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## Radioimmunotherapy of Metastatic Disease - Studies of Alpha- and Beta-particle-Emitting Radionuclides in a Preclinical Model

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# Radioimmunotherapy of Metastatic Disease

Studies of Alpha- and Beta-particle-Emitting  
Radionuclides in a Preclinical Model

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DOCTORAL DISSERTATION

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Title and subtitle Radioimmunotherapy of Metastatic Disease. Studies of Alpha- and Beta-particle-Emitting Radionuclides in a Preclinical Model		
Abstract <p>In radioimmunotherapy, monoclonal antibodies (mAbs) are used as a targeting agent carrying a toxic payload consisting of radionuclides. This treatment allows the irradiation of small lesions, even on the microscopic level. In the present work, two radionuclides with different properties were used. Lutetium-177 (<sup>177</sup>Lu) is a beta-particle-emitting radionuclide (t<sub>1/2</sub>=6.7 days) and astatine-211 (<sup>211</sup>At) emits alpha particles (t<sub>1/2</sub>=7.2 h). These radionuclides were used to label a mAb targeting the Lewis Y antigen expressed on many carcinomas. A syngeneic rat colon carcinoma model was used in all the studies. The aim of the work described in this thesis was to evaluate the therapeutic effects and toxicity of mAbs labeled with different activities of these two radionuclides, administered separately, or administration of <sup>177</sup>Lu-mAbs, followed by administration of the unlabeled mAbs, <sup>177</sup>Lu-mAbs or <sup>211</sup>At-mAbs. The intratumoral distribution of radioimmunoconjugates was examined over time using digital autoradiography and was related to tumor histology.</p> <p>The results show that the tumor response was dose dependent after treatment with <sup>177</sup>Lu-mAbs alone. Both evaluated activities of <sup>211</sup>At-mAbs resulted in similar tumor response rate as the highest tested activity of <sup>177</sup>Lu-mAbs (400 MBq/kg). Metastases were detected in approximately half the animals, regardless of the radionuclide or administered activity. The toxicity was deemed tolerable as the numbers of white blood cells and platelets recovered to initial levels.</p> <p>Administration of the minimal effective activity of <sup>177</sup>Lu-mAbs followed by treatment with unlabeled mAbs, <sup>177</sup>Lu-mAbs, or <sup>211</sup>At-mAbs resulted in a small increase in the number of tumors showing complete response. No effect was seen on the development of metastases. The additional treatment with unlabeled mAbs did not show any toxic effects. Repeated treatment with <sup>177</sup>Lu-mAbs resulted in a prolonged period of low white blood cell counts and a second decrease in platelet counts. Sequential administration of minimal effective activities of first <sup>177</sup>Lu-mAbs and then <sup>211</sup>At-mAbs also resulted in prolonged myelotoxicity, but with faster recovery of the white blood cells than following the repeated treatment with <sup>177</sup>Lu-mAbs.</p> <p>Studies on the intratumoral distribution of the radioimmunoconjugates are important for our understanding of the response to treatment. The results showed that the activity was initially located in the tumor margins, and did not reach the more central parts until 24 h after administration. The distribution of activity was heterogeneous despite the expression of the antigen on all tumor cells. The tumor histology changed from dense tumor growth to areas of stromal tissue in some tumors after 24 h, while other tumors continued to grow. Calculations of the dose rate indicate that the tumors are treated most efficiently during the first 24 h after injection.</p> <p>In summary, these results show that single treatment with alpha- and beta-particle-emitting radionuclides results in comparable toxicity at activities giving the same rate of tumor response. The studies on combined treatment showed that although a higher number of tumors showed complete response, there was no effect on the development of metastatic disease or survival.</p>		
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Radionuclides in a Preclinical Model

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# List of original papers

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

- I. Repeated radioimmunotherapy with  $^{177}\text{Lu}$ -DOTA-BR96 in a syngeneic rat colon carcinoma model  
**Sophie E Eriksson**, Tomas Ohlsson, Rune Nilsson, and Jan Tennvall  
*Cancer Biotherapy and Radiopharmaceuticals*, 27:134-140, 2012
- II. Treatment with unlabeled mAb BR96 after radioimmunotherapy with  $^{177}\text{Lu}$ -DOTA-BR96 in a syngeneic rat colon carcinoma model  
**Sophie E Eriksson**, Tomas Ohlsson, Rune Nilsson, and Jan Tennvall  
*Cancer Biotherapy and Radiopharmaceuticals*, 27:175-182; 2012
- III. Successful radioimmunotherapy of established syngeneic rat colon carcinoma with  $^{211}\text{At}$ -mAb  
**Sophie E Eriksson**, Tom Bäck, Erika Elgström, Holger Jensen, Rune Nilsson, Sture Lindegren, and Jan Tennvall  
*EJNMMI Research*, 3:23, 2013
- IV. Sequential administration of  $^{177}\text{Lu}$ -mAb and  $^{211}\text{At}$ -mAb in a syngeneic rat colon carcinoma model  
**Sophie E Eriksson**, Tom Bäck, Erika Elgström, Tomas Ohlsson, Holger Jensen, Rune Nilsson, Sture Lindegren, and Jan Tennvall  
*Manuscript*
- V. Changes in relation to tumor histology over time in a syngeneic rat colon carcinoma model. The intratumoral distribution of radiolabeled  $^{177}\text{Lu}$ -BR96 mAbs  
Anders Örbom\*, **Sophie E Eriksson**\*, Erika Elgström, Tomas Ohlsson, Rune Nilsson, Jan Tennvall, and Sven-Erik Strand  
\* Regarded as joint first authors  
*Accepted for publication in Journal of Nuclear Medicine* 2013

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# Abbreviations

BN	Brown Norway (rat strain)
CEA	Carcinoembryonic antigen
CT	Computed tomography
DNA	Deoxyribonucleic acid
DOTA	1,4,7,10-tetraazacyclododecane tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
i.v.	Intravenous
IA	Injected activity
IgG	Immunoglobulin G
$K_d$	Equilibrium binding constant
LeY	Lewis Y antigen
mAb	Monoclonal antibody
MALDI-MS	Matrix-assisted laser desorption ionization mass spectroscopy
p.i.	Post injection
PBS	Phosphate buffered saline
PET	Positron emission tomography
PSMA	Prostate-specific membrane antigen
RAHA	Rat anti-human antibody
$S_{2Gy}$	Surviving fraction after irradiation with 2 Gy
SPECT	Single-photon-emission computed tomography
VEGF	Vascular endothelial growth factor

# Summary

In radioimmunotherapy, monoclonal antibodies (mAbs) are used as a targeting agent carrying a toxic payload consisting of radionuclides. Systemic administration of such radioimmunoconjugates will result in the accumulation of radioactivity in tumor lesions expressing the target antigen. This treatment allows the irradiation of small lesions, even on the microscopic level.

In the present work, two radionuclides with different properties were used. Lutetium-177 ( $^{177}\text{Lu}$ ) is a beta-particle-emitting radionuclide with a physical half-life of 6.7 days and a maximal range in soft tissue of 1.8 mm. Astatine-211 ( $^{211}\text{At}$ ) has a physical half-life of 7.2 h and emits alpha particles with a maximal range of 70  $\mu\text{m}$ . These radionuclides were used to label a mAb targeting the Lewis Y (LeY) antigen expressed on many carcinomas as well as in some normal tissues in both humans and rats. A syngeneic rat colon carcinoma model was used in all the studies. The aim of the work described in this thesis was to evaluate the therapeutic effects and toxicity of mAbs labeled with different activities of these two radionuclides, administered separately, or administration of  $^{177}\text{Lu}$ -mAbs, followed by administration of the unlabeled mAbs,  $^{177}\text{Lu}$ -mAbs or  $^{211}\text{At}$ -mAbs. The intratumoral distribution of radioimmunoconjugates was examined over time using digital autoradiography and was related to tumor histology.

The results show that the tumor response was dose dependent after treatment with  $^{177}\text{Lu}$ -mAbs alone. The minimal effective activity of  $^{177}\text{Lu}$ -mAbs, defined as the lowest activity resulting in complete response of inoculated in five out of six rats, was determined to 400 MBq/kg body weight. Both evaluated activities of  $^{211}\text{At}$ -mAbs resulted in similar tumor response rate as the minimal effective activity of  $^{177}\text{Lu}$ -mAbs. Metastases were detected in approximately half the animals, regardless of the radionuclide or administered activity. The toxicity was deemed tolerable as the transient weight loss was less than 10% 2-3 days after treatment, and the numbers of white blood cells and platelets recovered to initial levels. The time to full recovery was longer after the administration of  $^{177}\text{Lu}$ -mAbs than after  $^{211}\text{At}$ -mAbs.

Administration of the minimal effective activity of  $^{177}\text{Lu}$ -mAbs followed by treatment with unlabeled mAbs,  $^{177}\text{Lu}$ -mAbs, or  $^{211}\text{At}$ -mAbs resulted in a small increase in the number of tumors showing complete response. No effect was seen on the development of metastases. The additional treatment with unlabeled mAbs

did not show any toxic effects. Repeated treatment with  $^{177}\text{Lu}$ -mAbs resulted in a prolonged period of low white blood cell counts and a second decrease in platelet counts. Sequential administration of minimal effective activities of first  $^{177}\text{Lu}$ -mAbs and then  $^{211}\text{At}$ -mAbs also resulted in prolonged myelotoxicity, but with faster recovery of the white blood cells than following the repeated treatment with  $^{177}\text{Lu}$ -mAbs.

Studies on the intratumoral distribution of the radioimmunoconjugates are important for our understanding of the response to treatment. The results showed that the activity was initially located in the tumor margins, and did not reach the more central parts until 24 h after administration. The distribution of activity was heterogeneous despite the expression of the antigen on all tumor cells. The tumor histology changed from dense tumor growth to areas of stromal tissue in some tumors after 24 h, while other tumors continued to grow. Calculations of the dose rate indicate that the tumors are treated most efficiently during the first 24 h after injection.

In summary, these results show that single treatment with alpha- and beta-particle-emitting radionuclides results in comparable toxicity at activities giving the same rate of tumor response. The studies on combined treatment showed that although a higher number of tumors showed complete response, there was no effect on the development of metastatic disease or survival.

# Introduction

Primary tumors are commonly treated by surgery, often in combination with external-beam radiation therapy, usually resulting in good loco-regional control of tumor growth. However, most cancer-related deaths today are caused by metastatic disease [1, 2].

## Metastatic disease

The metastatic process is initiated by detachment of the tumor cell from adjacent cells and the extracellular matrix and is followed by migration within the tissue causing invasive tumor growth. In the next step, the cell enters the circulation by passage between the endothelial cells of a blood or lymphatic vessel [2, 3]. Only a small fraction of circulating tumor cells will form metastases [2], since most circulating tumor cells are apoptotic or already dead [3]. Occasionally, circulating tumor cells will be trapped in the microvascular bed in a distant organ [2, 3]. In many cases, the organ can be indicated by the lymphatic and venous drainage of the tumor [3-5]. The next step is extravasation into the tissue [2, 3]. In order to form a metastasis, the new microenvironment must be adapted to support tumor growth [6, 7]. Residual tumor cells can be dormant for long periods without proliferating, and this quiescence is sometimes promoted by the microenvironment itself [6, 8]. Other mechanisms resulting in clinically undetectable tumors are lack of angiogenesis and immunological clearance [6]. Metastatic growth is accelerated after inactivation of metastatic suppressor genes. The genetic differences between primary tumors and metastases suggest that they progress in parallel, rather than linear, as previously proposed [6, 9, 10]. This means that the metastatic process may be initiated long before the primary tumor is detected and treated [3, 10].

Despite the fact that the differences between primary tumors and metastases are well documented [9], the same treatment is often given for metastatic disease as for primary tumors [1]. The benefit of treating metastases in late stages of the disease has been found to be poor and, therefore, efforts have been made to develop adjuvant treatment protocols [11]. It is not known whether the same mechanisms of resistance are responsible for treatment failure in metastatic

disease as in primary tumors [11]. The biology of dormant tumor cells seems to differ from that of both primary tumors and metastases [8]. In the case of targeted therapies, the targeted pathway might not be active, or the antigen may not be expressed in dormant tumor cells [11], resulting in the cells being refractory to treatment [8]. Since primary tumors and metastases often diverge early, systemic treatment should be directed at mutations or changes occurring early in the development of the disease [11]. Adjuvant therapy has been shown to be ineffective in preventing metastatic disease and prolonging overall survival in many cases [8], indicating that the eradication of dormant tumor cells might require novel targets [12].

## Targeted therapies

The identification of tumor cell properties that differ from those of normal cells has resulted in therapies directed at changed signal pathways and other markers in the tumor cell membrane, or the microenvironment [13, 14]. Several tyrosine-kinase inhibitors and mAbs are directed at growth signaling pathways, and will reduce tumor cell growth by the inhibition of signal transduction. In addition, mAbs can induce an immune reaction such as antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity upon binding to the cell [15]. The first mAbs produced by the hybridoma technique were murine [16], resulting in the development of human anti-mouse antibodies when used in clinical studies, and thus a reduction in the therapeutic effect. The mAbs developed for therapeutic use are now engineered to contain human rather than murine sequences of amino acids. In chimeric mAbs, the constant regions of both the heavy and the light chains have been replaced with the corresponding human sequences. In humanized mAbs, all but the complementarity-determining regions are of human origin. Further advances in genetic engineering now allow the production of completely human mAbs. Since molecular size affects distribution in the body, antibody fragments and engineered constructs have also been evaluated for use in cancer treatment [15, 17].

## Radioimmunotherapy

The therapeutic potential of mAbs can be enhanced by loading them with a toxic moiety, such as a potent drug or a radionuclide. Antibody-drug conjugates, consisting of cytotoxic drugs linked to mAbs, must in general be internalized to release the active drug [18]. Clinical studies have shown that antibody-drug

conjugates have greater efficacy than the corresponding unlabeled mAbs [19]. In radioimmunotherapy, the mAbs are loaded with a radionuclide that causes cellular damage in tumor lesions. Both kinds of labeled antibodies cause toxicity: radioimmunotherapy mainly results in myelotoxicity from irradiation of the red bone marrow, while antibody-drug conjugates are more prone to cause liver toxicity due to hepatic degradation [20]. Two radioimmunoconjugates,  $^{90}\text{Y}$ -ibritumomab tiuxetan and  $^{131}\text{I}$ -tositumomab, have been approved by the U.S. Food and Drug Administration for the treatment of B-cell non-Hodgkin lymphoma, and they have also been found to be clinically efficacious in patients who have previously undergone other modes of treatment [21]. Despite their heterogeneous uptake [22], good results have been achieved in the treatment of lymphoma, which can be partly explained by high radiosensitivity, good vascularization, and the homogeneous expression of the target antigen. In comparison, the results obtained in studies on solid carcinomas have been disappointing. Solid tumors often exhibit heterogeneous drug uptake and require higher doses [23]. Most early studies on the effects of radioimmunotherapy on solid tumors were conducted in patients with large tumor burdens who had previously undergone several kinds of treatment, and this might also have contributed to the poor outcome and the high toxicity [24]. There has recently been renewed interest in radioimmunotherapy for the treatment of microscopic cancer of the colon, breast, prostate and ovaries, among others [23], and several mAbs and target antigens are currently being evaluated [14].

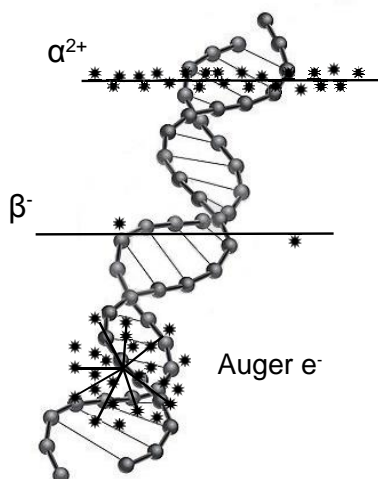
## Radionuclides suitable for radioimmunotherapy

Ionizing irradiation causes cellular damage mainly by inducing DNA breaks, resulting in cellular death if the lesions are recognized by the cell as being beyond repair. Irradiation also stimulates signaling between cancer cells, enhancing the response, a phenomenon known as the bystander effect [25]. External-beam radiation therapy has been well established as a loco-regional cancer treatment modality for over a century. However, the treatment of micrometastatic disease may require the irradiation of large volumes of the body, and external radiation therapy is therefore not suitable for the treatment of disseminated disease. In systemic radiotherapy, a radionuclide is administered to the patient, which then accumulates in the tumor lesions resulting in local irradiation. Compared with external radiation therapy, the dose rate achieved by radionuclides is low [26]. This will result in an increase in the fraction of cells able to repair damage, but also redistribution in the cell cycle to more radiosensitive phases. In the simplest form of systemic radiotherapy, the radionuclide itself targets the site of the lesion, for example, the use of  $^{131}\text{I}$  for differentiated thyroid cancer and  $^{223}\text{Ra}$  (Alpharadin)

for the treatment of bone metastases in prostate cancer patients. In other cases, carrier molecules such as peptides and mAbs are used for targeting.

The radionuclides most often used for therapy are beta emitters, which cause cell damage by the emission of an electron. Their linear energy transfer is low, which means that they cause sparse ionization along their path through tissue (Figure 1). Beta particles will mainly cause DNA single strand breaks, either by a direct hit, or mediated by the formation of reactive oxygen species thus implying that hypoxia reduces tumor cell radiosensitivity. Other radionuclides emit alpha particles, which consist of a helium nucleus, and have a high linear energy transfer, resulting in a high probability of DNA double strand breaks upon passage through the cell nucleus (Figure 1). This means that a few direct hits by an alpha particle can cause cell death, while thousands of beta particles would be needed to cause similar damage [26]. Auger electron emitters have also been evaluated preclinically for use in radioimmunotherapy [27, 28]. These radionuclides emit several electrons that have a very short particle path length in tissue, and must therefore be transported to the cell nucleus to cause damage or death. A list of radionuclides currently being used and candidates considered for radioimmunotherapy is presented in Table 1.

In general, the longer path length of beta particles will result in the irradiation of not only the targeted cell, but also surrounding cells. This so-called crossfire effect will compensate to some degree for heterogeneous tumor uptake, but may also result in the irradiation of normal tissue when the tumor lesion itself is very small.



**Figure 1.** Illustration of the ionization densities of different types of emitted particles in the context of the DNA molecule. Modified from [26].

In such cases, alpha-particle-emitting radionuclides are considered more appropriate due to their short path length. However, a disadvantage is the few available alpha emitters, with either a short physical half-life limiting their usefulness in the clinical setting, or daughter nuclides that might cause toxicity if redistributing to healthy tissue.

**Table 1.** Physical properties of radionuclides used or currently being considered for radioimmunotherapy

<b>Radionuclide</b>	<b>Therapeutic particle</b>	<b>Physical half-life</b>	<b>Maximal range in tissue</b>	<b>Comment</b>
Yttrium-90	$\beta$	2.67 d	11.3 mm	
Iodine-131	$\beta$	8.0 d	2.3 mm	
Lutetium-177	$\beta$	6.7 d	1.8 mm	
Rhenium-188	$\beta$	17.0 h	10.1 mm	
Astatine-211	$\alpha$	7.2 h	70 $\mu\text{m}$	
Lead-212	$\alpha$	10.6 h	<100 $\mu\text{m}$	In vivo generator of $^{212}\text{Bi}$
Bismuth-213	$\alpha$	46 min	84 $\mu\text{m}$	3 daughter radionuclides
Radium-223	$\alpha$	11.4 d	<100 $\mu\text{m}$	6 daughter radionuclides
Actinium-225	$\alpha$	10.0 d	<100 $\mu\text{m}$	6 daughter radionuclides
Thorium-227	$\alpha$	18.7 d	<100 $\mu\text{m}$	7 daughter radionuclides
Indium-111	Auger	3.0 d	<100 nm	
Iodine-125	Auger	60.5 d	<100 nm	



# Aims of this work

The general aim of the work presented in this doctoral thesis was to investigate ways of improving the therapeutic outcome of radioimmunotherapy with alpha- and beta-particle-emitting radionuclides in a preclinical model. The specific aims were:

- to determine the minimal effective activity of  $^{177}\text{Lu}$ -DOTA-BR96 in order to reduce toxicity, allowing for a second administration of radioimmunotherapy (Paper I),
- to investigate the therapeutic effect of  $^{211}\text{At}$ -mAbs on solid colon tumors (Paper III),
- to evaluate the therapeutic benefit of additional treatment with the unlabeled (Paper II) and radionuclide-labeled mAb BR96 (Papers I and IV) after successful radioimmunotherapy with the minimal effective dose of  $^{177}\text{Lu}$ -DOTA-BR96, and
- to study the intratumoral distribution of radioactivity using digital autoradiography, and to compare the distribution over time with tumor histology (Paper V).

# The tumor model

## The antibody and its target

The mAb BR96 (Seattle Genetics, Inc.) was first developed in a murine form by the immunization of mice with human breast cancer cells [29], before conversion of the hybridoma cell line to produce the chimeric murine/human version [30] used in these studies. The dissociation constant was determined to be 4 nM (Paper I). The mAb BR96 targets the LeY antigen expressed on several human carcinomas, for example, breast, gastrointestinal, pancreatic, non-small-cell lung, cervical, and ovarian, as well as some melanomas [29]. LeY (also known as CD174) is a blood-group related [31] tetrasaccharide expressed on both glycolipids and glycoproteins in the cell membrane [32]. In adults, LeY is mainly found in healthy epithelial linings of the gastrointestinal tract [29] and on some hematopoietic progenitor cells [33].

## Radiochemistry

The radiochemical method used to prepare radioimmunoconjugates is selected based on the physiochemical properties of the radionuclide. Radiometals such as yttrium and lutetium require a chelate molecule to be conjugated to the protein. Halogens such as iodine can bind directly to the protein by a redox reaction, but might be released from the tumor cell as a result of dehalogenation. Radiometals, on the other hand, will remain within the tumor cell after internalization, thus increasing the probability of a therapeutic effect. In this work, the radiometal  $^{177}\text{Lu}$  and the halogen  $^{211}\text{At}$  were used. Since traditional halogenation with  $^{211}\text{At}$  will result in low yield and poor stability of the product, the mAb was first conjugated with an activated tin ester that has suitable properties for astatination.

BR96 was conjugated with the chelate S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) (Macrocyclics, Dallas, TX), as described by Forrer *et al.* [34]. Briefly, the antibody was first transferred to a sodium carbonate buffer by repeated centrifugation through an Amicon-15 filter. The DOTA chelate was added at a molar ratio of 3 DOTA moieties to 1

immunoglobulin G (IgG) moiety, and the solution was incubated for 1 h at 37°C. The conjugate was then transferred to an ammonium acetate buffer, and the final concentration was adjusted to 10 mg BR96/mL. The number of DOTA moieties per IgG moiety was determined using matrix-assisted laser desorption ionization mass spectroscopy, (MALDI-MS) and by dividing the increase in molecular mass by the mass of the DOTA moiety. The immunoreactivity was determined by saturation binding curve analysis, with BN7005-H1D2 cells as target antigen and increasing concentrations of BR96 and DOTA-BR96 (40 ng/mL-40 µg/mL). After incubation at room temperature for at least 1 h, the bound mAbs were detected by horseradish peroxidase (HRP)-conjugated anti-human IgG, and the equilibrium binding constant ( $K_d$ ) was calculated. The immunoreactivity is given by the ratio:  $K_d(\text{BR96})/K_d(\text{DOTA-BR96})$ .

Two different conjugations were used: the first batch contained 2.3 DOTA moieties per IgG moiety, and had an immunoreactivity of 0.90 (Papers I and II). The second batch contained 2.4 DOTA moieties per BR96 moiety, and had an immunoreactivity of 0.86 (Papers II, IV, and V).

Labeling was performed with  $^{177}\text{LuCl}_3$  (PerkinElmer, Boston, MA) by heating the DOTA-BR96 in 0.25 M ammonium acetate buffer (pH 5.3) and the radionuclide solution to 45°C for 10 min. The mAb solution was then added to the vial containing the radionuclide and incubated at 45°C for 15 min. The reaction was quenched by incubation with excess chelate (DTPA) for 5 min. The radioimmunoconjugate was then diluted to 375 µg/mL in 1% human serum albumin (Baxter Medical AB, Kista, Sweden). The radiochemical purity was determined by high-performance liquid chromatography (HPLC) with a  $7.8 \times 300$  mm molecular sieving column (SEC S3000; Phenomenex, Torrance, CA) eluted with 0.05 M sodium phosphate at a rate of 1.0 mL/min. Specific activities and radiochemical purity are reported in the papers.

The  $^{211}\text{At}$  was produced by irradiation of stable bismuth using the  $^{209}\text{Bi}(\alpha,2n)^{211}\text{At}$  reaction at the Cyclotron and PET Unit, Rigshospitalet, Copenhagen, Denmark. The target was then transported to the Department of Nuclear Medicine at Sahlgrenska University Hospital, Gothenburg, Sweden, where the astatine was dry distilled into a chemically useful form, as described previously [35]. The labeling of BR96 with  $^{211}\text{At}$  was performed as described by Lindegren *et al.* [36]. In brief, the BR96 was first incubated with the *N*-succinimidyl 3-(trimethylstannyl) benzoate reagent for 30 min. The immunoconjugate was then added to a vial containing the radionuclide. *N*-iodosuccinimide was added and the reaction was terminated after 1 min by the addition of sodium ascorbate. The  $^{211}\text{At}$ -BR96 fraction was isolated into PBS using a Sephadex-15 column. Bovine serum albumin was added to prevent radiolysis.

In all *in vivo* studies, the final volume of the injected radioimmunoconjugate was 0.4 mL, and the amount of mAb was adjusted to 150 µg per animal, resulting in approximately 0.6 mg/kg body weight. The radioimmunoconjugates were administered intravenously using a cannula. The activity in the syringes was measured before and after injection to calculate the injected activity.

## The cell line

The BN7005 cell line originates from a colon carcinoma induced by 1,2-dimethylhydrazine in a Brown Norway (BN) rat [37]. The H1D2 clone was established after limiting dilution of BN7005 in the absence of selection pressure [38]. The cell line has a short doubling time during exponential growth *in vitro* of approximately 10 h. The LeY antigen is expressed on most cells *in vitro*, and the BR96 mAb can be internalized upon binding, as demonstrated previously [39].

The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 14 mg/L gentamicin, at 37°C in a humidified environment containing 5% CO<sub>2</sub>, and detached by trypsin treatment at passage. Cells in the exponential growth phase were used in all experiments.

## Radiosensitivity of the cell line *in vitro*

Radiation sensitivity varies between tumors and has a major impact on the tumor response to external-beam radiation therapy. In the BN colon carcinoma model, radioimmunotherapy results in complete response, rather than delayed tumor growth as seen in most other tumor models. The radiosensitivity of the cell line BN7005-H1D2 was determined to exclude the possibility that the results of treatment were due to radiation hypersensitivity.

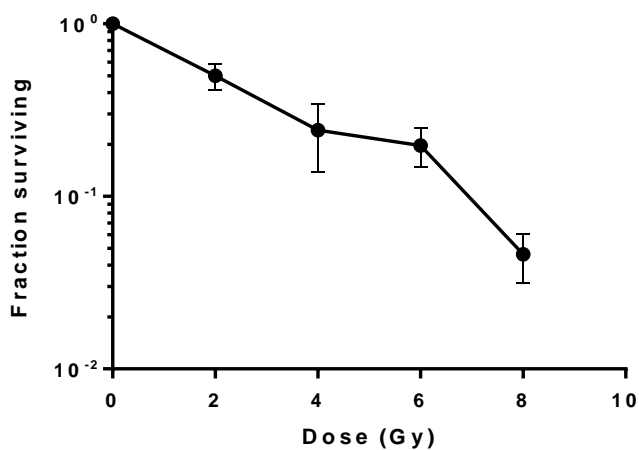
The radiosensitivity was determined using a colony forming unit assay, as described by Michel *et al.* [40]. Briefly, cells were irradiated with 2, 4, 6, or 8 Gy of <sup>137</sup>Cs in a Gammacell® 3000 Elan blood irradiator (MDS Nordion, Ottawa, Canada) during exponential growth. The cells were then detached, counted and diluted before seeding of 0.39-400 cells/well in 96-well plates. After one week, the number of wells with at least one colony consisting of >50 cells was determined,

and the initial concentration of viable tumor cells was calculated with Quality software<sup>1</sup>. The data were then fitted by non-linear regression (second-order polynomial using GraphPad Prism 5.02 Software), and the surviving fraction was calculated.

The surviving fraction of the BN7005-H1D2 cell line after irradiation with 2 Gy ( $S_{2Gy}$ ) was determined to be 0.55 (range 0.50-0.60; Figure 2). Human cell lines of colorectal origin have been reported to have a mean  $S_{2Gy}$  of 0.46 when several cell lines were compared [41]. Hence, the BN7005-H1D2 cell line is considered to have intermediate radiosensitivity, and is somewhat less sensitive to irradiation than corresponding human cell lines.

## The rats

Male Brown Norway rats were used in all studies (Harlan Laboratories, Inc.). As in humans, the LeY antigen is expressed in some normal tissues in these rats, mainly in the epithelium of the gastrointestinal tract [42]. Rats of this strain are fully immunocompetent, meaning that syngeneic tumor grafts can interact with stromal cells and the immune system [43].



**Figure 2.** Surviving fraction of BN7005-H1D2 cells after external beam irradiation. Error bars denote standard deviation.

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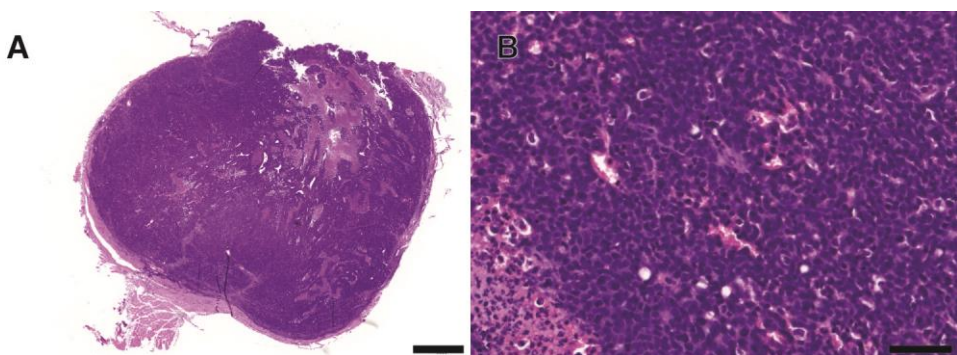
<sup>1</sup> <http://ubik.microbiol.washington.edu/computing/quality/jquality.htm>

The animals were housed under standard conditions, with free access to fresh water and standard pellets. Animals were sacrificed with an overdose of isoflurane when tumor growth exceeded the maximal permitted size ( $20 \times 20$  mm), if their general state of health was affected (signs of metastatic disease or severe weight loss  $>15\%$  of normal body weight), or at the end of the study. All experiments were conducted in compliance with Swedish legislation on animal protection, and were approved by the Regional Ethics Committee on Animal Experiments.

## The tumors

Tumor cells were inoculated between the peritoneum and the abdominal muscle (sometimes referred to as sub-peritoneal). This is an invasive procedure performed under aseptic conditions, requiring anesthesia with isoflurane and analgesia with buprenorphine. The skin and the muscle were opened 2-3 cm along the mid-ventral line, homeostatic forceps were used to gently lift the muscle wall on the right side, and the cell suspension ( $3 \times 10^5$  cells in 0.05 mL) was carefully injected underneath the peritoneum from the inside of the abdomen. As the syringe was removed, the cells were prevented from leaking out by pressing the needle tracks. The muscle and the skin were then closed with sutures.

These tumors grow rapidly, and formed a solid tumor with an approximate diameter of 10 mm after two weeks (*i.e.* the time for administering the radioimmunoconjugates). The growing tumors can easily be palpated and measured with a digital caliper, without the need for imaging or any invasive procedure such as laparotomy. The tumor volumes were calculated as  $\text{length} \times \text{width}^2 \times 0.4$ , as has been validated previously [39].



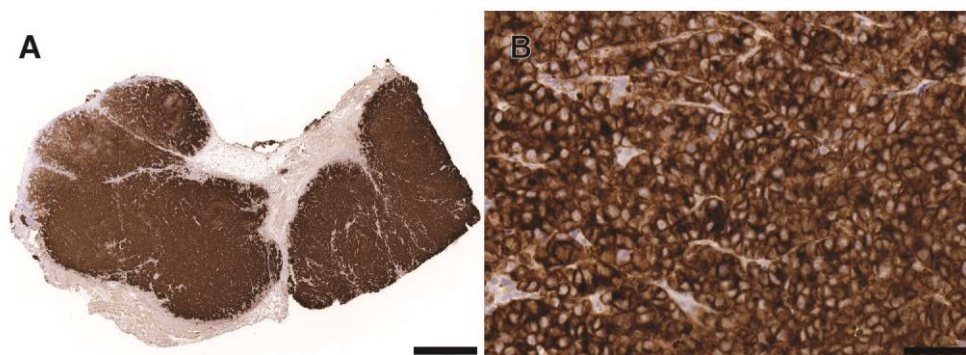
**Figure 3.** Untreated tumor tissue 14 days after inoculation with tumor cells. Scale bars 1 mm (A) and 50  $\mu\text{m}$  (B).

These tumors bear a close resemblance to poorly differentiated human colon adenocarcinoma according to clinical pathologists. The histology of these tumors consists of dense growth of tumor cells with infiltrating stroma, often with necrotic regions of various sizes (Figure 3). This differs from tumors of the same cell line inoculated subcutaneously, which generally have a large necrotic core. The presence of stromal and vascular interaction and the ability to form metastases makes this syngeneic tumor model more relevant than many subcutaneous xenograft models, which often lack these properties [43].

## Antigen expression in tumors

The antigen expression is of major importance for the results of treatment, especially when radionuclides with a short particle range are used. The expression of LeY in tumors was visualized using immunohistochemistry. Both snap-frozen tumors and tumors first fixed in 4% paraformaldehyde with subsequent embedding in paraffin were evaluated. Snap-frozen tissues were fixed in 4% formalin after cryosectioning. Paraffin-embedded tissues require rehydration and heat-induced antigen retrieval at pH 6 before staining. In the staining procedure, sections were first incubated with unlabeled BR96 and then with an anti-human antibody conjugated with HRP. When the substrate diaminobenzidine is added, a brown precipitate is formed indicating the site of the antigen. The other tumor structures are stained with hematoxylin for histological examination.

Evaluation of LeY expression in untreated tumors excised on the day on which radioimmunoconjugates were first administered to the other animals (14 days after inoculation with tumor cells) demonstrated homogeneous distribution over the sections, with complete membrane staining of all tumor cells (Figure 4). The antigen expression in tumors excised 24 h after i.v. administration of 0.1-10 mg/kg



**Figure 4.** LeY antigen expression in untreated tumors 14 days after inoculation with tumor cells. Scale bars 1 mm (A) and 50 μm (B).

body weight unlabeled BR96 was unaffected, apart from reduced staining in the tumor margins and small, more central areas of the tumor (Paper V). This was probably a result of antigens being blocked by the *in vivo* administered mAbs, rather than a change in expression due to selection. The same pattern was observed in tumors excised 48 h after injection of 0.1 mg/kg BR96. However, a change in histology, from dense growth of tumor cells observed in untreated tumors to granulation tissue (*i.e.* newly formed stromal tissue) and recent necrosis, was observed in tumors from animals given 1.0 or 10 mg/kg BR96. At the same time, the LeY antigen was only detected in small areas. The possibility of antigen blocking by the *in vivo* administered mAbs cannot be ruled out as an explanation of this reduced antigen expression. However, the histological examinations clearly indicated that BR96 itself could have a therapeutic effect, possibly via an immune reaction mediated through the Fc receptor [44, 45]. This effect has been seen previously in this animal model after the administration of 15 mg/kg BR96 [46]. In that study, tumors in four of six rats went into transient complete response, but all animals were sacrificed within 80 days due to local recurrence or metastatic disease.

## Biodistribution

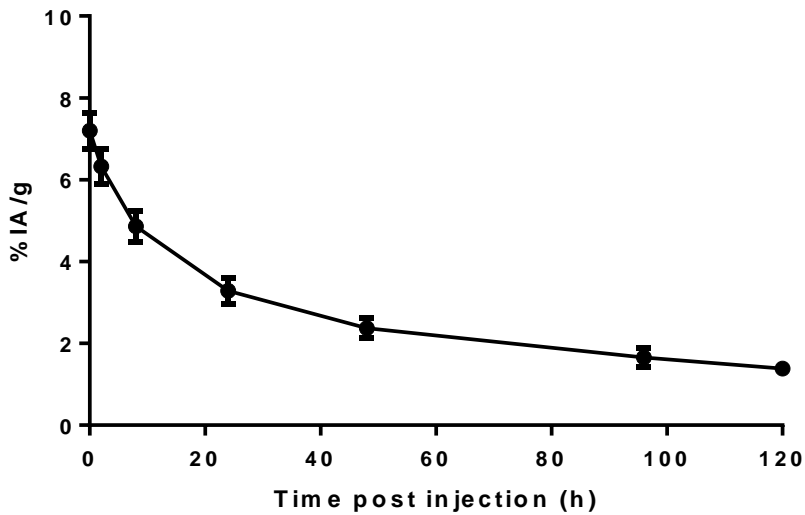
The biodistribution of a medical compound is of importance since it affects the possibility of achieving a therapeutic effect, as well as the toxicity. Ideally, a high proportion of the injected radioimmunoconjugates should accumulate in the tumor before most of the radionuclide has decayed, while the uptake in normal tissue should be minimal. The pharmacokinetics, *i.e.* the rate of clearance from the blood, should be slow enough to allow tumor uptake, but this will also have a major impact on bone marrow toxicity.

The pharmacokinetics and biodistribution were evaluated by injection of a sub-therapeutic activity, 50 MBq/kg  $^{177}\text{Lu}$ -BR96, followed by measurements of the activity in tissue samples excised at several time points, using a NaI(Tl) scintillation well counter.

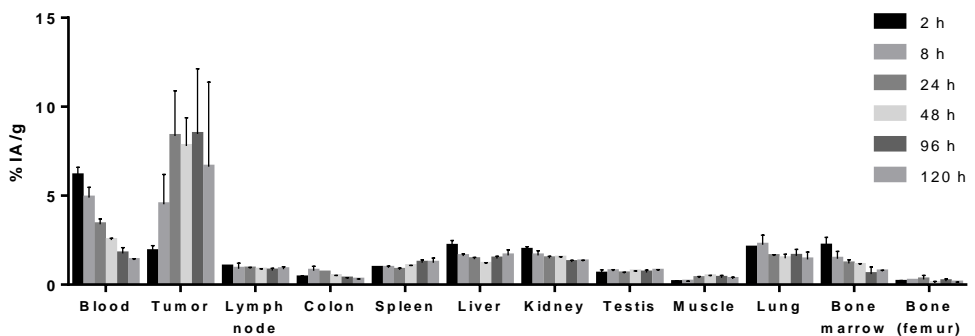
The blood clearance is seen in Figure 5. The pharmacokinetics is often divided into two phases: redistribution from blood plasma into other tissues (called the alpha phase), and elimination from the blood (the beta phase). The decay-corrected half-lives for these two phases were found to be  $t_{1/2\alpha}=5.6$  h and  $t_{1/2\beta}=32.5$  h. The maximal tumor uptake, approximately 8% of the injected activity (IA) per g blood, was detected 24 p.i., and remained at the same level until 96 h p.i. (Figure 6). The activity in blood-rich organs, *i.e.* the liver, kidneys, lungs and red bone marrow, reached approximately 2% IA/g at 2 h p.i. and then generally decreased. It should



be noted that tumor uptake in rats is generally lower than tumor uptake in mice, due to the different distribution volumes [47].



**Figure 5.** Pharmacokinetics (blood clearance) after injection of  $^{177}\text{Lu}$ -BR96, expressed as the % injected activity (IA) per gram blood.



**Figure 6.** Biodistribution of  $^{177}\text{Lu}$ -BR96 in organs at different times p.i. Error bars denote standard deviation.

## Autoradiography

Imaging modalities such as positron emission tomography (PET) and single-photon-emission computed tomography (SPECT) in combination with computed tomography (CT) can be used to study the distribution of a radiolabeled substance in animals and patients *in vivo*. These techniques can be of great help in the evaluation of new compounds during drug development. In order to be able to compare the drug distribution within the tumors directly with the histological findings, an *ex vivo* method such as autoradiography is needed. Traditionally, a film emulsion is irradiated by a radioactive tissue sample resulting in a high-resolution image of the distribution of radioactivity. One limitation of this method is that it does not always allow quantitative measurements. In many cases, the film autoradiography has been replaced by storage phosphor screens, which provide more convenient handling but lower resolution. In order to be able to perform dosimetric calculations based on autoradiography, a solid-state detector system was used in the present studies (Biomolex700 Real-Time Digital Imager, Biomolex AS, Norway). The emitted particles are detected in a double-sided silicon strip detector with an intrinsic spatial resolution of 50  $\mu\text{m}$ . The recorded events are then analyzed and displayed as a digital image using software developed in IDL 6.4 (ITT Visual Information Solutions, Boulder, CO), with corrections for dead or miscalibrated strips, and for radioactive decay.

## Intratumoral distribution

Tumor drug uptake depends on several factors. Firstly, the properties of the targeting molecule will affect uptake and retention. The size of the molecule is important since small molecules will be cleared from the circulation rapidly, while large molecules exhibit slow uptake and intratumoral transport. Fragmentation and engineering of mAb constructs have been performed in order to find the ideal balance between uptake and clearance, however, it has been predicted that IgG has the optimal size for tumor uptake [48].

High affinity will result in strong binding to available antigens close to perfused blood vessels, thus forming a binding-site barrier restricting further uptake [49]. In fact, higher uptake is seen for mAbs with moderate affinity [50]. Wittrup *et al.* predicted that a binding-site barrier effect is unlikely when  $K_d > 1 \text{ nM}$  [48], and this was therefore not a major factor in the present work as BR96 has an equilibrium binding constant of 4 nM. An advantage of IgG molecules is that they can form bivalent bonds, which will result in better tumor retention than with fragments such as scFv [51]. The mAb dose will also affect the intratumoral distribution.

Low doses will result in the accumulation of mAbs in the tumor periphery in small lesions (<1 mm in diameter) [52], and close to blood vessels in larger tumors [53, 54]. In general, a higher dose will result in an overall higher tumor uptake [55]. It should be noted that the optimal mAb dose has not been determined in the BN rat tumor model. The antigen expression will also affect the intratumoral distribution, as a lower amount of antigen will require a lower concentration of the targeting molecule to become saturated [56].

The concentration of the drug taken up in a tumor is inversely proportional to the tumor volume, *i.e.*, higher drug concentrations are detected in small tumors [57-59]. Insufficient uptake has been observed for the traditional cytostatic drug doxorubicin [60], as a result of the tumor properties such as blood vessel distribution. Tumor blood vessels are often immature and more permeable than normal blood vessels [61], which results in a high interstitial fluid pressure reducing convection, thereby lowering the tumor uptake of drugs [62]. On the other hand, vascular permeability will result in an increase in the accumulation of drugs regardless targeting: a mechanism called enhanced permeability and retention [63]. Normalization of blood vessels by, for example, treatment with anti-vascular endothelial growth factor (VEGF) compounds, will lower the interstitial fluid pressure [64], increasing drug uptake [65], despite the fact that the number of blood vessels may decrease [66]. The extracellular matrix can also affect the tumor uptake of drugs. Treatment with collagenase has been shown to lower the interstitial fluid pressure, resulting in higher and more homogeneous uptake of mAbs [67, 68]. Such methods will probably not be implemented in the clinic since collagenase is not tumor specific and may increase the number of metastases.

The distribution of radioimmunoconjugates within tumors will have a significant effect on the outcome of treatment. Previous studies have mainly been performed with anti-carcinoembryonic antigen (CEA) antibodies in xenograft models of colon carcinoma for up to 48 h p.i. [57, 59, 69-72]. In the current work, the tumor uptake in the syngeneic rat colon carcinoma model was monitored for up to one week. The distribution was studied at sub-therapeutic activities, 25-50 MBq/kg, to circumvent the effects of irradiation interfering with the results. The study was performed in two parts, and the administered activity was lowered in the second experiment to further decrease the effects of irradiation. The time points were also adjusted to obtain better observations of the changes in activity distribution (Paper V).

After injection of the radioimmunoconjugate, three to six tumors were excised 2, 8, 24, 48, 72, 96, 120, and 168 h p.i., cut in halves, and snap-frozen, as described in Paper V. The tumors were cryosectioned, and 10-h measurements were made on three sections per tumor with the digital autoradiography system. Adjacent sections were stained with Mayer's hematoxylin and Chromotrope 2R for histo-

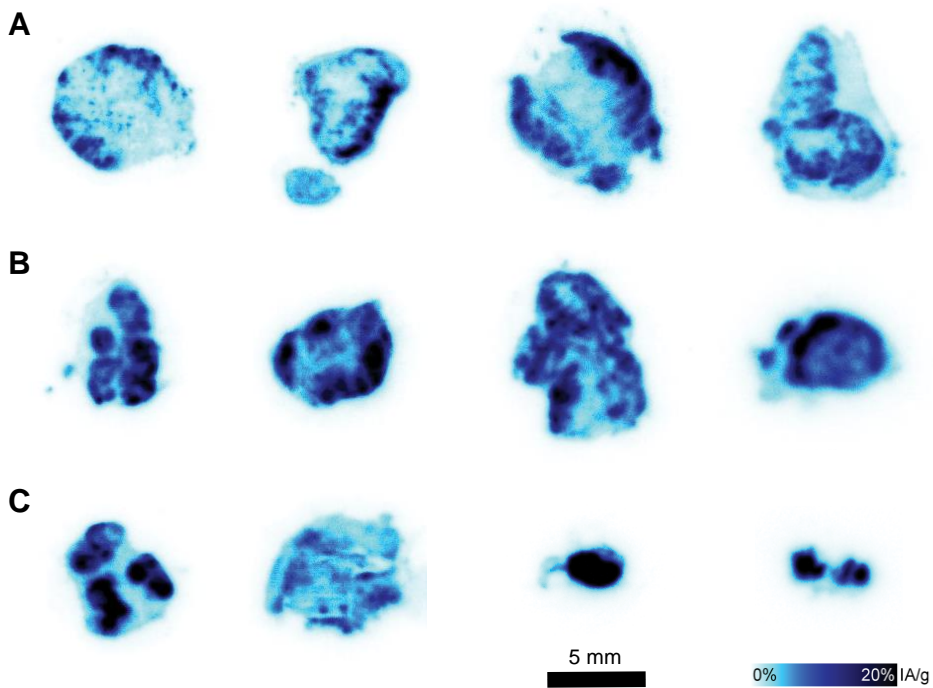
logical examination. In addition, immunohistochemistry was used for detection of the target antigen, blood vessels, and proliferating cells on additional sections from the same series. Blood samples drawn 2 min p.i. and before sacrifice were used for activity measurements, together with tumor halves not used for cryosectioning.

In tumors excised 2 and 8 h p.i., the activity was mainly located in the tumor margins (Figure 7A). The LeY antigen was detected in the membranes of all tumor cells in these tumors. Histological examination revealed dense tumor growth with necrosis in the central parts, as in untreated tumors. The tumors were well vascularized, although it is not known whether the vessels were functional or not. Proliferating cells were detected in all areas of the tumors, as expected considering the rapid tumor growth. After 24-48 h, the activity was found in more central areas, but higher levels were still detected in the periphery of most of the samples (Figure 7B). These tumors were also well vascularized. The tumor histology showed a change from dense tumor growth to areas of granulation tissue, somewhere between 24 and 48 h. The cells in areas of granulation tissue lacked the target antigen. This observation corresponds to the findings in tumors from animals treated with unlabeled BR96. At 24 h p.i., the activity hotspots were correlated with areas of dense tumor growth, which is in contrast to the correlation of hotspots with granulation tissue observed 48 h p.i. The dose of BR96 used for radioimmunotherapy, 0.6 mg/kg body weight radioimmunoconjugate (150  $\mu$ g for rats with an approximate body weight of 250 g), was between the evaluated doses of unlabeled BR96. However, the therapeutic effects seen in these experiments were probably the result of an immunological response, since the administered activities were lower than the activities seen to result in complete response of the tumors (see the chapter on Single Radioimmunotherapy).

In studies of anti-CEA mAbs in xenograft models, a high uptake has been seen 24 h p.i. in intrahepatic tumors [57, 59, 72], which is in line with the results of the present study. Uptake has been found to be lower at the same time point in subcutaneous tumors [55, 71]. In another study, the activity was found to be located close to blood vessels 24 h p.i., but the distribution became more homogenous 48 h p.i. [69]. Although the tumors of the BN rat model resemble poorly differentiated human colon adenocarcinoma, the observed intratumoral distribution of activity was more like that in well-vascularized and differentiated tumors than poorly differentiated tumors with abnormal vasculature in xenograft studies [71].

Large variations were seen 72 h p.i. and later (Figure 7C). For example, 11 of the 18 tumors had decreased in volume, while three had continued to grow. This will most likely affect the evaluation of activity uptake, making it difficult to draw any conclusions from the data. The activity in tumors that continued to grow was

generally lower than in tumors that had decreased in size after the treatment. Changes in tumor volume will affect the correlation between absorbed dose calculations and treatment outcome [73]. The small tumors contained few blood vessels, a low fraction of proliferative tumor cells, and a large proportion of granulation tissue and stroma. The activity was often accumulated in areas of granulation tissue, which has not been seen in other studies. The opposite was observed in the large tumors, which had dense areas of proliferating tumor cells with maintained antigen expression. In xenograft models, the accumulation of activity has been observed in necrotic areas 48 h p.i. and later, although this was probably due to the pooling of free iodine in blood [57, 70].



**Figure 7.** Activity distribution in tumors excised 8 h p.i. (A), 24-48 h p.i. (B), and 72-168 h p.i. (C).

# Single radioimmunotherapy

Radioimmunotherapy is most commonly given as a single dose. The choice of radionuclide is of importance for the therapeutic efficacy. Beta-particle-emitting radionuclides have a longer particle range resulting in crossfire, which may compensate for heterogeneous tumor uptake. On the other hand, alpha-particle-emitting radionuclides are more efficient in causing lethal cell damage. The beta-emitter  $^{177}\text{Lu}$  has a maximal particle path length of 1.8 mm in soft tissue and a physical half-life of 6.7 days. It is considered a suitable candidate for radioimmunotherapy, and is also being evaluated for peptide receptor radionuclide therapy [74]. The alpha-emitting radionuclide  $^{211}\text{At}$  has a particle path length of less than 70  $\mu\text{m}$  and a physical half-life of 7.2 h. Both these radionuclides were evaluated for radioimmunotherapy in the rat colon carcinoma model with regard to their therapeutic effects and toxicity.

## Study design

The maximal tolerable dose<sup>2</sup>, defined as the highest injected activity allowing 100% survival without any signs of infection, bleeding, or diarrhea, and with <20% body weight loss, has previously been determined to be 600 MBq/kg body weight of  $^{177}\text{Lu}$ -labeled BR96 in the syngeneic rat model [75]. At that activity, complete response was observed in all animals, but with subsequent development of metastases in half of the animals. In order to decrease the toxicity resulting from a single treatment and repeated radioimmunotherapy, the minimal effective activity, defined as the lowest administered activity resulting in complete response in five out of six animals, was determined in the present work. Four different activities of  $^{177}\text{Lu}$ -BR96 between zero and the established maximal tolerable dose were evaluated: 60, 135, 270, and 400 MBq/kg body weight (Paper I). DOTA-BR96 was used as a control.

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<sup>2</sup> The maximal tolerable dose is an established concept in pharmacology, and here refers to the activity rather than the absorbed dose.

A suitable activity of  $^{211}\text{At}$ -BR96 was calculated based on biodistribution data for  $^{177}\text{Lu}$ -BR96, the absorbed dose to red bone marrow from  $^{177}\text{Lu}$ -BR96, and data on the maximal tolerable dose of  $^{211}\text{At}$  to bone marrow, as described in Paper III. The resulting activity was 5 MBq/animal (*i.e.* about 20 MBq/kg body weight)  $^{211}\text{At}$ -BR96. The effect of 2.5 MBq (*i.e.* about 10 MBq/kg) was also investigated, and 150  $\mu\text{g}$  unconjugated BR96 was used as a control. The characteristics of the animals and tumors on the day of treatment (day 0) are listed in Table 2.

After injection of the radioimmunoconjugate, body weight and tumor volume were determined twice per week. Myelotoxicity was monitored by counting blood cells with a Vet 530CA Medonic Cell Analyzer (Boule Medical, Stockholm, Sweden) twice per week for the first four weeks p.i., and then once per week until the end of the study. Blood samples were obtained by inserting a cannula in the tail artery and collecting 0.2 mL blood in EDTA-coated test-tubes. The study was ended 100 days p.i., as it had been seen in a previous study lasting 180 days, that metastatic disease was detectable within 100 days p.i. [75].

## Toxicity resulting from treatment

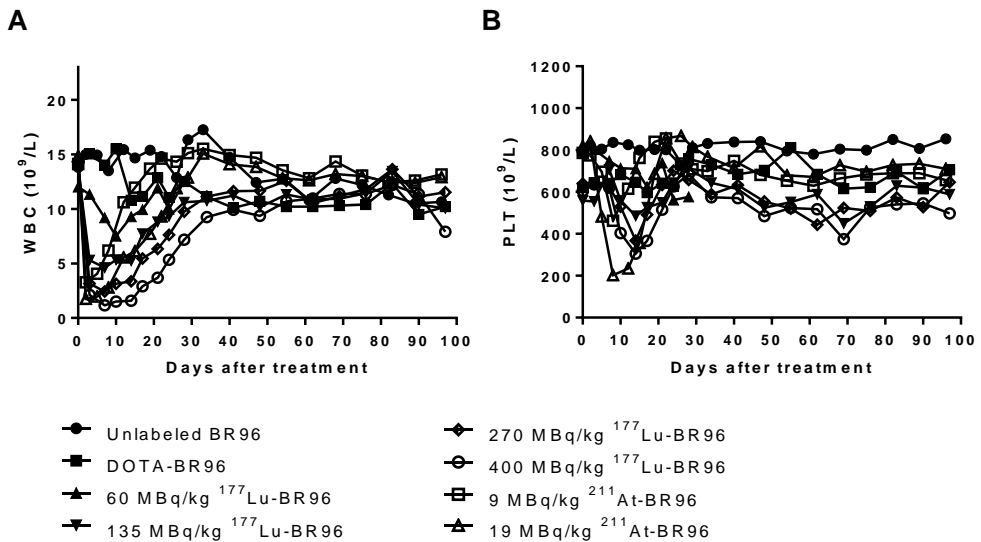
Body weight was affected in a dose-dependent manner with both the radionuclides studied ( $p=0.0002$ , one-way ANOVA), with up to 9% loss on the first occasion (2-3 days) p.i. Rats given unlabeled BR96 had gained 1% in body weight at the corresponding time point. All animals recovered from the weight loss. Later in the studies, weight loss was related to tumor burden and the detection of metastatic disease.

**Table 2.** Characteristics of the various groups of animals on the day of administration of radioimmunotherapy (median and range)

<b>Group</b>	<b>Body weight (g)</b>	<b>Tumor volume (mm<sup>3</sup>)</b>	<b>IA/kg body weight (MBq/kg)</b>
Unlabeled BR96 (n=6)	291 (282-300)	535 (282-640)	-
DOTA-BR96 (n=5)	256 (229-267)	551 (32-720)	-
<b><math>^{177}\text{Lu}</math>-BR96</b>			
60 MBq/kg (n=6)	243 (236-266)	936 (486-2462)	64.4 (54.9-71.7)
135 MBq/kg (n=6)	272 (256-283)	806 (440-1037)	131.4 (120.5-140.2)
270 MBq/kg (n=6)	235 (234-242)	650 (294-1014)	276.3 (266.1-280.2)
400 MBq/kg (n=6)	246 (240-254)	580 (292-979)	389.7 (384.4-395.6)
<b><math>^{211}\text{At}</math>-BR96</b>			
9 MBq/kg (n=6)	271 (269-278)	540 (256-640)	9.3 (9.0-9.6)
19 MBq/kg (n=6)	261 (247-266)	370 (176-520)	19.3 (18.3-19.9)

In radioimmunotherapy, bone marrow is the dose-limiting organ. The effects on bone marrow were measured by counting red blood cells, white blood cells and platelets in peripheral blood. The number of red blood cells was stable during the experiment, apart from the acute decrease associated with ascites due to abdominal metastases. White blood cell counts decreased rapidly after the injection of the radioimmunoconjugates (Figure 8A), nadirs being detected 2 days after the injection of either activity of  $^{211}\text{At}$ -BR96, 10 days after the injection of 60 MBq/kg  $^{177}\text{Lu}$ -BR96 and 7 days after the injection of 135-400 MBq/kg  $^{177}\text{Lu}$ -BR96. The number of white blood cells recovered faster following treatment with  $^{211}\text{At}$ -BR96 than with  $^{177}\text{Lu}$ -BR96, despite similar values of the nadir. This may be due to the difference in physical half-life of the two radionuclides, as  $^{211}\text{At}$  has a shorter half-life ( $t_{1/2}=7.2$  h) than  $^{177}\text{Lu}$  ( $t_{1/2}=6.7$  days). The number of platelets also decreased in a dose-dependent manner after treatment (Figure 8B), to approximately 50% of the initial value, on day 14 after the highest activity of  $^{177}\text{Lu}$ -BR96, to 50% on day 8 after administration of the lower activity of  $^{211}\text{At}$ -BR96, and to 25% on day 8 after treatment with the higher activity of  $^{211}\text{At}$ -BR96. The number of platelets returned to the initial value within one month in all animals.

Evaluation of liver and kidney toxicity after treatment with the higher activity of  $^{211}\text{At}$  (20 MBq/kg body weight) showed transient grade 1 liver toxicity (National Cancer Institute Common Terminology for Adverse Events version 4.0), but no effect on kidney function. Previous evaluation of toxicity after treatment with 600 MBq/kg  $^{177}\text{Lu}$ -BR96 did not reveal any toxicity above grade 1 in any organ [75].



**Figure 8.** White blood cell (WBC) counts (A) and platelet (PLT) counts (B) after radioimmunotherapy, used for monitoring of myelotoxicity.



**Table 3.** Response to treatment after a single administration of radioimmunotherapy

Group	Complete response	Stable disease	Progressive disease	Local recurrence	Metastases	Complete response seen on day
Unlabeled BR96 (n=6)	1/6	0/6	5/6	0/1	5/6	12
DOTA-BR96 (n=5)	0/5	0/5	5/5	n/a	2/5	-
<b><sup>177</sup>Lu-BR96</b>						
60 MBq/kg (n=6)	0/6	0/6	6/6	n/a	5/6	-
135 MBq/kg (n=6)	2/6	4/6*	0/6	0/2	4/6	7, 7
270 MBq/kg (n=6)	4/6	1/6	1/6	1/4	3/6	3, 7, 14, 14
400 MBq/kg (n=6)	5/6	1/6	0/6	1/5	4/6	7, 10, 14, 14, 14
<b><sup>211</sup>At-BR96</b>						
9 MBq/kg (n=6)	5/6	0/6	1/6	1/5	3/6	5, 5, 8, 12, 26
19 MBq/kg (n=6)	5/6	1/6	0/6	1/5	2/6	5, 8, 12, 22, 26

\* One tumor was stable for 76 days and then started to progress rapidly.

The absorbed dose of <sup>177</sup>Lu-BR96 was determined using a method described by Larsson *et al.* [76], and resulted in an absorbed dose of 0.55 Gy/MBq in the tumor and 0.40 Gy/MBq in bone marrow.

## Therapeutic response

A dose-dependent tumor response was seen following the administration of <sup>177</sup>Lu-BR96 (Table 3). The minimal effective activity of <sup>177</sup>Lu-BR96 was determined to be 400 MBq/kg, as this administered activity resulted in complete response of local tumors in five out of six rats. This activity is  $0.67 \times$  the maximal tolerable dose [75], thus showing that additional radioimmunotherapy is possible. Both the activities of <sup>211</sup>At-BR96 evaluated (9 and 19 MBq/kg) resulted in complete response of local tumors in five out of six animals (Table 3), and the lower activity, of 9 MBq/kg, was thus established as the minimal effective activity. In comparison, the DOTA-BR96 itself did not result in any therapeutic response, while 150  $\mu$ g unlabeled BR96 (0.6 mg/kg body weight) resulted in complete response in one out of six tumor-bearing animals. The first day on which the tumor was non-palpable ranged from as early as three days up to 26 days after injection; the longest time was found in animals treated with <sup>211</sup>At-BR96. Recurrence of local

disease was observed in several groups, showing that not all the tumor cells had been eradicated by the radioimmunotherapy.

Metastases were found in approximately half the animals (Table 3), with a higher incidence among animals given unlabeled BR96 and the lowest activity of  $^{177}\text{Lu}$ -BR96. There was a tendency towards a reduction in tumor growth rate in these groups, allowing metastases to reach detectable sizes before sacrifice. However, the number of animals in each group was too small to draw any reliable conclusions. The main location of metastases was lymph nodes, but tumor growth was also detected in the liver and in the abdomen. The rate of survival was significantly higher in animals receiving the higher activities of  $^{177}\text{Lu}$ -BR96 and  $^{211}\text{At}$ -BR96 ( $p < 0.0001$ , log-rank test; Figure 9). Since the tumors grow rapidly in animals not given radioimmunotherapy, these rats might have been sacrificed before metastases could be detected. Thus, the number of untreated animals developing metastatic disease was not determined. In addition, it has not yet been investigated when during primary tumor growth dissemination starts, or whether the rapid regression after radioimmunotherapy affects dissemination in any way in the BN tumor model.

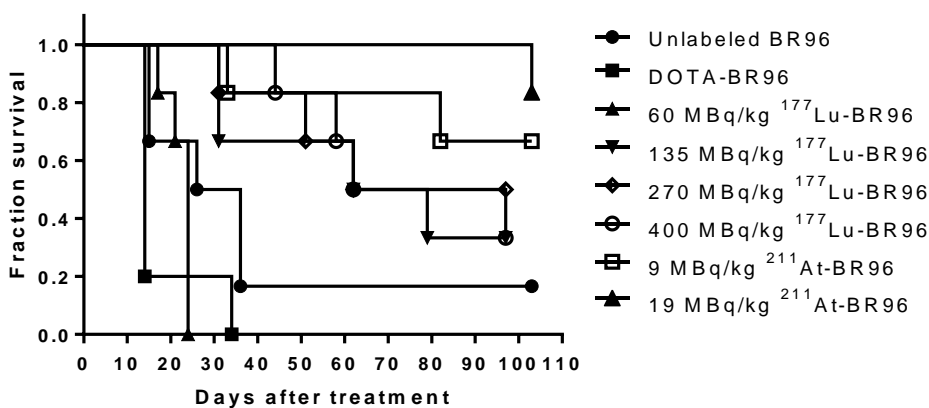
All tumor findings evaluated immunohistochemically expressed the target antigen. After 400 MBq/kg  $^{177}\text{Lu}$ -BR96, more than half of the samples showed complete membrane staining on at least 50% of the tumor cells, and on more than 90% in one third of the samples. Samples from animals treated with  $^{211}\text{At}$ -BR96 showed more than 50% antigen-expressing tumor cells in half of the samples. However, the number of positive cells was less than 10% in one third of the tumor tissues from these groups. In comparison, all tumor samples from the group treated with unlabeled BR96 showed complete membrane staining of at least 50% of the tumor cells, and staining of more than 90% of the tumor cells in half of the samples. This indicates that a second treatment directed at the same target might be more favorable after  $^{177}\text{Lu}$ -BR96 than  $^{211}\text{At}$ -BR96.

In clinical studies, radioimmunotherapy of colon carcinoma has often resulted in poor response [24], probably due to the relatively large tumor volumes in those studies. Radioimmunotherapy is more appropriate as an adjuvant treatment, or for the treatment of small tumor lesions. This approach has been evaluated in rats with liver metastases, where  $^{177}\text{Lu}$ -mAbs were seen to delay tumor growth in animals with microscopic tumors, but had no effect in animals with larger lesions [77]. Radioimmunotherapy with  $^{177}\text{Lu}$ -mAbs prolonged survival after resection of macroscopic liver metastases in the same syngeneic animal model [78]. The same group demonstrated that adjuvant treatment with  $^{177}\text{Lu}$ -mAbs in a model of anastomosis prevented local recurrence [79] and prolonged survival [80]. Prolonged survival was also observed in a clinical study employing radioimmuno-

therapy with  $^{131}\text{I}$ -anti-CEA mAbs after resection of liver metastases, compared to a control group treated with liver resection only [81].

The therapeutic effect of  $^{211}\text{At}$ -mAbs has not previously been examined in any colon carcinoma models. Preclinical studies on ovarian cancer have shown growth inhibition of subcutaneous xenograft tumors by i.v. radioimmunotherapy [82] and eradication of peritoneal tumor growth when the radioimmunoconjugate was administered intraperitoneally [83-85]. The positive results have been translated to a phase I study with i.p. administration of  $^{211}\text{At}$ -radioimmunoconjugates against ovarian carcinoma [86].

When  $^{177}\text{Lu}$ -labeled mAbs and the alpha-emitting  $^{213}\text{Bi}$ -labeled mAbs were compared in a murine xenograft model of peritoneal growth of gastric cancer,  $^{177}\text{Lu}$ -mAbs were found to be more toxic than  $^{213}\text{Bi}$ -mAbs at activities resulting in corresponding treatment effects [87]. The difference in myelotoxicity was probably caused by the much longer physical half-life of  $^{177}\text{Lu}$  (6.7 days) than the short half-life of  $^{213}\text{Bi}$  (46 min). The corresponding observation was made in the present work, with  $^{211}\text{At}$ -mAbs causing less myelotoxicity and faster recovery than  $^{177}\text{Lu}$ -mAbs at activities resulting in the same tumor response.



**Figure 9.** Survival after single treatment with the radioimmunoconjugates  $^{177}\text{Lu}$ -BR96 or  $^{211}\text{At}$ -BR96.

# Combination treatment

The development of metastatic disease after a single treatment with  $^{177}\text{Lu}$ - or  $^{211}\text{At}$ -BR96 indicates that one treatment is not sufficient to eradicate all the tumor cells. In the clinical setting, radioimmunotherapy of lymphoma is combined with unlabeled mAbs, in that a pre-dose of unlabeled mAb is given to block binding to healthy cells. Since unlabeled mAbs also have an anti-tumor effect [44], it may be possible to enhance the effect of radioimmunotherapy by treatment with unlabeled mAbs afterwards. Fractionation, *i.e.* giving repeated small doses, has been proposed as a means of increasing the total administered activity, increasing the tumor uptake, and reducing toxicity [88]. A combination of radionuclides with different properties has also been proposed as a way of enhancing the effect of treatment and to target lesions of various sizes [89].

In the present studies, therapeutic effects and the toxicity of unlabeled BR96,  $^{177}\text{Lu}$ -BR96, or  $^{211}\text{At}$ -BR96 given after treatment with  $^{177}\text{Lu}$ -BR96 were evaluated in the syngeneic rat tumor model. A regimen preventing the development of rat anti-human antibodies (RAHAs) was also established in order to prevent a decrease in the efficacy of the second dose. To decrease toxicity caused by free  $^{211}\text{At}$ , a halogen blocking agent was given.

## Prevention of RAHAs

The immune system is programmed to recognize and destroy anything that is of foreign origin. The development of human anti-mouse antibodies against therapeutic antibodies of murine origin has been seen in clinical studies [90, 91]. This will result in elimination of the drug, thereby decreasing the therapeutic response. As a consequence, most mAbs approved for clinical use today, or being developed, are either partly (chimeric or humanized) or completely human.

An alternative way of preventing an immune response to the radioimmunoconjugate is to treat the patient with an immunosuppressive agent. In the present studies, Cyclosporin A was used as it has previously been proven to prevent an immune response in clinical studies of radioimmunotherapy [92-94] and in pre-clinical studies of immunotoxins in rats [95]. It inhibits the activation of T-cells by

binding to a protein in the signaling pathway responsible for the production of pro-inflammatory cytokines, and is widely used to prevent the rejection of organ transplants [96].

The rats were given 10 mg/kg body weight Cyclosporin A (Sandimmun®, Novartis) for five consecutive days, starting one day before injection of the first radioimmunoconjugate. To verify the effect, plasma samples were drawn three days before and ten days after injection of the radioimmunoconjugate (Paper II). The presence of RAHAs was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) method. First, 96-well plates were coated with DOTA-BR96. The plasma samples were then added to the coated wells, allowing binding of the RAHAs, which were then detected by the addition of an anti-rat IgG, which in this case was conjugated to an enzyme (HRP). The enzyme catalyzes the formation of a colored product when the substrate is added, and the increase in absorbance of the product can be determined using a photometer. The concentration of RAHAs is then calculated by comparing the absorbance of the sample to that of a sample with known concentration measured simultaneously. The results showed that the concentration of RAHAs before administration of the radioimmunoconjugate was approximately 2 µg/mL plasma. Treatment with Cyclosporin A resulted in 1.7 µg RAHAs /mL ten days after radioimmunotherapy, while animals not given any immunosuppressive treatment had on average 79 µg RAHAs/mL plasma, which was significantly higher ( $p < 0.0001$ , Mann–Whitney test); see Paper II, Figure 3. The sustainability of the effect was tested by analyzing plasma samples drawn two weeks after the administration of unlabeled BR96, which was given as a consolidation therapy two weeks after the radioimmunotherapy. The results showed plasma levels of RAHAs comparable to those in animals not given any Cyclosporin A ten days after radioimmunotherapy. This implies that Cyclosporin A should be administered prior to all stages of treatment except the last one. In addition, the levels of unlabeled BR96 in animals given Cyclosporin A treatment together with radioimmunotherapy were compared three days after administration with those in animals not given any immunosuppressant treatment at the time of radioimmunotherapy. This was done using a similar ELISA method, in which anti-human IgG was used to coat the wells and HRP-conjugated anti-human IgG was used for detection. The plasma concentration of unlabeled BR96 was significantly lower in animals not given Cyclosporin A (66 µg/mL vs. 82 µg/mL,  $p = 0.0021$ , Mann–Whitney test). Since the treatment proved to be effective, Cyclosporin A was also used in studies of repeated administration of radioimmunoconjugates (Papers I and IV).

Tumors regressed more slowly in animals treated with Cyclosporin A ( $p = 0.0002$ , Mann–Whitney test). The first day with a non-palpable tumor was recorded 10-28 days after radioimmunotherapy, compared to 3-10 days in animals not given Cyclosporin A, in the same experiment (see Paper II, Figure 1). One possible

explanation of the delay in regression is that the immune defense involved in the degradation of dead tumor cells is not activated to the same extent when the T-cells are suppressed. Cyclosporin A has been reported to act synergistically with immunotoxins in a previous preclinical study on metastatic cervix carcinoma, thereby prolonging survival [95]. In another study, in which 20 mg Cyclosporin A/kg body weight was administered to rats for 30 days, both local tumor growth and the number of metastases increased [97]. In the present work (Paper II), the Cyclosporin A treatment did not affect the number of animals showing complete response, recurrence of local disease, or metastatic disease.

In a previous study of immunoconjugates in the BN rat tumor model, the 15-deoxyspergualin used to suppress the immune response was seen to act synergistically with the drug conjugate [42]. Cyclosporin A was considered preferable in the present work since synergistic effects would have confounded the evaluation of the additional administration of unlabeled and radiolabeled mAbs after radioimmunotherapy.

## Study design

The activities administered in these studies were based on the results of single treatment with  $^{177}\text{Lu}$ -BR96 and  $^{211}\text{At}$ -BR96 described above (Papers I and III). The intention was to first induce initial regression of tumors approximately 10 mm in diameter with a high proportion of complete responses, by administering the minimal effective activity 400 MBq/kg body weight  $^{177}\text{Lu}$ -BR96. Additional treatment was administered when any remaining tumors were small; the timing depending on the need for bone marrow recovery. The same dose of unlabeled BR96 previously found to result in a transient tumor response [46], 15 mg/kg body weight, was given two weeks after radioimmunotherapy (Paper II). A second administration of  $^{177}\text{Lu}$ -BR96, with an activity of either 150 or 350 MBq/kg, equivalent to 0.9 and 1.25 times the maximal tolerable dose in single treatment, was given three weeks after the first treatment (Paper I). The second administration of  $^{177}\text{Lu}$ -BR96 was delayed compared to that of unlabeled mAbs to allow further bone marrow recovery. In the last study, either 5 or 10 MBq/kg body weight  $^{211}\text{At}$ -BR96 was administered 25 days after the first radioimmunotherapy, either with or without halogen blocking (Paper IV). Halogen blocking was performed by intraperitoneal administration of 35 mg/kg sodium perchlorate, and has been used previously to lower the uptake of free astatine in the thyroid, stomach and lungs [98, 99]. The group not given halogen blocking was included since no such toxicity was noted in rats treated with a single administration of  $^{211}\text{At}$ -BR96 without halogen blocking (Paper III).

**Table 4.** Characteristics of the groups of animals on the day of treatment with 400 MBq/kg <sup>177</sup>Lu-BR96 (median and range)

Group	n	Body	Tumor	IA (MBq)	IA/kg
		weight (g)	volume (mm <sup>3</sup> )		(MBq/kg body weight)
400 MBq/kg <sup>177</sup> Lu-BR96	29	251 (203-307)	486 (115-1490)	100.4 (95.22-108.0)	403.0 (323.0-515.8)
+ CsA	31	258 (219-288)	520 (255-920)	100.5 (89.48-107.6)	395.9 (332.6-475.6)
+ 15 mg/kg BR96	19	255 (228-291)	680 (130-1487)	99.88 (90.22-106.1)	386.7 (343.2-440.0)
+ 150 MBq/kg <sup>177</sup> Lu-BR96	10	238 (215-270)	235 (11-726)	99.89 (95.27-104.4)	419.4 (372.5-485.4)
+ 350 MBq/kg <sup>177</sup> Lu.BR96	10	227 (204-245)	294 (11-680)	102.4 (94.86-105.9)	449.2 (417.4-502.8)
+ 5 MBq/kg <sup>211</sup> At-BR96	16	257 (224-268)	743 (324-1094)	98.58 (95.91-104.9)	382.6 (361.8-444.8)
+ 10 MBq/kg <sup>211</sup> At-BR96	14	262 (244-273)	726 (384-1149)	99.56 (93.12-104.9)	377.1 (347.5-422.8)
+ 10 MBq/kg <sup>211</sup> At-BR96, no halogen blocking	15	262 (247-282)	600 (230-968)	103.3 (98.50-110.4)	390.5 (369.0-434.0)

CsA = Cyclosporin A

The characteristics of the various groups of animals on the day of the first and second treatments are listed in Tables 4 and 5, respectively. Cyclosporin A was administered as described above in order to avoid the development of RAHAs. In the first two studies (Papers I and II), the immunosuppressant was only given to animals receiving a second administration of mAbs, as this would be unnecessary in animals only receiving one treatment. However, in a later study (Paper IV), the immunosuppressant was given to all animals, including the reference group, in an attempt to reduce the differences between the groups. Data from animals given 400 MBq/kg <sup>177</sup>Lu-BR96 with and without Cyclosporin A treatment are presented separately in all tables and figures.

Tumor volume and body weight were recorded twice per week throughout the studies. Myelotoxicity was monitored by blood cell counts using the Medonic Cell Analyzer. The blood samples were drawn through a cannula inserted into the tail artery, and 0.2 mL blood was drawn into EDTA-coated test-tubes. This was done twice per week for the first four weeks after the last administration, and then once per week until the end of the study. The animals were monitored for approximately 100 days after the second treatment. Tumor findings were recorded at autopsy.

**Table 5.** Group characteristics at the time of the second administration of mAbs. Unlabeled BR96 was given on day 14, <sup>177</sup>Lu-BR96 on day 21, and <sup>211</sup>At-BR96 on day 25 after the first treatment with 400 MBq/kg <sup>177</sup>Lu-BR96.

Group	Body weight (g)	Complete response	IA (MBq)	IA/kg (MBq/kg body weight)
400 MBq/kg <sup>177</sup> Lu-BR96	n/a	26/29 (90%)	-	-
+ CsA	285 (226-322)	25/31 (81%)	-	-
+ 15 mg/kg BR96	267 (235-310)	10/19 (53%)	-	-
+ 150 MBq/kg <sup>177</sup> Lu-BR96	261 (231-291)	9/10 (90%)	37.56 (35.11-40.91)	149.4 (127.6-168.1)
+ 350 MBq/kg <sup>177</sup> Lu-BR96	252 (230-269)	8/10 (80%)	88.46 (82.78-89.51)	351.7 (310.1-384.8)
+ 5 MBq/kg <sup>211</sup> At-BR96	289 (240-305)	12/16 (75%)	1.5 (1.3-1.7)	5.2 (4.4-6.8)
+ 10 MBq/kg <sup>211</sup> At-BR96	293.5 (263-317)	12/14 (86%)	2.9 (2.7-3.1)	9.8 (8.9-10.7)
+ 10 MBq/kg <sup>211</sup> At-BR96, no halogen blocking	295 (279-309)	12/15 (80%)	3.1 (3.0-3.3)	10.8 (9.9-11.3)

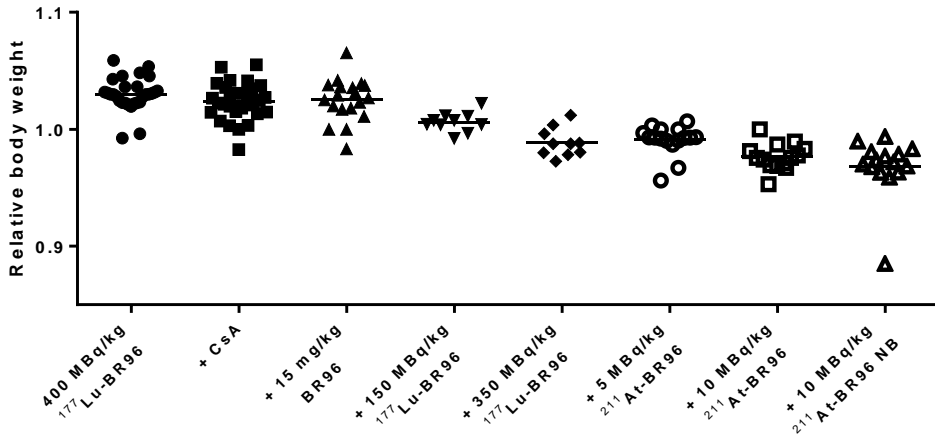
CsA = Cyclosporin A

## Toxicity resulting from treatment

Body weight decreased by less than 10% after the administration of 400 MBq/kg <sup>177</sup>Lu-BR96; the nadir being seen on the first occasion (3 days) after treatment. The second administration of 350 MBq/kg <sup>177</sup>Lu-BR96 resulted in a smaller decrease in body weight compared to the first treatment. Animals given the lower activity of <sup>177</sup>Lu-BR96 increased in weight after treatment, although not as much as the reference group. The decrease in body weight after treatment with <sup>211</sup>At-BR96 was dose-dependent, and was more pronounced than in the group given the higher activity of <sup>177</sup>Lu-BR96 (Figure 10).

Severe loss of body weight was seen without the detection of metastases at autopsy after treatment (Table 6). About half of the animals in the group given 10 MBq/kg <sup>211</sup>At-BR96 without halogen blocking lost so much weight that they had to be sacrificed, weight loss starting between days 35 and 59 (Paper IV, Figure 2). Administration of halogen blocking decreased the number of animals showing severe loss of body weight, and only three out of 14 animals in the group given 10 MBq/kg <sup>211</sup>At-BR96 with halogen blocking had to be sacrificed before the end of the study (weight loss starting between days 42 and 52). Severe weight loss was

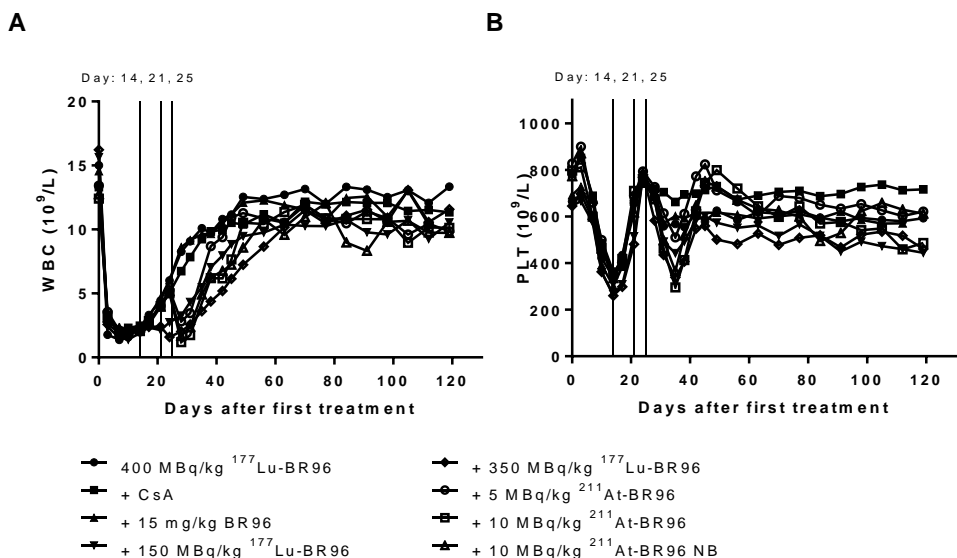




**Figure 10.** Difference between body weight 3 days after administration of the second treatment and on the day of treatment. CsA = Cyclosporin A; NB = no halogen blocking

also seen in the group given the lower activity of  $^{211}\text{At-BR96}$ , and the groups given a second treatment with  $^{177}\text{Lu-BR96}$ . In addition, severe weight loss was noted in the reference group given Cyclosporin A. The mechanisms causing the weight loss is not yet determined. The small intestine was histologically examined in some of the animals suffering from weight loss and compared to the reference group, but there were no signs of pathological changes in the villi or crypts in any of the animals examined

Both white blood cell and platelet counts decreased after radioimmunotherapy (Figure 11). The first administration of 400 MBq/kg  $^{177}\text{Lu-BR96}$  resulted in an immediate decrease in white blood cells; the nadir being seen on days 7-10 p.i. The reference groups not given any further antibody treatment and the group given 15 mg/kg unlabeled BR96 on day 14 started to recover after day 14, and their white blood cell counts stabilized between days 38 and 50 p.i. Administration of 150 MBq/kg  $^{177}\text{Lu-BR96}$  resulted in a minor decrease in white blood cell count, and delayed the recovery to baseline levels to day 56. Treatment with the higher activity of  $^{177}\text{Lu-BR96}$ , 350 MBq/kg and with  $^{211}\text{At-BR96}$  resulted in a decrease in white blood cell count, with a nadir 3 days p.i. After treatment with 350 MBq/kg  $^{177}\text{Lu-BR96}$  the white blood cell count reached stable levels on day 63. This was later than after treatment with  $^{211}\text{At-BR96}$ , and can probably be explained by the much longer physical half-life of  $^{177}\text{Lu}$ . Treatment with  $^{211}\text{At}$  resulted in a dose-dependent decrease in white blood cell count, which was recovered on days 45 (5 MBq/kg  $^{211}\text{At-BR96}$ ) and 49-52 (10 MBq/kg  $^{211}\text{At-BR96}$  with and without halogen blocking).



**Figure 11.** Myelotoxicity in terms of white blood cell counts (A) and platelet counts (B), after the different combinations of treatment. NB = no halogen blocking

The number of platelets decreased after treatment with 400 MBq/kg  $^{177}\text{Lu-BR96}$ , with the nadir on day 14. A small rebound effect was seen on day 24 before stabilization in groups not given a second treatment with radioimmunoconjugates. Although  $^{177}\text{Lu-BR96}$  and  $^{211}\text{At-BR96}$  were administered on different days after the first treatment, the second nadir was detected on day 35 in all groups. The decrease was dose dependent, with the lower activities of both  $^{177}\text{Lu-BR96}$  and  $^{211}\text{At-BR96}$  resulting in a smaller decrease than the higher activities. The number of platelets returned to initial values at the same time in all groups, namely on day 45. A late decrease in platelet counts was observed in one animal given 150 MBq/kg  $^{177}\text{Lu-BR96}$ , and in two animals given 10 MBq/kg  $^{211}\text{At-BR96}$ , starting on days 77, 98, and 63, respectively. This was associated with a decrease in red blood cell count in one of the animals. The red blood cell count was unaffected by the treatments in all other animals.

## Therapeutic response

The initial treatment with 400 MBq/kg  $^{177}\text{Lu-BR96}$  resulted in a high number of complete responses before administration of the second treatment (Table 5). The lower rate of response noted in the group given unlabeled BR96 (53%) can at least partly be explained by the earlier recording of this, as the unlabeled treatment was

given on day 14 (*i.e.* at least one week earlier than treatment with radiolabeled BR96). The reference group in the same experiment was not given any Cyclosporin A, which was found to delay tumor regression, as discussed above. In all other groups, the proportion of animals showing complete response of the inoculated tumor was at least 75%.

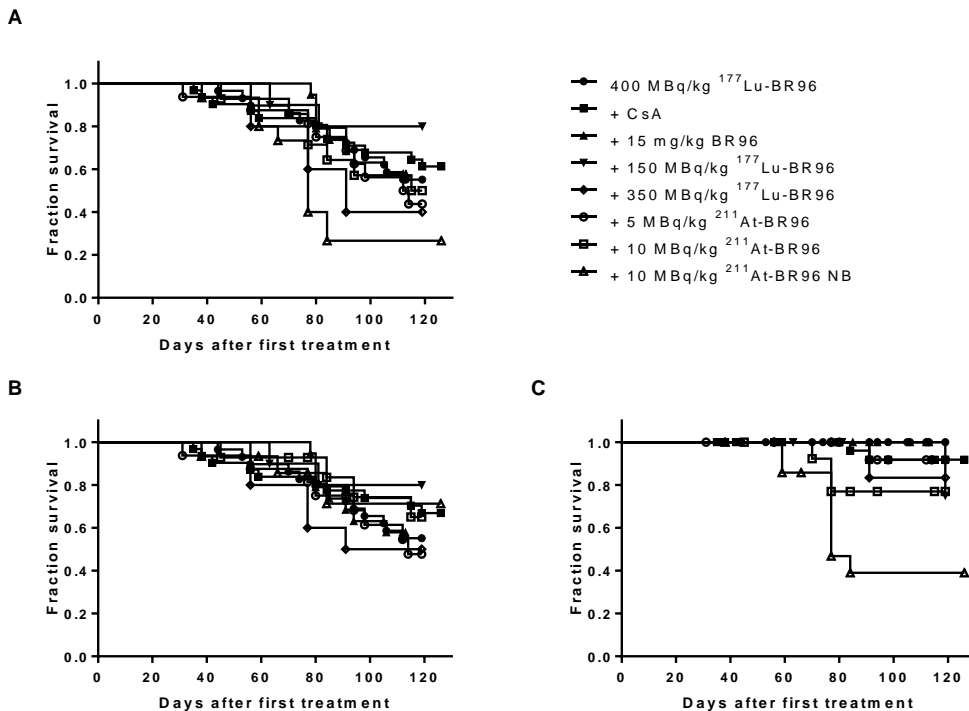
After the second treatment, the numbers of animals exhibiting complete response of the inoculated tumors increased in all groups (where possible), and no statistically significant differences were seen between the groups (Table 6). The remaining tumors, classified as progressive disease, all responded to the second treatment initially, but then started to grow again, in some cases requiring sacrifice of the animals before the end of the study. Recurrence of inoculated tumors was detected in six animals (Table 6).

Metastases were detected in all groups after treatment (Table 6). The proportion of affected animals ranged from 20-50%, but the differences were not statistically significant. The metastases were mainly found in the lymph nodes, lungs, liver, mesentery, and throughout the abdomen. The location did not vary between treatments or experiments. The lack of therapeutic effect on metastases was first thought to be an effect of the particle range of  $^{177}\text{Lu}$  being too long, resulting in energy deposition outside of the microscopic metastases. However, using the alpha-particle emitter  $^{211}\text{At}$  instead did not reduce the frequency of metastases nor the time at which they were detected. This indicates that radioimmunotherapy is not suited for the treatment of dormant tumor cells or non-vascularized metastases.

**Table 6.** Tumor response after all administrations of mAbs

<b>Group</b>	<b>Complete response</b>	<b>Progressive disease</b>	<b>Local recurrence</b>	<b>Metastases</b>	<b>Severe body weight loss</b>
400 MBq/kg $^{177}\text{Lu}$ -BR96	27/29 (93%)	2/29 (7%)	1/27 (4%)	14/29 (48%)	0/29 (0%)
+ CsA	27/31 (87%)	4/31 (13%)	1/27 (4%)	10/31 (32%)	2/31 (6%)
+ 15 mg/kg BR96	19/19 (100%)	0/19 (0%)	3/19 (16%)	9/19 (47%)	0/19 (0%)
+ 150 MBq/kg $^{177}\text{Lu}$ -BR96	10/10 (100%)	0/10 (0%)	1/10 (10%)	2/10 (20%)	2/10 (20%)
+ 350 MBq/kg $^{177}\text{Lu}$ .BR96	9/10 (90%)	1/10 (10%)	0/9 (0%)	5/10 (50%)	1/10 (10%)
+ 5 MBq/kg $^{211}\text{At}$ -BR96	14/16 (88%)	2/16 (13%)	0/14 (0%)	8/16 (50%) <sup>1</sup>	1/16 (6%)
+ 10 MBq/kg $^{211}\text{At}$ -BR96	13/14 (93%)	1/14 (7%)	0/13 (0%)	5/14 (36%) <sup>1</sup>	3/14 (21%)
+ 10 MBq/kg $^{211}\text{At}$ -BR96, no halogen blocking	14/15 (93%)	1/15 (7%)	0/14 (0%)	3/15 (20%) <sup>2</sup>	8/15 (53%)

<sup>1</sup>The value for the reference group in this experiment was 6/16 (38%). <sup>2</sup>The value for the reference group in this experiment was 4/15 (26%). CsA= Cyclosporin A



**Figure 12.** Survival of all animals (A), animals with tumor burden at sacrifice (B), and animals without tumor burden at sacrifice (C), after the different combinations of treatment. CsA = Cyclosporin A; NB = no halogen blocking

Survival was not prolonged by any of the additional treatments (Figure 12). The tumor-dependent survival (censored from toxicity-related death) was not significantly different between the groups subjected to various combinations of radioimmunotherapy. However, the severe weight loss seen in some animals resulted in a significantly worse survival in the group given  $^{211}\text{At}$ -BR96 without halogen blocking ( $p < 0.0001$ ) when animals sacrificed due to tumor burden were censored.

Antigen expression in tumors and metastases was investigated using immunohistochemistry. Generally, more than 50% of the tumor cells were positive in more than half of the samples. No differences were found between the groups within the individual experiments, indicating that the second administration of radioimmunotherapy did not result in further selection of antigen-negative cells.

If the initial treatment had resulted in a significant decrease in antigen expression, changing the mAb and target antigen in the second treatment would probably have increased the therapeutic effect. In the case of heterogeneous expression of the antigen, a mixture of targeting molecules directed towards different antigens could be used in an attempt to achieve better tumor uptake and therapy. It has been

shown that treatment with two different mAbs and a peptide, all labeled with  $^{213}\text{Bi}$ , resulted in improved tumor response in a xenograft model of prostate cancer [100]. However, such a combination is far from clinical implementation due to the few approved radioimmunoconjugates.

Fractionated treatment with an  $^{131}\text{I}$ -radioimmunoconjugate has been reported to result in better response of human colon xenografts in mice than a single administration [101]. Studies on the treatment of liver metastases in a xenograft model with three weekly injections of  $^{131}\text{I}$ -anti-CEA mAbs showed longer disease-free survival in mice with smaller metastases than in those with larger metastases [102]. The opposite has also been reported: the therapeutic efficacy in murine xenografts was found to be lower using fractionated radioimmunotherapy with  $^{131}\text{I}$ -labeled anti-CEA mAbs than when the total activity was given in one dose [103]. In a phase I study, repeated treatment with both  $^{177}\text{Lu}$ - and  $^{90}\text{Y}$ -labeled anti-prostate-specific membrane antigen (PSMA) was found to be tolerable regarding myelotoxicity, when  $0.5 \times$  the maximal tolerable dose of  $^{177}\text{Lu}$  and  $1 \times$  the maximal tolerable dose of  $^{90}\text{Y}$  were given per treatment [104].

Despite being proposed as a means of improving radionuclide therapy [89], few studies have been carried out on the effects of combinations of different radionuclides. The only studies published to date were on the combination of the two beta-emitting radionuclides  $^{177}\text{Lu}$  and  $^{90}\text{Y}$ , used to label somatostatin analogs. Both one and two tandem administrations of the two radiopeptides showed prolonged survival in rats with subcutaneous pancreas tumors than single and repeated administration of either of the radiopeptides [105]. A tendency towards prolonged overall survival has also been found in a clinical study following tandem administration of the two radiopeptides compared to administration of the  $^{90}\text{Y}$ -peptide alone [106]. Preliminary data from a clinical study on repeated treatment with both  $^{90}\text{Y}$ -labeled peptides and  $^{177}\text{Lu}$ -labeled peptides at fixed activities have shown limited toxicity and objective response in 67% of the patients [107]. Cyclic administration of up to four doses of  $^{90}\text{Y}$ -labeled peptides and  $^{177}\text{Lu}$ -labeled peptides has also been evaluated in patients with neuroendocrine tumors, and was reported to result in improved overall survival compared to patients receiving corresponding cycles of  $^{90}\text{Y}$ -peptides alone [108].

# Conclusions

Studies have been carried out on radioimmunotherapy with beta- and alpha-particle emitting radionuclides to determine differences in therapeutic effects and toxicity between the radioimmunoconjugates. Different combinations of radioimmunoconjugates and unlabeled mAbs were also compared. The main conclusions that can be drawn from this work are presented below.

The minimal effective activity of  $^{177}\text{Lu}$ -mAbs was determined to 400 MBq/kg in the rat colon carcinoma model, thus allowing for a second administration of radioimmunotherapy after induction of complete response. Radioimmunotherapy with both activities of the alpha-particle-emitting radionuclide ( $^{211}\text{At}$ -labeled mAbs) resulted in similar tumor response to that seen with the minimal effective activity of the beta-particle emitter ( $^{177}\text{Lu}$ -labeled mAbs). This implies that alpha emitters can be used for the treatment of tumors with a diameter of about 10 mm, despite generally being considered unsuitable for lesions of that size. The administered activity and choice of radionuclide did not affect the number of animals developing metastases.

Radioimmunotherapy with the alpha emitter  $^{211}\text{At}$  caused less toxicity than the beta emitter  $^{177}\text{Lu}$  at activities resulting in similar therapeutic effects. This is probably due to the shorter range and physical half-life of  $^{211}\text{At}$ , and further supports use of alpha emitters.

A second administration of either unlabeled mAbs or radiolabeled mAbs did not prevent the development of metastatic disease or prolong survival. This indicates that dormant tumor cells and non-vascularized metastases are not easily targeted. As previously discussed, this might be the result of a lack of vasculature and other biological differences between microscopic metastases and growing tumors [8]. It is clear that further evaluation and a better understanding of the properties of dormant tumor cells are required if we are to develop effective targeted therapies for these kinds of cells.

Treatment with two administrations of the same or different radioimmunoconjugates resulted in increased toxicity, seen as severe body weight loss in some animals. Elucidation of the mechanisms involved in weight loss is necessary in order to gain a better understanding of radiotoxicity.

The intratumoral uptake and distribution of radioimmunoconjugates is a dynamic process. Treatment of the tumor cells was most efficient during the first 24 h p.i., thus indicating that a radionuclide with a shorter physical half-life than  $^{177}\text{Lu}$  may be as effective. This could partly explain the efficacy of  $^{211}\text{At}$ . The activity remained in the areas where tumor cells had been eradicated.

# Future perspectives

The various forms of combined treatment did not prove to be effective in the studies presented in this thesis. The treatment protocols, in particular the timing of the second administration, could perhaps be modified to enhance the efficacy. This is especially true for unlabeled mAbs, where the temporal immune suppression by radioimmunotherapy may impair the therapeutic mechanisms. A longer delay in administration of the second dose of radioimmunotherapy may also reduce the toxicity, and result in better uptake when the metastases are vascularized.

To further evaluate the suitability of radioimmunotherapy in metastatic disease, an orthotopic animal model, with several tumor nodules of various sizes in the liver or lungs, could be developed and used. It would be very interesting to compare the efficacy of  $^{211}\text{At}$ -labeled and  $^{177}\text{Lu}$ -labeled radioimmunoconjugates in such models.

One obstacle that must be overcome regarding the use of  $^{211}\text{At}$  is its limited availability, as only a few cyclotrons are currently producing this radioisotope. The few alpha-emitting radionuclides that are being considered for radioimmunotherapy all have limitations, such as short half-life or daughter nuclides that may result in toxicity. Further studies are required to demonstrate which of these radionuclides can be used for targeted therapies.

As in the animal model used here, metastases are not found in all patients, and these patients will be seriously over-treated if a radioimmunoconjugate is administered as an adjuvant treatment. In order to use radioimmunotherapy in the treatment of microscopic disease, it is necessary to develop predictive markers for the identification of patients who will benefit from such treatment. It is not currently known why some patients develop metastases, while others do not. Since approximately half of the animals in the animal model used in this work demonstrated metastases after radioimmunotherapy, this model can be used to study the mechanisms involved in metastatic development, including the immune system.

Radioimmunotherapy has not yet been approved for the treatment of solid tumors. Several clinical studies are ongoing [14] and will hopefully demonstrate whether radioimmunoconjugates are effective in the clinic. Their efficacy will depend on several factors, such as expression of the target antigen, the choice of radionuclide, and the identification of suitable patients. Improved results may be obtained by



combining mAbs directed against different antigens and/or labeled with radio-nuclides with different properties. Combinations with other forms of treatment, such as chemotherapy, immunotherapy, and therapy with tyrosine kinase inhibitors may further enhance efficacy, as has been demonstrated with external radiotherapy.

# Populärvetenskaplig sammanfattning

Cancer behandlas i första hand med kirurgi, cellgifter och extern strålbehandling. Under senare år har man dessutom utvecklat så kallade målsökande behandlingar, som binder till en viss struktur i tumören. De kan verka på olika sätt: antingen genom att blockera en signal i cellerna och på så vis hindra tumören från att växa, eller genom att stimulera patientens immunförsvar så att det bryter ner tumören. Man kan också använda dessa tumörbindande läkemedel som bärare av mycket giftiga ämnen för att styra dessa gifter till tumören och samtidigt försöka skydda frisk vävnad. Vid radioimmunoterapi använder man sig av antikroppar som känner igen strukturer på tumörcellernas yta som bärare av radioaktiva ämnen. På så vis kommer radioaktiviteten att ansamlas i tumören efter injektion och bestråla den inifrån. Detta kan framförallt vara användbart när det finns många små tumörer utspridda i kroppen, så kallade dottersvulster (metastaser). Den huvudsakliga biverkningen vid denna typ av behandling är att antalet blodkroppar sjunker till följd av att den känsliga röda benmärgen utsätts för bestrålning. Detta gör att man blir mer infektionskänslig och lättare får blödningar, och därför är det viktigt att man inte ger för mycket behandling.

Våra studier baseras på en djurmodell med råttor som bär tjocktarmstumörer på insidan av bukväggen. I studierna har vi använt radioaktiva ämnen med olika egenskaper. Lutetium-177 avger små partiklar, elektroner, som kan transporteras upp till 1,8 mm i vävnad. En fördel med denna räckvidd är att tumörceller som inte själva bundits av bärarmolekylen ändå kan bli bestrålade. Astat-211 avger lite större partiklar, heliumkärnor, när det sönderfaller. Dessa transporteras mycket kort väg, upp till 0,07 mm, och orsakar många skador på sin väg. Därför anses astat-211 passa bättre för att behandla mycket små tumörer. Vi började med att testa minsta mängd radioaktivitet som behövdes för att få tumörerna i fem av sex djur att försvinna, detta bestämdes till 400 MBq/kg kroppsvikt lutetium-antikropp och 10 MBq/kg kroppsvikt astat-antikropp. Samtidigt följdes antalet blodkroppar noggrant för att vara säkra på att djuren återhämtades efter biverkningarna. I båda fallen utvecklade ungefär hälften av djuren så kallade metastaser efter behandlingen. Vi fortsatte våra studier med att testa om man kunde behandla först med lutetium-antikropp så att tumören försvann och sedan ge ytterligare en behandling för att förlänga effekten och försöka hindra att tumören spridde sig. De extra behandlingar som testades var samma antikropp fast utan radioaktivitet, en andra

behandling med lutetium-antikropp och behandling med astat-antikropp. Mätningar av blodkroppar visade att det inte blev några ytterligare biverkningar av antikropp utan radioaktivitet och att även om antalet blodkroppar minskade igen efter en andra behandling med radioaktivitet så återhämtades djuren. Dock var det några djur som gick ner mycket i kroppsvikt efter en andra behandling med radioaktivitet, framförallt när lutetium-antikropp och astat-antikropp kombinerats. Det tyder på att det finns andra organ som också är känsliga för strålningen, vilket måste kartläggas ordentligt innan man går vidare och utvecklar behandlingen i klinik. Den extra behandlingen minskade inte antalet djur med metastaser och förlängde inte heller överlevnaden. Vi tror att detta kan bero på att de tumörceller som var kvar vid den andra behandlingen var svårtillgängliga eller saknade strukturen som antikroppen binder till.

För att kunna förklara varför vi har så goda effekter av behandlingen har vi tagit ut tumörer vid olika tidpunkter efter injektion och kartlagt hur radioaktiviteten fördelas i förhållande till bland annat blodkärl och strukturen som antikroppen binder till. Resultaten visar att radioaktiviteten först finns i de yttre delarna av tumören för att tränga längre in med tiden. Samtidigt ändras tumören så att strukturen som antikroppen binder till försvinner i bestrålade områden och i områden med mycket radioaktivitet bildas ärrvävnad i samband med att tumörcellerna dör.

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