The Gastrin-Releasing Peptide Receptor as Target for Molecular Imaging and Therapy of Prostate Cancer using Radiolabelled Bombesin Analogues

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THE GASTRIN-RELEASING PEPTIDE RECEPTOR AS TARGET FOR MOLECULAR IMAGING AND THERAPY OF PROSTATE CANCER USING RADIOLABELLED BOMBESIN ANALOGUES

De GRP receptor als doel voor moleculaire beeldvorming en therapie van prostaatkanker door middel van radioactief gebonden bombesine analogen

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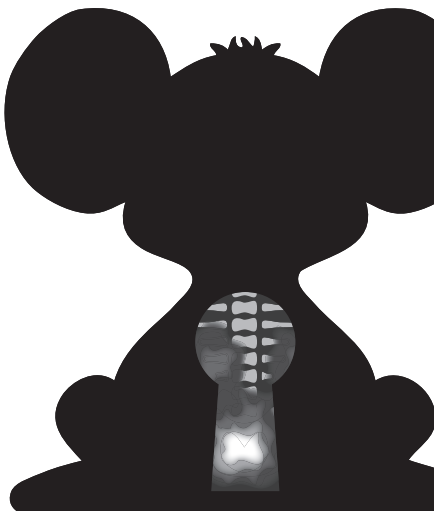
CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS



The lack of accurate imaging options for prostate cancer (PC) remains an unresolved issue. It hampers the clinical management of disease, including staging of primary PC and localisation of PC tumour recurrence. Another major concern in PC management is the absence of curative treatment options for metastasised disease.

The field of nuclear medicine provides opportunities that may fill these limitations in PC management by employing radiopharmaceuticals, such as radiolabelled peptide-based biotracers, that can be visualised by the nuclear imaging modalities single photon emission tomography (SPECT) and positron emission tomography (PET). Furthermore, such radiolabelled peptides may be applied to offer targeted peptide receptor radionuclide therapy (PRRT). In the case of PC, bombesin (BN) is a promising receptor-targeting peptide due to its high affinity to Gastrin-Releasing Peptide Receptors (GRPR) overexpressed on the membranes of PC cells. The use of radiolabelled BN analogues has been proposed for improvement of imaging and staging of PC as well as for treatment of localised or occult PC metastases.

PROSTATE CANCER; INCIDENCE AND PROGNOSIS

PC is a major health problem in the Western world due to its high incidence and significant overall mortality [1, 2]. While localised PC can be treated with curative intent, disseminated PC is a deadly disease, leaving only palliative treatment options [3]. Therefore, it is crucial to accurately determine the extent of the disease at presentation in order to choose the best strategy for treatment. In The Netherlands the incidence of PC in 2009 was 10166 men and mortality was 2492 [4]. PC confined to the prostate can be innocent; remaining indolent during life, and with less than 3% the lifetime risk of PC-related death is low [5, 6]. Autopsy studies have shown that a significant proportion of prostate tumours will never become clinically relevant [7]. This clearly shows that aggressive (radical) treatment is not always needed in case of PC. Indeed, incidental PC was found in 33% of male donors aged 60-69, and 46% of men over age 70 [8].

DETECTION OF PC USING PSA

In earlier years when public awareness was little and (opportunistic) screening was unusual, most PC patients presented with symptoms of disseminated late stage disease. Nowadays, due to increased alertness, higher responsiveness, and improved screening options, PC is predominantly diagnosed at an early stage. Detection of PC is based on clinical examination, in particular digital rectal examination (DRE) of the prostate[9, 10], laboratory levels of prostate specific antigen (PSA) in blood and ultimately histopathology on prostate biopsies needed for PC diagnosis.

Blood levels of PSA, a glycoprotein originating from the prostate gland, are usually increased in PC patients compared to the healthy population. It is widely used as a screening marker for PC [5, 11, 12]. However, the use of PSA for screening has its limitations as it is prostate-specific, but not a cancer-specific protein. Elevated PSA blood levels may also be caused by other non-cancerous prostate gland conditions such as prostatitis and benign prostatic hyperplasia (BPH) [13, 14]. As a consequence, the specificity of PSA for the diagnosis of PC is rather low, leading to many false positive results in case of prostatitis or BPH. At a PSA cut-off level of 4.0 ng/mL specificity for PC of up to 70% has been reported [15].

Since the introduction of PSA it is increasingly being used for PC screening. Obviously this has led to a tremendous rise in prostate biopsies. Approximately 50% of men with screen-detected PC carry an indolent form of PC that would have never caused any symptoms [16]. The European Randomised Study of Screening for PC (ERSPC) showed that PSA-based screening does reduce PC-death rate by up to 31%, but emphasised at the same time that this goes at the expense of overdiagnosis and overtreatment [17, 18].

RISK STRATIFICATION OF PC

An important challenge in PC management is to distinguish patients who need invasive therapy from patients who need no intervention at all. Current Dutch PC guidelines [19] agreed to stratify PC patients into 3 risk groups based on the EAU/ESTRO criteria [20]:

- Low; T1c-T2a, Gleason score <7, initial PSA (iPSA) <10 ng/mL

- Intermediate; T2b-c or Gleason score =7 or iPSA 10-20 ng/mL
  (two of these factors is high risk)

- High; T3 or Gleason score >7 or iPSA >20 ng/mL
  (one of these factors is high risk)

The stage of cancer is a description of the extent of cancer spread. In case of PC the TNM (Tumour, Node, Metastasis) staging system, shown in Table I, is used. There are two types of PC staging; the clinical stage and the pathological stage. An important element of PC risk stratification is the clinical T-stage. It is an estimate of local PC extent obtained previous to curative therapy. T-stage is based on the results of DRE, PSA level, transrectal ultrasound (TRUS) findings and results of PC tumour observed in tissue from transurethal resection (TUR).

Grading of PC as expressed in the Gleason score is another decisive determinant in risk stratification. Pathologists assign one grade between 0-5 to the most common tumour growth pattern, and a second to the next most common tumour growth pattern. The two grades are added together resulting in the Gleason Score. Lower Gleason scores represent more differentiated tumours [21]. The last criterion required for the assessment of PC risk is the PSA value in blood before any form of treatment, called iPSA.

Table 1

Stage T1	**Tumour present, but not detectable clinically or by imaging**
T1a	Tumour incidentally found in less than 5% of prostate tissue resected
T1b	Tumour incidentally found in greater than 5% of prostate tissue resected
T1c	Tumour detected by prostate biopsy performed for elevated PSA
Stage T2	**Organ-confined disease**
T2a	Tumour located in half or less than half of one prostate lobe
T2b	Tumour located in more than half of one prostate lobe
T2c	Tumour located in both prostate lobes
Stage T3	**Extra-prostatic disease**
T3a	Extra capsular extension including microscopic bladder neck involvement
T3b	Tumour invading the seminal vesicle(s)
T4	Tumour invading surrounding organs
N	**Regional lymph nodes**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Lymph node metastases
M	**Distant metastasis**
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Modified table on the 2009 Tumour, Node, Metastasis (TNM) classification of prostate cancer.

Statistically derived tools, called nomograms, have been composed to predict the chance of disease recurrence in patients with clinically localised PC before radical treatment for clinical use. The Most accurate predictions have been obtained by combining the predictive parameters iPSA, T-stage and Gleason score. Based on these parameters the probability of PC recurrence after initial therapy with curative intent has been reported correctly in up to 79% by Kattan or Partin nomograms [22, 23].

THERAPY

Localised PC

PC confined to the borders of the prostate can often be treated curatively. Treatment choice is mainly based on risk stratification. Due to the low lifetime risk to die from PC and because of the currently observed overtreatment of PC with ensuing side effects, active surveillance is nowadays offered to patients with low-risk PC [24, 25]. Patients under active surveillance are closely monitored for disease progression by frequent serum PSA analyses and re-

peat prostate biopsies. When, despite the initial classification of low risk, clinical signs like PSA and re-biopsy show changes that lead to reclassification towards a more aggressive phenotype curative radical treatment is suggested and often initiated.

Localised intermediate or high risk PC mostly requires radical treatment [20]. Options of radical treatment are surgical excision of the prostate (open, laparoscopic or robot-assisted laparoscopic prostatectomy with or without lymph node dissection), or radiotherapy by brachytherapy (radioactive seeds or high dose rate; HDR) or external beam [26-34]. Various treatment modalities have shown excellent survival of patients with localised PC, while high risk patients showed more frequently PC recurrence [26-34]. Since randomised controlled trials are lacking, no high-quality evidence exists to support any given treatment modality over another [35]. It has until now been assumed that both surgery and radiotherapy show similar survival rates. Radical treatment needs to be well-considered because it is associated with significant morbidity, such as erectile dysfunction (up to 90%) and urinary incontinence (up to 74%), leading to a decrement in the quality of life [36]. Besides these morbidities radiotherapeutic options are also associated with functional bowel problems [37].

Disseminated PC

Growth of prostate epithelial (tumour) cells depends on a functional androgen axis with physiological levels of androgens[38]. The first and so far most effective choice of treatment of disseminated PC is endocrine deprivation therapy aiming at ceasing tumour growth by reducing circulating testosterone levels or blocking the androgen receptor [39, 40]. Different forms of endocrine therapy exist, all aim at reducing circulating androgen levels by surgical bilateral orchidectomy, receptor blockade by anti-androgens, and finally chemical castration by luteinizing hormone-releasing hormone (LHRH) receptor antagonists or LHRH receptor agonists or estrogens. Despite extensive research and numerous clinical trials, no differences in terms of survival have been observed between the different forms of endocrine treatment [19, 39, 41-43].

Eventually PC relapse occurs in the majority of patients due to progressive tumour growth despite endocrine ablation therapy. The exact mechanism of this progression towards so-called castration resistant PC (CRPC) is unknown although several theories have been proposed [44, 45]. At the point of CRPC, palliative treatment options are scarce leaving a median survival of 10 months [46-48]. Although chemotherapeutic successes have for long been very poor, in 2004 the use of docetaxel in CRPC became standard treatment following the results of two large randomised phase 3 trails. They showed a significant survival benefit (of 2 to 3 months) as well as a significant improvement in quality of life after docetaxel-based chemotherapy over the standard control treatment group receiving mitoxantrone / prednisone [49, 50].

DEVELOPMENTS IN RADIOLOGY AND NUCLEAR MEDICINE FOR IMAGING OF PC

Accurate anatomical discrimination between local, locally invasive or metastasised PC remains a non-resolved clinical issue in PC patients. Conventional imaging techniques, such as transrectal ultrasound (TRUS), computerised tomography (CT), Magnetic Resonance Imaging (MRI) and bone scintigraphy, have limited added value since their accuracy is low, especially in low-risk patients [51-54]. Evidence-based guidelines of the American College of Radiology Appropriateness Criteria, updated in 2009, indicate that no conventional imaging options are appropriate for treatment planning in early PC patients with T1-2, Gleason score ≤6, PSA <10 and <50% biopsy cores positive [55]. In order to improve staging of primary PC and localisation of PC tumour recurrence research on imaging modalities is ongoing in both nuclear medicine and radiology.

The field of radiology has predominantly focussed on developments in ultrasound and MRI [52, 56-60]. So far, only MRI combined with an endorectal coil (to obtain high quality images of the area surrounding the rectum) was proven convincingly promising correctly detecting up to 93% of seminal vesicle invasion of PC [60]. For this reason Dutch guidelines agreed that endorectal MRI ≥1.5 tesla can be of additive value for tumour staging in patients with intermediate or high risk PC [19].

Nuclear medicine applies radiopharmaceuticals for imaging and therapy of various diseases. For 3-dimensional imaging of radioactivity distribution after injection of the radiopharmaceutical, the very sensitive imaging techniques SPECT and PET are being employed [61, 62]. SPECT is based on detection of radionuclides emitting single photons while PET can visualise positron emitting radionuclides. Radiolabelled metabolic tracers to detect PC (bone) metastasis are frequently applied in nowadays clinical practice [63, 64]. Dutch guidelines recommend a bone scan for PC patients with initial PSA ≥ 20ng/ml, locally advanced disease, Gleason score ≥ 8 or skeletal-related symptoms [19]. Besides their use in metastatic (bone) imaging metabolism-based PET tracers, such as ^{18}F-FDG, ^{11}C-choline and ^{11}C-acetate, are extensively being studied for their potential in PC staging [52, 65].

In addition, a strong focus is directed towards the development of radiopharmaceuticals that target PC specifically. These tracers may be applied for both staging and treatment of PC. An example is the monoclonal antibody ^{111}In-capromab pendetide (Prostascint) directed to the prostate-specific membrane antigen. This radiopharmaceutical has been approved by the American Food and Drug Administration (FDA) for targeted diagnostic imaging and staging of LN metastasis of PC [66, 67].

The present thesis focuses on the preclinical evaluation of novel and promising PC-targeting ligands based on BN or Gastrin-Releasing Peptide (GRP). These ligands can target the GRPR,

membrane receptors overexpressed on PC cells [68, 69]. Radiolabelled BN and GRP-based peptide analogues show high affinity to GRPR leading to excellent tumour-specific binding [70]. Preclinical and clinical studies dealing with GRPR-based imaging are reviewed in chapter 2 of this thesis.

PEPTIDE RECEPTOR-BASED PC TARGETING USING BOMBESIN ANALOGUES; AIMS OF THIS THESIS

Application of BN analogues radiolabelled with positron or gamma radiation emitting radionuclides for targeted GRPR imaging may help to improve both accurate staging of primary PC and sensitive localisation of PC tumour recurrence [70]. Labelled with cytotoxic beta or alpha emitting radionuclides BN analogues may even be employed for PRRT to treat both known and occult PC metastases or for focal PC therapy. The preclinical studies in this thesis aimed at further analysing the GRPR as potential target for molecular imaging and radionuclide therapy of PC using BN analogues and had the following objectives:

- To select the optimal BN analogue for GRPR targeting.

- To study GRPR expression in patient materials, human PC cell lines and xenografts and the influence of different biological and hormonal conditions.

- To study the impact of various experimental conditions on BN analogue pharmacokinetics in mice.

- To validate the diagnostic value of a selected BN analogue comparing peptide-receptor-based targeting with metabolism-based targeting in a mouse model.

The research described in this thesis has been performed as part of a collaboration between the Department of Urology and the Department of Nuclear Medicine at Erasmus MC Rotterdam. The Department of Urology has long-standing expertise in clinical and fundamental research of PC. The research group has established 13 unique human PC xenograft models and has the availability over a large panel of in vitro cell lines. Also, an extensive biorepository of human tissue samples from patients with long-term clinical follow-up has been established over the years.

The Department of Nuclear Medicine of Erasmus MC in Rotterdam was one of the pioneers in development and introduction of peptide receptor-mediated diagnostic and therapeutic radiopharmaceuticals for neuroendocrine tumours. The research team played a pivotal role in development and research leading to the first radiolabelled peptide approved by the FDA in 1994, called OctreoScan, an ^{111}In-labelled somatostatin analogue [71]. Currently one of the focuses of this team is directed towards BN research. For this thesis, the department radiolabeled different peptide analogues and performed preclinical evaluation studies, including

in vivo imaging using state-of-the-art SPECT-CT and PET animal imaging devices.

OUTLINE OF THIS THESIS

The introduction of this thesis (**chapter 1**) focuses on PC and its clinical issues. It enunciates the need of new biomarkers for detection and monitoring of PC and introduces the promising BN peptide analogues in short. **Chapter 2** provides a general and more detailed overview of preclinical and clinical literature on the GRPR as a target for imaging and radionuclide therapy of PC using radiolabelled BN analogues. The potential of BN analogues in early diagnosis, monitoring and therapy of PC is discussed.

A valid comparison of performance of different radiolabelled BN analogues is hampered by many protocol variations between the preclinical studies performed. In **chapter 3,** we compared the PC targeting efficacy of five promising radiolabelled BN analogues for SPECT imaging under standardised experimental conditions in a mouse study. From this study we could conclude that the GRPR-antagonist Demobesin-1 and the GRPR-agonists AMBA and Pesin were the most promising analogues.

Since the majority of patients with disseminated PC will eventually need hormonal therapy, it is essential to identify the impact of hormonal ablation treatment on GRPR expression. Earlier preclinical studies from our group showed downregulation of GRPR-expression after androgen withdrawal [72]. In the literature low GRPR expression was suggested in CRPC as well [69]. In **chapter 4** we explored androgen regulation of GRPR expression in three in vivo human PC xenograft models (PC82, PC295 and PC310) in more detail. This study revealed expression of GRPR in these androgen-responsive human PC xenografts to be significantly reduced by androgen ablation and to be reversed after restoring the hormonal status of the animals.

Further studies to unravel the effect of hormonal manipulation and AR status on GRPR expression in PC are described in **chapter 5**. We used the clinically relevant androgen-responsive and GRPR-expressing human VCaP cell line and studied GRPR expression in human tissue samples at various clinical stages. In contrast to our earlier observations described in chapter 4 BN-specific binding in VCaP was not significantly androgen regulated suggesting overexpression of GRPR in more advanced PC can escape 'normal' androgen regulation control mechanisms rendering it constitutively activated. From human tissue samples higher overall median GRPR-specific uptake was seen in PC compared to benign tissue, though lower PC outcomes showed considerable overlap with GRPR expression levels in benign tissue samples.

Chapter 6 identifies the impact of various experimental conditions on pharmacokinetics using ^{111}In-AMBA in nude mice bearing human PC xenografts.

Effects of experimental characteristics like HPLC peptide purification of the (radiolabelled) peptide, variations in administrated specific activity and saturation of the GRPR by predosing with BN prior to administration of radiolabelled BN were studied. This study showed that not only the chemical structure of BN analogues determines its GRPR binding, but also demonstrates significant effect on BN-binding to GRPR of HPLC peptide purification, variabilities in specific activity, variations in experimental conditions and saturation of the GRPR by predosing with BN prior to administration of radiolabelled BN.

Metabolism-based targeting of PC is being studied as a strategy for PC imaging. In **chapter 7** we describe the comparison of BN peptide receptor-mediated targeting using ^{68}Ga-AMBA with metabolism-based targeting by ^{18}F-choline for PET imaging of PC. This experimental study suggests peptide-receptor based imaging to be superior to metabolism-based imaging for PET visualisation of PC xenografts.

This thesis concludes with the **Summary and Future Directions chapter,** where the study results are reviewed and discussed. A final statement is given on their clinical relevance and the prospective of BN-related peptides for use in GRPR-based PC-targeted SPECT/PET imaging.

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CHAPTER 2

PEPTIDE RECEPTOR IMAGING OF PROSTATE CANCER WITH RADIOLABELLED BOMBESIN ANALOGUES

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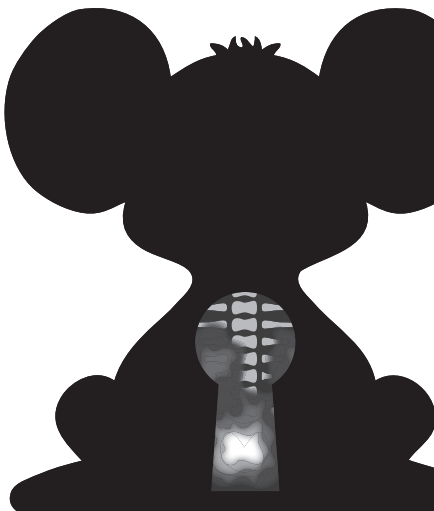


ABSTRACT

Prostate Cancer (PC) is a type of cancer that is often diagnosed at very early stages due to improved detection among man in the Western World. Current imaging techniques are not optimal to determine extent of minimal early stage PC even though this is of great clinical importance. Human PC and high-grade PIN have shown high Gastrin-Releasing Peptide Receptor (GRPR) expression, while normal prostate tissue and BPH revealed to be predominantly GRPR-negative. Radiolabelled Gastrin-Releasing Peptide (GRP) or bombesin (BN) analogues targeting the GRPR can be used as non-invasive tools to diagnose, monitor and potentially treat PC. These BN analogues have already proven to be able to image PC in both tumour-bearing mice and clinical patients showing no important side effects.

It is desirable that new peptides require fast-track standardised comparative testing in relevant PC models to select the best performing BN analogues for further evaluation in patients. Although knowledge about GRPR expression and development of new BN analogues can be extended, it is time to study performance of BN analogues for peptide-receptor based imaging in patients validating results of PC imaging using histopathology as a golden standard.

INTRODUCTION

The medical specialism of nuclear medicine focuses on the application of radiolabelled tracers for scintigraphic imaging or radionuclide therapy of disease. Molecular nuclear medicine holds the unique potential of being able to find, diagnose and treat disease as well as to monitor treatment response.

The current evolution of knowledge in molecular biology has resulted in new targets to detect human cancer specifically. New developments in (radio) chemistry have improved molecular delivery of radionuclides to disease-target sites. Consequently this has resulted in the generation of novel tracers. Technical developments in scintigraphic instruments and reconstruction software have improved imaging modalities allowing small-animal scintigraphic techniques useful for experimental nuclear research of, especially rodent, disease models. See for review[1].

In oncology radioactive iodine introduced in the early 1950s and the widely used ^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) have been developed as indicators of cancer cells. A promising opportunity for nuclear applications in oncology lies in the development of radiolabelled peptides that target receptors for imaging and therapy. This technique is based on targeting specific receptors that are overexpressed in tumour compared to normal

tissue with highly selective radiolabelled peptides for specific imaging and monitoring. Linked to appropriate therapeutic radionuclides these peptides can also be used as radiotherapeutics in peptide receptor radionuclide therapy (PRRT).

In order to bring peptide-receptor based modalities into the clinic, radiopharmaceuticals with high affinity and high specificity for preferably cancer-specific receptors are required allowing visualisation and quantification of radioactivity in the tumour in a reproducible and repeatable manner. See for review[2]. Somatostatin is a well-known peptide, of which analogues have been implemented successfully in the clinics to visualise and treat various neuroendocrine tumours[3, 4].

This review focuses on the use of the Gastrin-Releasing Peptide Receptor (GRPR) as a target for imaging and radionuclide therapy of prostate cancer (PC) using radiolabelled bombesin (BN) analogues. The potential of these peptides for their use in early diagnosis, monitoring and therapy of PC will be discussed.

PROSTATE CANCER

PC is the most frequently diagnosed cancer among men in the Western world and is the third most common cause of death[5]. Specific antigen (PSA) PSA has been increasingly used to detect PC[6], although it has limited diagnostic specificity and prognostic value[7]. The impact of PSA-based screening on survival has recently been reported by the European Randomised Study of screening for PC (ERSPC) study showing a significant reduction in death from prostate cancer, but at the cost of overtreatment[8]. Due to PSA-based screening, the number of patients that are detected with early disease is rising. Although, final diagnosis of PC is made by histopathological confirmation of transrectal ultrasound-guided prostate biopsies, staging of the disease is essential for the decision for the most accurate treatment. It discriminates between organ-confined disease, in which local therapy such as surgery or radiation may still be beneficial, and PC beyond the confines of the gland for which a systemic approach like hormonal therapy is the first choice of treatment. None of the currently used imaging modalities are sufficiently reliable to determine the extent of disease in early detected PC[9]. Non-invasive sensitive imaging strategies to accurately diagnose, stage and monitor PC are therefore essential. Radiolabelled peptide-based imaging by scintigraphy may be the alternative application to fill this gap and improve diagnostic sensitivity for early PC. Also, PRRT may be an alternative tumour-specific targeting strategy in progressive patients with metastatic, therapy-resistant PC.

PET metabolic radiotracers such as ^{18}F-FDG, ^{11}C-choline and ^{11}C-acetate have been intensively studied for imaging PC. ^{18}F-FDG was found to have a low accuracy in primary staging of PC mainly due to low metabolic glucose activity and urinary excretion of the metabolic tracer [10]. Choline and acetate PET was reported useful for staging of LN disease[10, 11].

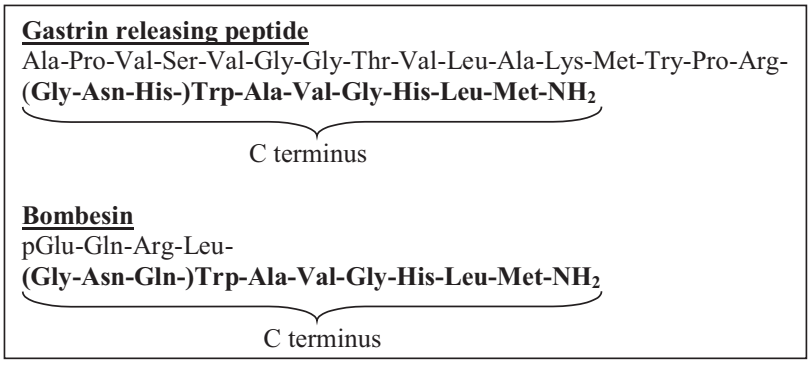
ProstaScint (^{111}In-capromab pendetide), a monoclonal antibody against prostate-specific membrane antigen (PSMA) as

a target, is the only nuclear imaging application for PC-specific imaging. It has been approved by the American Food and Drug Administration and is put to practice for diagnostic imaging and staging of LN metastasis of PC[12].

An alternative target for PC imaging may be the GRPR using radiolabelled BN analogues.

on the cell membranes of prostatic intraepithelial neoplasias (PIN), primary PC and invasive prostatic carcinomas, whereas normal prostate tissue and, in most cases, benign prostate hyperplasia (BPH) were predominantly GRPR-negative[13]. The underlying molecular mechanisms of aberrant GRPR expression and/or activation in human PC are unknown at present. GRP interacts with GRPR inducing cell growth in various

Figure 1



Amino acid sequence of the 27-amino acid neuropeptide GRP and its mammalian homologue; the linear tetradecapeptide BN.

BN FOR PEPTIDE-TARGETED IMAGING OF PC

Gastrin-Releasing Peptide (GRP) is a 27-amino acid neuropeptide that is the mammalian homologue of the linear tetradecapeptide BN originally isolated from the skin of the frog; Bombina bombina. GRP binds selectively to the GRPR. It shares homology with BN at the amidated C terminal sequence in the final 7 amino acids (Figure 1).

Using tumour autoradiography it has been reported that in human samples GRPRs are expressed at high density tumours including PC[14]. The relation between GRPR and PC stage is still uncertain. PC in xenografts derived from late stage androgen-independent disease showed lower expression of the GRPR than xenografts established from early androgen-dependent PC[15]. Besides in PC GRPR is also over expressed in several other human tumour types and metastases including breast-, colon-, lung-, ovary-, renal, CNS and head or neck squamous cancer[14, 16].

Four subtypes of the cell surface BN receptor are known. Among them three are mammalian: the NMB receptor

(BB_1), Gastrin-Releasing Peptide Receptor (GRPR/BB_2) and BN receptor subtype 3 (BRS-3/BB_3). A fourth receptor, BB_4, is only found in amphibians[14, 17]. The only well characterised receptor to which GRP and BN bind with high affinity is the GRPR (BB2).

RESEARCH ON GRPR AND BN-ANALOGUES

The finding of GRPR overexpression in PC and other cancer types stimulated the search for BN/GRP peptide analogues that could bind with high potency to the GRPR. GRP-antagonistic analogues have been developed to realise antiproliferative effects and indeed this has led to promising growth-inhibitory effects in human GRPR-positive PC cell lines and human PC3-bearing mice[18]. Besides this approach it was also proposed that GRP analogues could be used as molecular tracers for imaging and treatment of PC tumours, when those analogues would be linked to a radioactive agent[13, 19]. This application has been studied in both preclinical and clinical settings.

Synthetic BN analogues can be categorized into two different types based on their structures. Type A-analogues are truncated with only a portion, usually BN[7-14] at the C terminus, of the peptide retained. This C terminal sequence is generally thought to be essential for receptor recognition, signal transduction, and biologic function (Figure 1). Type B analogues on the other hand are synthesized in full length. In these analogues usually one or more amino acid residues are selectively replaced.

A type A-analogue is generally thought to be favourable while it is more stable than the full-length type B tetradecapeptides for in vivo applications and still binds to the GRPR adequately[20, 21]. For radiolabelling of GRP-analogues biomolecules are generally designed in a way to keep the labelling site at distance from the receptor-binding site, but at the same time tag the radiometal into the molecule in an irreversible way leading to stable radiolabelled derivatives.

PRECLINICAL

The development of new analogues is mostly aimed at improving the sensitivity and specificity of GRPR targeting. Several new BN-analogues have been developed and tested for their potential in early diagnosis, monitoring and therapy in vivo. In a preclinical setting general peptide characteristics such as stability, biodistribution and toxicity were often tested using the human PC3 cell line and the experimental PC3 bearing xenograft model.

A potent ^{99m}Tc-based BN analogue designed for GRPR based targeting and tested in the PC3 xenograft model with the highest absolute tumour-uptake in animals described in literature, is Demobesin-1[22]. Other promising BN-analogues that were tested in preclinical studies using PC3 bearing mice include the DOTA chelated compounds AMBA

Table 1

Analogue	Ref(s)	Radionuclide	Chelator	Linker	Amino acid sequence													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
Native BN	[14]				pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
Demobesin-1	[22]	^{99m}Tc	N_4	BzDig						**DPhe**	Gln	Trp	Ala	Val	Gly	His	Leu-**NHEt**	
AMBA	[23,38]	^{177}Lu	DOTA	G-4-aminobenzyl							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
Pesin	[24]	$^{68/67}$Ga, ^{177}Lu	DOTA	$dPEG_4$							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
MP2653	[25]	^{111}In	DTPA						**ACMPip**	**Tha**	Gln	Trp	Ala	Val	**β-Ala**	His	**Tha**	**Nle-NH_2**
[DTPA1,Lys3 *(*Pm-DADT*)*, Tyr4]BN*	[26]	^{99m}Tc	Pm-DADT**		pGlu	Gln	**Lys**	**Tyr**	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
Aoc-BN(7-14)	[27]	^{64}Cu	DOTA	Aoc							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
[Lys3]BN	[28]	^{18}F	FB		pGlu	Gln	**Lys**	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
RP527	[33]	^{99m}Tc	N_3S	Gly-5aVa							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
[Leu13] BN	[34-37]	^{99m}Tc		Aca***	**Cys**	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
MP2248	[39]	^{111}In	DTPA		**Pro**	Gln	Arg	**Tyr**	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
DOTABOM	[40]	^{68}Ga	DOTA															

** In this compound DTPA is not used as a chelator for radionuclide complexation, but as a built-in pharmacokinetic modifier to reduce hepatobiliary clearance.*
*** Linked to Lys on position 3.*
**** In between amino acids 1 and 2.*

Table 1

Amino acid sequence of native BN (14 amino acids) and the BN-analogues described. BN = bombesin, Ref(s) = reference(s).
Chelators: N4 = 6-{p-[(carboxymethoxy)acetyl]aminobenzyl}-1,4,8,11-tetraazaundecane, DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, DTPA = Diethylene triamine pentaacetic acid, Pm-DADT = diaminedithiol, FB = 4-Fluorobenzoyl, N_3S = dmgly-L-ser-L-cys(acm).
Linkers: BzDig = p-aminobenzyldiglycolic acid, PEG = polyethylene glycol, Aoc = 8 carbon linker, Gly-5aVa = 5-amino-valeroyl, Aca = 6-amino-n-hexanoic acid.
Introduced amino acids: Phe = Phenylalanine, ACMPip = 4-Amino-carboxymethylpiperidine (non-natural amino acid), Tha = β-(2-Thienyl)-alanine (non-natural amino acid), β-Ala = β-Alanine (non-natural amino acid), Nle = Norleucine, Lys = Lysine, Cys = Cysteine, Pro = Proline, Tyr = Tyrosine.

and Pesin, the DTPA chelated compound MP2653 (Compound 3 in Visser et al.) and [DTPA1, Lys3(Pm-DADT), Tyr4]BN (for amino acid sequence of native BN and BN-analogues see Table 1)[23-26].

Rogers et al. introduced the radiolabelled BN analogue ^{64}Cu-DOTA-Aoc-BN$^{7-14}$ as the first GRPR targeting radiopharmaceutical to use for PC-imaging with PET[27]. The BN-analogue ^{18}F-FB-[Lys^3]BN designed for GRPR-targeted PET has also been tested showing promising results for PC imaging[28].

At the moment a valid comparison of available analogues for PC detection is difficult as standardisation between the preclinical studies performed is lacking. Therefore we recently performed a standardised preclinical study comparing four DOTA-chelated BN agonists and one N4-chelated BN antagonist (manuscript submitted).

GRPR will internalise into the cell when it is activated by an agonistic ligand binding the receptor. Internalisation of the receptor-radioligand complex has always been thought to be a crucial step for optimal imaging and therapy. It would provide essential accumulation and retention of radioactivity in the cell, thus increasing the radioactive signal at the target site. Interestingly, high-affinity somatostatin receptor antagonists that poorly internalise into tumour cells have recently shown an equal or even higher tumour uptake and a higher retention rate in preclinical studies as opposed to agonists, which do internalise[29]. Cescato et al. showed that GRPR antagonists may be superior targeting agents compared to GRPR agonists as well[30]. These data suggest a change of paradigm in which the intensified use of antagonistic in preference to agonistic analogues would be justified.

PC3 and Du145 cell lines are commonly used in GRPR binding studies. Both cell lines are androgen-independent and show no expression of the androgen receptor or PSA; characteristics which are essential to PC patients[31]. On the other hand, both cell lines express high levels of GRPR[32]. Therefore they remain functional for use in GRPR based studies. In a panel of 12 established human PC xenograft models representing the different stages of human PC, Visser et al. showed that high GRP receptor density was only observed in androgen-dependent PC xenografts. If

this result can be translated to the clinical situation, it might indicate that high GRPR expression is predominantly present in the early, androgen-dependent, stages of PC and not in later stages. In addition, in this preclinical study, androgen ablation strongly reduced GRPR expression in androgen-dependent tumours indicating that GRPR expression in human PC is androgen-regulated[15]. Thus GRPR based imaging may be especially relevant in early PC and less suited for hormone-treated patients with late stage disease.

CLINICAL

Few BN-analogues have been studied in PC patients. Van der Wiele published clinical data on ^{99m}Tc-RP527 in four androgen independent PC patients with metastatic bone lesions. Selective uptake was observed in one patient and 50% of the bone lesions could be visualised by SPECT in this patient. No short-term adverse or subjective effects were described in any of the subjects[33].

The GRP analogue [Leu13]BN which complexes with ^{99m}Tc was described in an article by Varvarigou et al.[34]. Herein [Leu13]BN showed to be a promising BN-analogue in GRPR expressing malignancies other than PC. A first clinical study using ^{99m}Tc-[Leu13]BN for imaging in an androgen-dependent PC patient by De Vincentis et al. resulted in the visualisation of the primary PC in this patient without observing relevant side-effects[35]. Scopinaro et al. proceeded evaluating the same analogue in 8 PC patients and reported all 8 primary PCs to be visualized in the prostate fossa by SPECT while 2 patients with benign adenomas did not show uptake. In this study SPECT showed uptake in obturator nodes which was proven to be cancer-specific after histopathology in 3 patients. MRI or CT did not show these LN metastases[36]. After this study Vincentis et al. reported SPECT-detected PC in 12/12 patients with androgen-dependent PC and loco-regional LN visualisation in 4 patients. Eleven patients got operated and results were histologically confirmed by surgical specimens. No short-term adverse effects were stated[37]. In a phase I study in hormone refractory PC patients aiming at PC therapy, using the ^{177}Lu-labelled BN analogue AMBA, SPECT imaging revealed lesions in 5 out of 7 patients with adittional high pancreatic uptake of radioactivity[38]. For an overview of clinical PC-imaging studies see Table 2.

Recently, Froberg et al. reported high uptake of the BN-agonist MP2248 and antagonist Demobesin-1 in the pancreatic region of 4 PC patients. Retention in the pancreas after injection of ^{111}In-MP2248 was much longer than retention of ^{99m}Tc-Demobesin-1[39].

Only very few PET studies have been reported to date for visualisation and quantification of GRPR expression in PC patients. A clinical study using ^{68}Ga-DOTABOM has been described by Hofmann et al.[40].

Table 2

Overview of clinical studies using BN-analogues for PC-tumour imaging patients

Ref = reference, BN = bombesin, PC = prostate cancer, AI = androgen independent, AD = androgen dependent, $^{+}$ = visualized by scintigraphy, $^{-}$ = not visualized by scintigraphy, pt(s) = patient(s), h.c. = histologically confirmed, BC = breast carcinoma, BAp = benign adenoma of the prostate, LN = lymph node, EPS = extraprostatic spread.

Authors	Ref	Radionuclide	BN-analogue	Total activity (MBq)	Peptide mass	PC population in study	PC+	Extra
Van der Wiele et al.	[33]	^{99m}Tc (SPECT)	RP527	555	3 ng/kg	4 AI	1	In the one PC^{+} pt 50% of h.c. bone lesions visualized 4/6 BC pts^{+}
De Vincentis et al.	[35]	^{99m}Tc (SPECT)	[Leu13]BN	185	0,7 µg	1 AD	1	
Scopinaro et al.	[36]	^{99m}Tc (SPECT)	[Leu13]BN	185	0,7 µg	8 AD	8	3LN^{+} all h.c.; all negative MRI/CT 2 benign PC pts $^{-}$
De Vincentis et al.	[37]	^{99m}Tc (SPECT)	[Leu13]BN	185	0,7 µg	12 AD	12	2 BAp pts^{-} 4LN^{+} all h.c.; 3/4 positive CT/MRI PC^{+} h.c. in 11/12 pts
Bodei et al.	[38]	^{177}Lu (SPECT)	AMBA	1140-1940	Not mentioned	7 AI	5	Study primarily aimed for PC therapy
Hoffman et al.	[40]	^{68}Ga (PET)	DOTABOM	26-80	24 nmol	11 AD	11	3 Ln+ & 2 pts with EPS+ (all h.c.)

FUTURE PROSPECTIVES

Although expression of the GRPR in various tissue types and tumours has been studied it requires further investigation. In case of PC, knowledge about the androgen regulation of GRPR is of great clinical relevance, as it will strongly determine the potential use of BN-based imaging and therapy in the different stages of PC.

A valid comparison of BN-analogues for PC detection, based on literature, is difficult as standardisation between studies is lacking. Differences in potency between existing and future BN-analogues need to become clear. Standardised studies with appropriate design to compare analogues are therefore required.

The field of radiolabelled peptides for receptor-based targeting of PC is evolving. New BN-analogues are being designed in order to improve characteristics like specificity, sensitivity and stability in vivo. BN-peptides should have high affinity for to the GRPR, remain intact in vivo for a long time and their clearance from circulation should be fast. Furthermore, for peptide-receptor targeting, receptors of interest should ideally be highly expressed in the disease tissue only. High expression in (non-cancerous) non-targeted tissue will cause non-specific peptide binding resulting in a high background signal while scanning and toxicity to healthy tissue when therapy is concerned. When developing a BN-analogue, besides taking its amino acid sequence into consideration, attention should be paid to the selection of an appropriate chelator while this determines which radionuclides can be complexed with the analogue.

Recent findings suggest that antagonistic GRPR-based peptides show higher tumour uptakes and radioactivity retention in PC tissue compared to agonistic peptides with comparable binding affinities. This finding paves the way for intensified development of new BN-antagonists. Also from a pharmacological point of view this change in paradigm is favourable, as antagonists may not induce the endocrinological side effects known from agonists, such as stimulation of tumour growth. Especially when a therapeutic dose is taken into account, agonistic BN-peptides could cause (side-)effects for PC patients. More research needs to be done to reveal the underlying mechanism in binding difference between agonistic and antagonistic peptides. To improve peptide-receptor targeting and increase the dose in target cells it might be suggested to test a cocktail of BN-agonists and antagonists for simultaneous administration.

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CHAPTER 3

A STANDARDISED STUDY TO COMPARE PROSTATE CANCER TARGETING EFFICACY OF FIVE RADIOLABELLED BOMBESIN ANALOGUES

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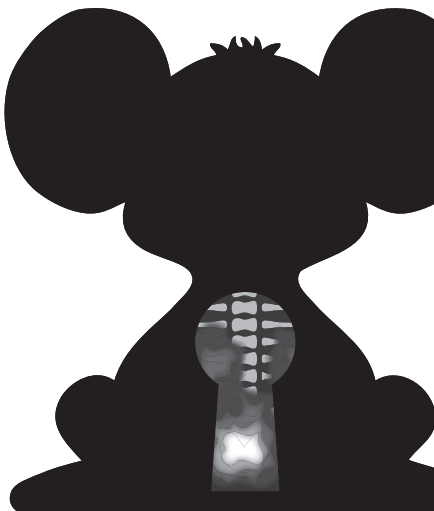


ABSTRACT

PSA-based screening of prostate cancer (PC) has dramatically increased early diagnosis. Current imaging techniques are not optimal to stage early PC adequately. A promising alternative for PC imaging is peptide based scintigraphy using radiolabelled bombesin (BN)-analogues that bind to Gastrin-Releasing Peptide Receptors (GRPR) being overexpressed in PC. When labelled to appropriate radionuclides BN targeting of GRPRs may also provide applications for peptide radionuclide receptor therapy (PRRT). Assessment studies under identical experimental conditions allowing a reliable comparison of the potential of such analogues are lacking. This study was performed to evaluate and directly compare five promising radiolabelled BN analogues for their targeting efficacy of PC under standardised conditions.

BN agonists ^{111}In-DOTA-Pesin, ^{111}In-AMBA, ^{111}In-MP2346 and ^{111}In-MP2653 and one antagonist ^{99m}Tc-Demobesin-1, were evaluated in GRPR-overexpressing human PC-3 tumour bearing mice performing peptide stability in vivo, biodistribution and GRPR targeting potential by animal SPECT/CT imaging and ex-vivo autoradiography.

HPLC analysis of blood showed intact Demobesin-1 at 5 and 15 min after injection (64.1%±1.6 and 41.0%±01, respectively) being much less for the other compounds. AMBA, the second most stable analogue, showed 36.1%±2.7 and 9.8%±1.1 intact peptide after 5 and 15min. PC-3 tumour uptake at 1 hour was comparable for Demobesin-1, AMBA, Pesin and MP2346 (3.0±0.4, 2.7±0.5, 2.3±0.5 and 2.1±0.9%ID/g, respectively), but very low for MP2653 (0.9±0.2%ID/g). In addition, MP2346 showed undesirable high uptake in the kidneys (7.9±1.9%ID/g) being significantly less for the other analogues. AMBA, MP2346 and Pesin revealed favourable increases in tumour to blood ratios over time while changes in tumour-to kidney and pancreas ratios for Demobesin-1 from 1 to 24h after injection were significantly better than for the other analogues. All analogues visualised PC-3 tumours by SPECT-CT and autoradiography.

In the present study the BN antagonist Demobesin-1 was the best performing analogue showing superior in vivo stability, highest tumour-uptake and retention while pancreatic and renal clearance were rapid. Pesin and AMBA were the best GRP-agonists with sufficient in vivo stabilities as well as high uptake and retention tumour. Based on these results all three analogues deserve further evaluation for clinical use in PC patients.

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among men in the Western world. It is the third most common cause of death[1]. After its introduction as a biomarker, the prostate specific antigen (PSA) has been increasingly used to detect PC[2] despite its limited diagnostic specificity and prognostic value[3]. The *European Randomised Study of Screening for PC (ERSPC) has* shown that screening for PC using PSA detection and digital rectal examination, has resulted in fourfold increase in the detection of PC[4]. To select an optimal therapeutic strategy for PC at time of diagnosis, it is essential to discriminate between organ-confined disease (with local therapy such as surgery or radiation therapy still likely to be beneficial) and PC beyond the confines of the gland (for which a systemic approach such as hormonal therapy is the first choice of treatment). Yet none of the standard imaging modalities are sufficiently reliable in determining the extent of minimal disease at time of diagnosis[5, 6]. New non-invasive sensitive imaging strategies to accurately diagnose, stage and monitor PC are therefore required. Radiolabelled peptide-based imaging by scintigraphy - single positron emission tomography (SPECT) or positron emission tomography (PET) - may be an interesting alternative to fill this gap and improve diagnostic accuracy for early PC.

Gastrin-releasing peptide receptors (GRPRs) are overexpressed in several primary human tumours and metastases[7]. Using tumour autoradiography, Markwalder and Reubi[8] reported that GRPRs are expressed in invasive prostate carcinomas and in prostatic intraepithelial neoplasms at high density, whereas normal prostate tissue and hyperplastic prostate tissue were predominantly GRPR negative. These findings suggest that GRPR may be used as a molecular basis for diagnosing and staging PC, similar to somatostatin receptors (SST) which are being successfully used in clinical practice[9-12].

Bombesin (BN) and its mammalian counterpart, gastrin-releasing peptide (GRP) bind to the GRPR and are involved in the regulation of a large number of biological processes. Four subtypes of the bombesin receptor are known of which the neuromedin B receptor (BB_1), GRPR (BB_2) and BN receptor subtype 3 (BRS-3 or BB_3) are mammalian receptors and the fourth subtype (BB_4), is found in amphibians only[7]. The carboxyl-terminal decapeptide of GRP is similar to that of the 14-amino acid amphibian BN peptide. BN possesses the same biologic and immunologic activities and has also got a high affinity for GRPR. Since BN is assumed to be more stable than GRP, developments have mainly been focused on BN analogues. BN peptides have been coupled to suitable chelators such as diethylenetriaminepenteacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-N,N',N'', N'''-tetraacetic acid (DOTA) and acyclic tetraamine (N_4, 1,4,8,11-tetraazaundecane functionalised at position 6 for binding to primary amine groups of peptides) to allow labelling with various radionuclides.

DOTA and DTPA coupled BN analogues enable high specific labelling with ^{111}In because of the superior cellular retention characteristics of this radionuclide[13]. The DOTA chelator can also be labelled with therapeutic radionuclides such as ^{90}Y, ^{213}Bi and ^{177}Lu, offering the possibility of using these BN analogues for therapeutic use. Additionally, replacement of the DTPA chelator with DOTA allows for radiolabelling with ^{68}Ga, making these peptides also suitable for PET imaging[14]. On the other hand, Demobesin-1 with its N_4 chelator enables labelling with ^{99m}Tc, the most widely-used radionuclide in nuclear medicine diagnosis.

The suitability of several radiolabelled BN analogues for PC targeting has been tested in preclinical and clinical studies (for review see [15]). However, a valid comparison of the potentials of the radiolabelled BN analogues described in the literature is considerably limited due to different evaluation protocols followed in individual preclinical studies. This situation strongly hampers the selection of the most suitable analogues for further clinical validation. Using GRPR overexpressing human PC-3 bearing SCID mice, we evaluated the PC-targeting efficacy of five promising radiolabelled BN analogues for SPECT imaging under identical experimental conditions. With BN agonists Pesin, AMBA, MP2346 and MP2653 and one antagonist Demobesin-1[16-20] we performed in vivo stability studies, biodistribution, SPECT/CT imaging and ex-vivo autoradiography along with haematoxylin-eosin (HE) staining.

MATERIALS AND METHODS

Analogues and Radiolabelling

The tested analogues were selected on the basis of their good PC targeting efficacy, as previously reported. In Figure 1A I the amino acid composition of BN and analogues studied are shown. AMBA[18] and Pesin[20] were kindly provided by Prof. Dr. Mäcke, University Hospital Basel, Switzerland. MP2346[16] and MP2653[17] were supplied by BioSynthema (St Louis, MO, USA). In this study radiolabelling of Pesin, AMBA, MP2346, and MP2653 was performed with $^{111}InCl_3$ (Covidien, Petten, The Netherlands), as described earlier[16, 21-23]. Incubation for the DOTA-peptides was performed at 80ºC for 20 min.

Demobesin-1 was a gift from Dr. T. Maina and Dr. B. Nock, Demokritos, Athens, Greece. This peptide was radiolabelled with ^{99m}Tc as previously described by Nock et al.[19, 23].

In order to prevent oxidation and radiolysis quenchers were added to all radiolabelled analogues. Quenchers used were ascorbate (Bufa BV, Uitgeest, The Netherlands), gentisic acid (Covidien, Petten, The Netherlands) and methionine (Fluka Biochemika, Buchs, Switzerland)[23].

Quality Control

The radiolabelling yield and radiochemical purity (RCP) of the labelled analogues were assessed by instant thin layer chromatography (ITLC) in a 1:1 methanol:ammonium acetate solution[19]

Figure 1 IA

Analogue	radionuclide	chelator	linker	Amino acid													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bombesin				pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
MP2653	^{111}In	DTPA						aCMpip	Tha	Gln	Trp	Ala	Val	b-Ala	His	Tha	Nle-NH_2
MP2346	^{111}In	DOTA		Pro	Gln	Arg	Tyr	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
Pesin	^{111}In	DOTA	$dPEG_4$							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
AMBA	^{111}In	DOTA	CH2CO-Gly-4-aminobenzyl							Gln	Trp	Ala	Val	Gly	His	Leu	Met-NH_2
Demobesin-1	^{99m}Tc	N_4	BzDig						DPhe	Gln	Trp	Ala	Val	Gly	His	Leu-NHEt	

I Amino acid sequence of native BN (14 amino acids) and the BN-analogues used in this study.
Chelators: N_4 = 6-R-1,4,8,11-tetraazaundecane, DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, DTPA = Diethylene triamine pentaacetic acid.
Linkers: R= BzDig = p-aminobenzyldiglycolic acid, PEG = polyethylene glycol.
Introduced amino acids: ACMpip = 4-Amino-carboxymethylpiperidine (non-natural amino acid), Tha = β-(2-Thienyl)-alanine (non-natural amino acid), β-Ala = β-alanine (non-natural amino acid), Nle = norleucine, Pro = proline, Tyr = tyrosine, Phe = phenylalanine.
** reference number from list references*

and by HPLC (see below). For ^{99m}Tc labelled analogue Demobesin-1, an extra ITLC with a 100% acetone solution was performed to quantify ^{99m}TcO2 content. After radiolabelling and cooling down to ambient temperature, 10 µL 4mM DTPA was added to the ^{111}In-DOTA peptides in order to avoid false positive quality control results e.g. due to colloid formation to complex any free ^{111}In[24, 25]. For the yield of incorporation of radioactivity a lower limit of 95% was set.

HPLC analysis of radiolabelled analogues was performed on a Waters Breeze HPLC system (Waters, Etten-Leur, The Netherlands) based on a 1525 binary HPLC pump connected to a Unispec MCA γ-detector (Canberra, Zellik, Belgium). A Symmetry C_{18} column (5 µm, 4.6 mm x 250 mm, Waters, Etten-Leur, The Netherlands) served as stationary phase eluted with a solvent system consisting of 0.1% trifluoroacetic acid (TFA) in H_2O (solvent A) and 0.1% TFA in methanol (solvent B) in a gradient protocol; 0-2 min 100% solvent A (flow rate 1 mL/min), 2-3 min 55% solvent B, 3-30 min 65% solvent B (flow rate 0.5 mL/min), 30-38 min 100% solvent B (flow rate 1 mL/min), 38-40 min 100% solvent B, 40-46 min 100% solvent A.

Radiochemical purity was estimated for each analogue by evaluating radioactivity peaks eluted from the HPLC column and calculating the area under the curve.

Cell culture

Human PC-3 cells were cultured in RPMI (LONZA, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) (GIBCO Invitrogen Co, Grand Island, NY, USA) and 5mL/500mL penicillin/streptomycin antibiotics (10.000 Units/mL penicillin, 10.000 Units/mL streptomycin; LONZA, Verviers, Belgium). Cells were grown in T175 Cellstar tissue culture flasks (Greiner Bio-one GmBH, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged using a *Trypsin - EDTA* solution (LONZA, Verviers, Belgium) 170000 U/L Trypsine-Versene and 200 mg/L EDTA. For the present study, cells were grown to near confluency and were than harvested and counted. Suspensions of cells were dissolved in PBS to yield approximately $6x10^6$ cells/0.15 mL for subcutaneous injection.

PC-3 xenograft

6 to 7-week old male Swiss nu/nu mice (Charles River, Kißlegg, Germany) were inoculated subcutaneously with PC-3 cells in the right shoulder. A maximum of 4 mice per cage were kept in 14 x 13 x 33.2 cm^3 individually ventilated cages (Techniplast), on sawdust (Woody-Clean, type BK8/15; BMI) under a 12-h light-dark cycle, at 50±5% relative humidity and controlled temperature (~22°C). Mice received irradiated chow and acidified drinking water ad libitum. Experiments were initiated when the tumour size reached a volume of 200-500 mm^3 (3-5 weeks after inoculation).

The experiment was approved by the Animal Experimental Committee (DEC) of the Erasmus MC and performed in agreement with The Netherlands Experiments on Animals Act (1977) and the European Convention for Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18 March 1986).

Peptide stability in vivo

Non tumour-bearing 6 to 7-week old male Swiss nu/nu mice (Charles River, Kißlegg, Germany) were injected intravenously into a lateral tail vein with 1 nmol radiolabelled analogue labelled with 30 MBq ^{111}In or 60 MBq ^{99m}Tc in a volume of 100 µL. Animals were sacrificed at 5 and 15 min after administration. For each time point 2 animals were used.

Blood was collected by orbita punction in an ethylenediaminetetraacetic acid (EDTA) tube to prevent clotting, and put on ice. Subsequently, samples were centrifuged for 5 min, 9500 g at +4 °C, after which the supernatant was collected and precipitated with ethanol (serum:ethanol 1:1). At HPLC run the final ethanol concentration was below 50%. Precipitated serum was then centrifuged for 5 min at 9500 g to dispose of large proteins. The supernatant containing the radiolabelled peptide was used for HPLC analysis. Results were indicated as percentage of intact radiolabelled peptide as determined from the HPLC analysis (% intact peptide of two mice per timepoint).

Biodistribution studies

PC tumour bearing mice were injected intravenously into a lateral tail vein with 10 pmol of analogue labelled with an activity of 0.5 MBq ^{111}In or 2.5 MBq ^{99m}Tc, in a volume of 0.1 mL. Animals were sacrificed at 1, 4, or 24 h after administration of test analogue. For each time point 4 animals were used. To discriminate between receptor-specific and non-specific binding, for each analogue three additional mice were co-injected with an excess of unlabelled [Tyr^4]BN (0.1 mg in 0.1 mL) and sacrificed 4 h post injection (pi). The tumours and selected tissues (blood, pancreas, kidney and colon) were excised, weighed, and counted for radioactivity in a γ-counter (LKB-1282-compugamma system, Perkin Elmer, Groningen, The Netherlands). Data were calculated as percentage of the injected dose per gram of tissue (% ID/g) using counts from 0.01 mL of the original injectate as reference.

SPECT-CT

SPECT-CT scans were performed with a four-headed multiplexing multi-pinhole NanoSPECT/CT™ (Bioscan Inc.,Washington D.C., USA). Each head was equipped with an application-specific tungsten-based collimator with nine pinholes of 1.4 mm diameter imaging a cylindrical field of view, providing a reconstructed resolution in the submillimeter range. For SPECT-CT a peptide amount of 0.25 nmol in 0.1 mL was injected intravenously. This is needed to enlarge peptide amount for tumour binding to achieve sufficient ra-

dioactivity for imaging. Total injected activities ranged from 23-31 MBq for the ^{111}In-analogues to 142 MBq for Demobesin-1. For each analogue, whole-body scans were performed at 1 and 4 h after injection of the radiolabelled analogue. An acquisition time of approximately 200 seconds per view was used, resulting in a total scanning time of about 1 h 30 min per animal. CT scans were performed with the integrated CT using a tube voltage of 45 kV and an exposure time of 1500 ms per view. After acquisition, the SPECT data were reconstructed with HiSPECT software (Scivis GmBH, Göttingen, Germany). For CT reconstruction a cone beam filtered back projection was employed. SPECT and CT data were automatically co-registered since both modalities shared the same axis of rotation. The fused data sets were analysed in the InVivoScope post-processing software (Bioscan Inc., Washington D.C., USA). Mice were sacrificed and their tumours were dissected and quickly frozen in liquid nitrogen for subsequent ex-vivo autoradiography.

Ex vivo autoradiography

Frozen PC-3 tumours derived from SPECT animals were cut into sections of 10 µm (Microm Cryo-Star HM 560 M, Walldorf, Germany) and mounted on glass slides (Superfrost plus slides, Menzelgläser, Braunschweig, Germany). Tumour sections were exposed to Super Resolution phosphor imaging screens (Packard Instruments Co., CT, USA) in X-ray cassettes. After 24-72 h screens were imaged by a Cyclone phosphor imager and analysed using the OptiQuant 03.00 image processing system (Perkin Elmer, Groningen, The Netherlands). After exposure, PC-3 sections were haematoxylin-eosin (HE) stained and the staining pattern was compared to the corresponding autoradiogram.

Statistics

Statistical analyses were performed using SPSS 11.01 (SPSS Inc., IL, USA). For this study we applied the Mann-Whitney U test. Statistical significance was assumed if p values were lower than 0.05.

RESULTS

Quality control

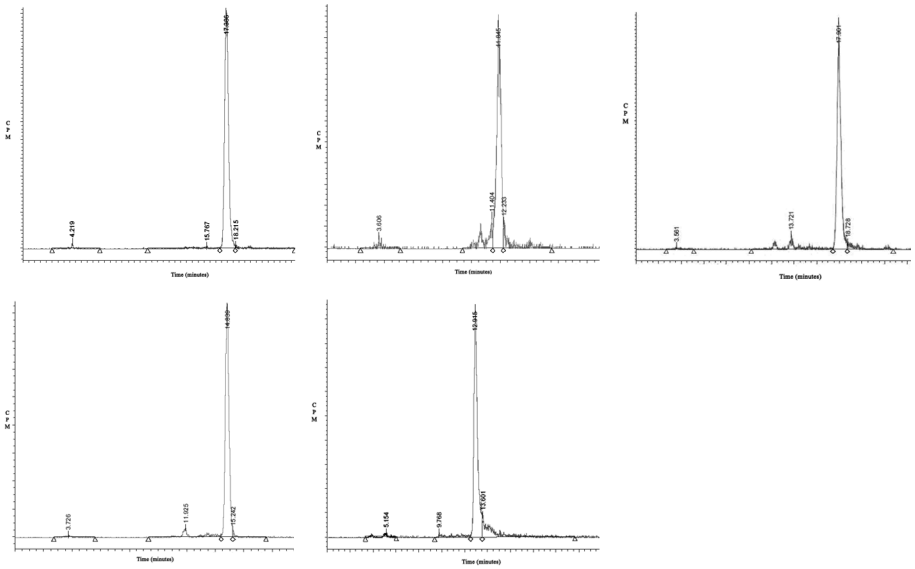
HPLC radiochromatograms for all analogues are presented in Figure 1B. Analyses of ITLC (data not shown) and HPLC indicated a labelling yield for all analogues of more than 97%. For Demobesin-1, formation of $^{99m}TcO_2$ colloid was <1% as concluded from ITLC analysis. RCP ranged from 74 to 93% for the various peptides.

Peptide stability in vivo

Peptide stability in vivo was highest for Demobesin-1 for both timepoints. At 5 min pi 64.1%±1.6 was still intact followed by AMBA (36.1%±2.7), Pesin (23.3%±1.4), MP2346 (21.2%±0.8), and MP2653 (9.8%±0.5). At 15 min pi

still 41.03%±0.11 of intact Demobesin-1 could be recovered with lower recoveries for AMBA (9.8%±1.1), MP2346 (3.4%±1.3), MP2653 (2.8%±0.4), and Pesin (1.8%±0.6) (Table 1). As derived from dose-calibrator measurements of activities in supernatant and serum it could be concluded that over 90% of total activity had been extracted from the serum after the precipitation procedure (data not shown).

Figure 1B



HPLC of all 5 radiolabelled analogues 1 h after labelling.

Table 1

In vivo stability of all analogues at 5 and 15 min pi injecting 1 nmol of radiolabelled analogue (30 MBq ^{111}In or 60 MBq ^{99m}Tc) i.v. in a volume of 0.1 mL in non-tumour-bearing nude mice. Results are indicated as % intact analogue from total injected analogue per mouse.

	mean (5 min)		mean (15 min)	
Analogue	Mouse 1	Mouse 2	Mouse 1	Mouse 2
^{99m}Tc-Demobesin-1	62.9	65.2	41,0	41.1
^{111}In-AMBA	34.2	38.0	9,0	10.6
^{111}In-Pesin	24.4	22.4	1.4	2.3
^{111}In-MP2346	21,8	20.6	4.3	2.4
^{111}In-MP2653	10.2	9.5	2.6	3.1

Biodistribution studies

Uptake results in various tissues for all radiolabelled analogues at all time points are summarised in Table 2. Figure 2 shows a graph of the tumour uptake results for all analogues at all timepoints. The highest PC-3 tumour uptake was obtained with Demobesin-1 (3.0±0.4 %ID/g) at 1h pi. The analogues AMBA, Pesin, MP2346, and MP2653 showed lower tumour uptakes at this time point: 2.7±0.5, 2.3±0.5, 2.1±0.9 and 0.9±0.2 % ID/g, respectively. The differences in tumour uptake at 4 h pi were consistent with those at 1 h. At 24 h, tumour retention of the ^{111}In-DOTA analogues AMBA and Pesin was highest: 1.6±0.4 and 1.2±0.2

Table 1

A. 1 hour pi (%ID/g ± SD)

Organ	**^{111}In-MP2653**	**^{111}In-MP2346**	**^{111}In-Pesin**	**^{111}In-AMBA**	**^{99m}Tc-Demobesin-1**
Blood	0.2±0.1	0.4±0.5	0.3±0.1	0.2±0.0	0.6±0.1
Pancreas	14.7±3.8	9.0±2.5	20.8±3.9	40.9±3.3	23.8±3.2
Kidneys	2.0±0.3	7.9±1.9	2.7±1.0	3.6±0.7	3.2±1.2
Colon	2.5±0.4	2.1±0.4	2.9±1.5	6.8±0.9	2.5±1.2
Tumour	0.9±0.2	2.1±0.9	2.3±0.5	2.7±0.5	3.0±0.4

B. 4 hour pi (%ID/g ± SD)

Organ	**^{111}In-MP2653**	**^{111}In-MP2346**	**^{111}In-Pesin**	**^{111}In-AMBA**	**^{99m}Tc-Demobesin-1**
Blood	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.0
Pancreas	11.4±2.5	8.9±2.1	17.5±2.8	40.5±7.6	4.1±1.6
Kidneys	1.2±0.4	10.6±1.6	1.5±0.4	3.4±1.4	1.1±0.2
Colon	1.6±0.6	1.8±0.7	3.6±0.4	5.6±1.2	1.2±0.3
Tumour	0.7±0.2	1.4±0.4	1.6±0.2	1.9±0.3	2.2±0.4

C. 24 hour pi (%ID/g ± SD)

Organ	**^{111}In-MP2653**	**^{111}In-MP2346**	**^{111}In-Pesin**	**^{111}In-AMBA**	**^{99m}Tc-Demobesin-1**
Blood	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pancreas	2.4±0.7	5.8±1.4	14.5±2.8	32.9±5.2	0.1±0.0
Kidneys	0.6±0.3	2.8±0.9	0.7±0.4	1.3±0.5	0.1±0.0
Colon	0.8±0.2	0.6±0.3	1.0±0.4	3.2±0.8	0.1±0.1
Tumour	0.4±0.1	0.7±0.4	1.2±0.2	1.6±0.4	0.4±0.1

Biodistribution data of all five radiolabeled analogues at time points A, B and C in PC3 tumour-bearing athymic nude mice injected with 10 pmol peptide, labelled with an activity of 0.5 MBq ^{111}In or 2.5 MBq ^{99m}Tc, in a volume of 0.1 mL into a lateral tail vein. Results are the mean and standard deviation of 4 mice per analogue per time point.

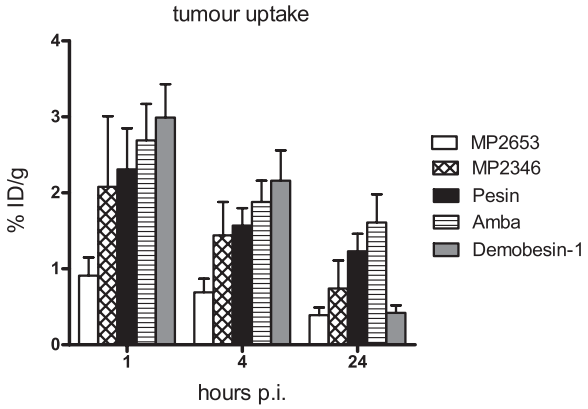


Figure 2
Tumour uptake for all analogues in PC-3 tumour-bearing athymic nude mice injected with 0.5 MBq ^{111}In or 2.5 MBq ^{99m}Tc bound to 10 pmol of peptide-conjugate, in a volume of 0.1 mL into a lateral tail vein sacrificed at 1, 4 and 24 h pi. Results are indicated as mean±standard deviation of 4 mice per analogue per time point.

%ID/g, respectively, with lower outcomes for MP2346, Demobesin-1 and MP2653 (0.7±0.4, 0.4±0.1 and 0.4±0.1 %ID/g, respectively). ^{99m}Tc labelled Demobesin-1 showed the most substantial decline in tumour uptake of 7.1 fold from 1 to 24 h, unlikely to what was observed for the other peptides. Tumour uptake of MP2653 was significantly lower than that of the other analogues at all time points.

AMBA showed the highest uptake in the GRPR expressing pancreas at 1 h pi (40.9±3.3 %ID/g). Lower uptake was found for Demobesin-1, Pesin, MP2653, MP2346 (23.8±3.2, 20.8±3.9, 14.7±3.8 and 9.0±2.5 %ID/g, respectively). Demobesin-1 showed a considerable wash-out from pancreas resulting in a 25-fold increase in tumour to pancreas ratio from 1 to 24 h pi. Increase in the tumour to pancreas ratio over time was significantly less for MP2653, Pesin, AMBA, and MP2346: 2.7, 1.0, 0.7 and 0.5 fold, respectively (Figure 3a).

In addition to the pancreas, also GRPR-specific specific uptake of radiolabel in colon was observed. Receptor specificity of peptide uptake was confirmed by co-injecting an excess of unlabelled [Tyr^4]BN resulting in strongly reduced uptake (>80%) of radiolabel in the colon. PC-3 tumours and GRPR-expressing pancreas showed a comparable blocking pattern for all analogues indicating GRPR-specific uptake. Colon uptake was highest for AMBA as compared to other analogues at all time points: 6.8±0.9 versus 2.9±1.5, 2.5±1.2, 2.5±0.4 and 2.1±0.4 %ID/g at 1h pi for Pesin, Demobesin-1, MP2653 and MP2346, respectively (Table 1).

The uptake of radiolabel by the kidneys was significantly higher for MP2346 (7.9±1.9 %ID/g at 1h) as compared to the other analogues at all time points: 3.6±0.7, 3.2±1.2, 2.7±1.0 and 2.0±0.3 %ID/g at 1h for AMBA, Demobesin-1, Pesin and MP2653, respectively (Table 1). The change in tumour to kidney ratio from 1 to 24 h pi was significantly higher for Demobesin-1 as compared to the other analogues: 3.6 fold versus

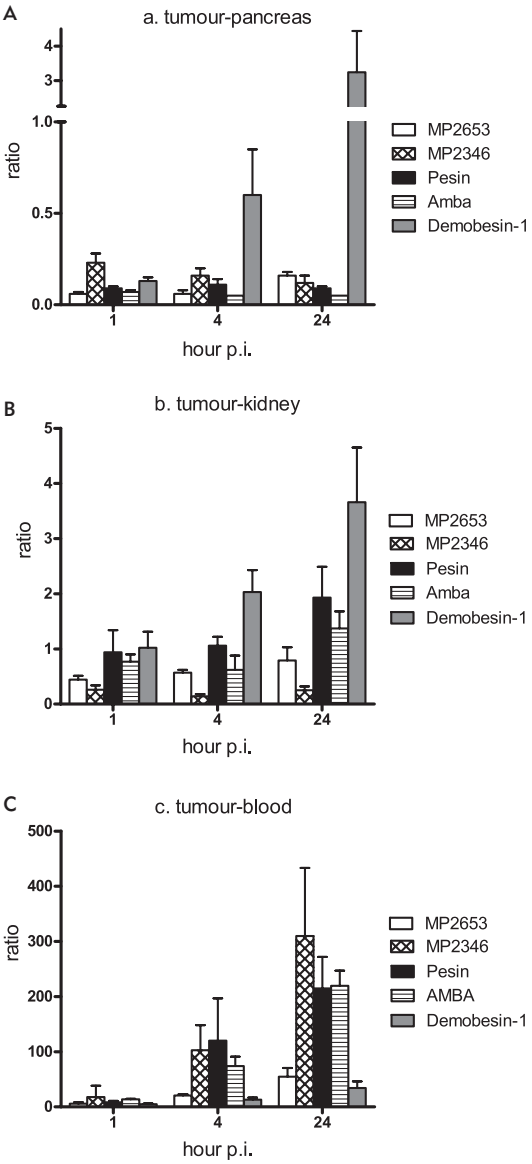


Figure 3

Ratios calculated from biodistribution in PC-3 tumour-bearing athymic nude mice injected with 0.5 MBq ^{111}In or 2.5 MBq ^{99m}Tc bound to 10 pmol of peptide-conjugate, in a volume of 0.1 mL into a lateral tail vein sacrificed at 1, 4 and 24 h pi.

Data show the tumour-blood ratio (a), tumour-pancreas ratio (b) and tumour-kidney ratio (c) at all time points for all radiolabelled analogues.

2.1, 1.8, 1.8 and 1.0 fold for Pesin, AMBA, MP2653, and MP2346, respectively (Figure 3b).

All peptides, except for MP2653 and Demobesin-1, showed high tumour to blood ratios indicating fast clearance from circulation. Pesin showed the largest increase in tumour to blood ratio of 14.4 fold from 1 to 4 h followed by MP2346, and AMBA (5.7 and 5.5, respectively) with the lowest ratios for MP2653 and Demobesin-1 (3.5 and 2.6, respectively) (Figure 3c).

SPECT-CT and ex vivo autoradiography

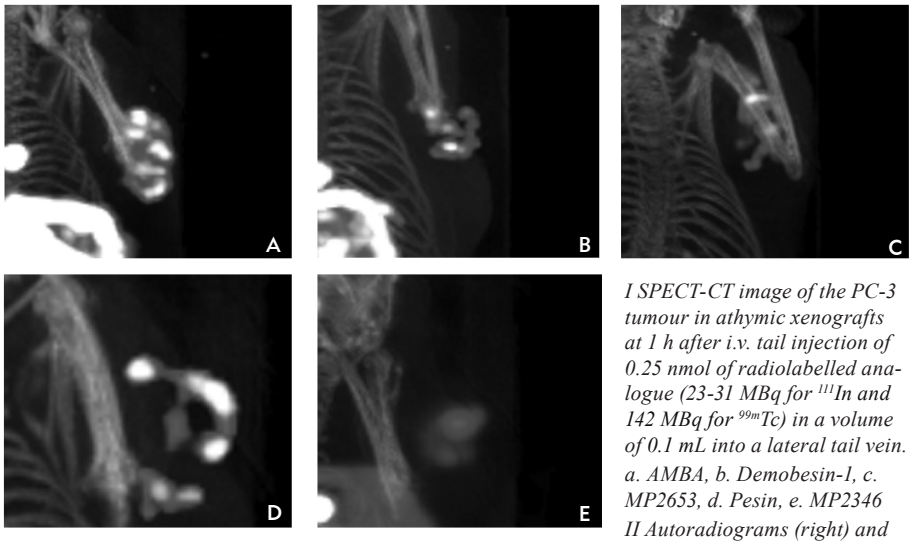
All analogues were able to image subcutaneous PC-3 tumours by SPECT-CT at 1 and 4 h pi (Figure 4 I). Additionally, ex vivo autoradiography of PC-3 tumours was performed at 1 and 4 h pi. Autoradiography revealed considerable heterogeneity of peptide binding within the tumour for all analogues with reduced binding in some (central) areas of the tumour as was also observed by in vivo SPECT-CT imaging as well. HE staining of consecutive slides revealed that this heterogeneous expression pattern coincided with re-

duced cell density in these tumour areas (Figure 4 II).

DISCUSSION

In order to initiate BN-based scintigraphic (SPECT) patient studies for the imaging of early PC, we reviewed preclinical studies from the literature as well as our own studies in search of a promising BN-analogue. Due to the lack of standardisation of experiments performed by various research groups, finding consistent comparisons - and thus a reliable choice of candidates - for further clinical validation, was very difficult. For this reason, we decided to set up a comparative study using a

Figure 4 I



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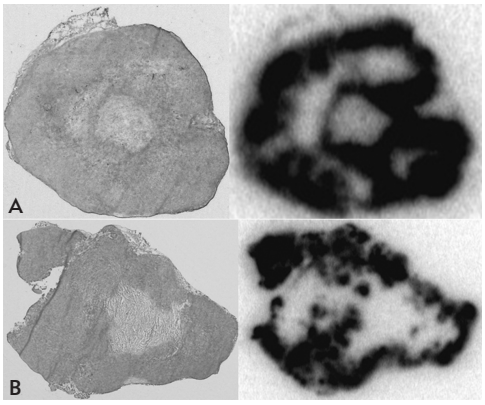

I SPECT-CT image of the PC-3 tumour in athymic xenografts at 1 h after i.v. tail injection of 0.25 nmol of radiolabelled analogue (23-31 MBq for ^{111}In and 142 MBq for ^{99m}Tc) in a volume of 0.1 mL into a lateral tail vein. a. AMBA, b. Demobesin-1, c. MP2653, d. Pesin, e. MP2346

II Autoradiograms (right) and corresponding haematoxylin-eosin stained sections (left) of a PC-3 tumour derived from a mouse 4 h after injection of MP2346 (a) and AMBA (b). Ex-vivo tumour sections were derived from PC-3 tumour-bearing athymic nude mice injected with 0.25 pmol peptide, labelled with 23-31 MBq for ^{111}In analogues in a volume of 0.1 mL into a lateral tail vein.

number of BN analogues under identical experimental conditions. Five radiolabelled analogues that had already been shown to have good GRPR affinities and targeting characteristics were selected for this study. Four of the radiolabelled BN analogues were GRPR agonists (AMBA, Pesin, MP2653, and MP2346) and one was an antagonist (Demobesin-1)[16-20].The choice for the BN agonist Pesin was based on studies that reported PC-3 tumour uptake of Pesin labelled with ^{67}Ga of 14.8 %ID/g 1 h after injection[20]. In another study, PC-3 tumour uptake of AMBA labelled with ^{177}Lu at 1 h pi was 6.4 %ID/g[18]. This analogue has already been applied in a therapeutic clinical study[26]. MP2346[16] and MP2653[17] labelled with ^{111}In have also shown high affinity for GRPR. All agonists were labelled with ^{111}In via a DTPA or DOTA.

The antagonist Demobesin-1 was included because of its very good profile (tumour uptake 1 h pi of 16.2 %ID/g in PC-3 bearing mice) shown in a preclinical study by Nock et al.[19]. Furthermore, recent preclinical studies using SST analogues suggest that radiolabelled antagonists are superior to agonists with regard to tumour uptake and retention of radioactivity[27]. Although it was shown that the antagonists did not trigger receptor internalisation in SST receptor expressing cells - a feature that was previously thought to be a crucial characteristic for good retention of tumour radioactivity - tumour values were reported to be higher for the antagonist than for the agonist. Also, the antagonists were shown washed out from normal organs much faster than agonists, which would potentially result in a reduction in radiotoxicity of normal organs. Recently, GRPR-based PC-3 tumour targeting using Demobesin-1 was also shown to be superior to an agonistic BN analogue with comparable receptor affinity[28].

The differences in half-life between the radionuclides we used required the inclusion of both early and late evaluation time points (1, 4 and 24 h pi). This enabled us to evaluate tissue and blood retention rates of the analogues over time. In the biodistribution studies we used 10 pmol of peptide per animal for all analogues. The peptide mass selected was based on the amounts reported in the literature[19, 20]. Also it was chosen while the peptide mass enables an appropriate labelling yield for biodistribution for all analogues[19, 20].

In the present study Demobesin-1 showed the highest tumour uptake at 1 and 4 h pi, although these values were not statistically significantly different from those of AMBA, Pesin, and MP2346. Tumour uptake of MP2653 was significantly lower when compared with that of all other analogues at all time points. Since sufficient tumour uptake is a prerequisite for diagnostic and therapeutic use, MP2653 was excluded from further evaluation. Although MP2346 showed good tumour-uptake, it had an unfavourable and significant higher uptake in the kidney when compared with the other analogues at all time points. This high renal uptake strongly limits its clinical use because of potential nephrotoxicity during PRRT. For this reason, MP2346 was

not further considered as best clinical analogue.

In contrast to the reduction in tumour uptake of Demobesin-1 from 3.0±0.4 %ID/g at 1 h to 0.4±0.1 %ID/g at 24 h pi, tumour retention of radioactivity after AMBA and Pesin injection remained fairly stable over this time period, indicating that these two analogues have potential for imaging at later time points. Also, this characteristic may be well suited for PRRT when BN analogues are coupled to therapeutic radionuclides for PRRT. Since a DOTA chelator allows AMBA and Pesin to bind a wide variety of radionuclides, both conjugates are suitable for imaging and PRRT purposes. Research on labelling N_4-chelated analogues with the beta-emitting ^{188}Re is ongoing.

The pancreas is the organ with the highest GRPR expression in mice. Its (non-intended) peptide uptake in relation to the targeted tumour uptake is of concern when evaluating GRPR targeted peptides. The tumour to pancreas ratio of Demobesin-1 showed an impressive and significant 4.6-fold increase from 1 to 4 h, which was much less marked in the other analogues (0.7-fold in AMBA and 1.2-fold in Pesin). In accordance with the study of Cescato et al.[28], the BN antagonist Demobesin-1 was rapidly washed out from the GRPR-expressing pancreas, while tumour uptake remained relatively high resulting in 25-fold higher tumour to pancreas ratios. We also observed this phenomenon in a patient study where we administered Demobesin-1 and compared it with an agonist ^{111}In-[DTPA-Pro^1,Tyr^4]bombesin (unpublished data). Besides uptake of radioactivity in pancreas, GRPR-specific uptake - as could be concluded from in blocking data - by the colon was also observed. Colon uptake is also highly relevant to PC imaging as it may interfere with detection of PC metastatic lesions at abdominal sites. Furthermore it may induce colon-radiotoxicity when employed for PRRT. Unfavourable GRPR-specific colon uptake was 2-fold higher for AMBA than for the other peptides.

Ideally, the tumour to blood ratios of analogues should increase over time with high retention in the tumour and declining activity in the circulation. Both AMBA and Pesin analogues showed large increases in tumour to blood ratios from 1 to 4 h (5.5-fold and 14.1-fold respectively). In contrast, Demobesin-1 showed the smallest change in this ratio (a 2.7-fold change between 1 and 4 h). The slower clearance of Demobesin-1 from the blood resulting in a lower tumour to blood ratio in comparison with the DOTA chelated peptides, may be related to the fact that the agonists are more hydrophilic and are therefore rapidly cleared by the kidney, whereas the lipophilic Demobesin-1 is excreted by the hepatobiliary tract as well. This could influence clearance kinetics. Rapid conversion to hydrophilic metabolites may also accelerate blood clearance of the agonists. Despite its favourable high tumour to blood ratio, the very high renal uptake of MP2346 remains a major disadvantage of this peptide.

Generally, peptides for use in peptide receptor-targeted scintigraphy

or therapy are chosen for their good BN receptor-targeting characteristics and in vitro stability. The in vitro serum half-life data of the BN peptides used in this study ranged from 3 hrs to several days[17-19, 21]. Peptide stability of analogues in vivo is, however, of much more relevance. For this reason, in the present study we determined the in vivo peptide stability of all analogues using HPLC. Peptide stability in vivo of all 4 agonists was very similar. Demobesin-1 was the most stable analogue. We can however not completely exclude the presence of unknown radiolabelled metabolites beside the intact peptide in the main peaks from HPLC. Despite the significantly longer in vivo peptide stability of Demobesin-1, tumour uptakes for this analogue were not significantly different from those of AMBA and Pesin. This implies that interactions between GRPR and BN occur rapidly. There was no direct correlation between in vivo peptide stability and analogue performance in mice.

The administration of pharmacologically-active agonistic peptides is known to cause endocrine and haemodynamic effects by the process of endocytosis[29]. Antagonists on the other hand do not have the potential to enter and activate cells and, as a consequence, pharmacological side effects may be less. This is especially relevant for PRRT where higher amounts of peptide are used than for imaging studies. Furthermore, several experimental studies have shown that BN antagonists induce anti-tumour effects[30-34], while BN agonists generally stimulate PC tumour growth[7, 35, 36]. Another advantage of Demobesin-1 is that it is highly selective for GRPR and has no affinity for other BN receptor subtypes, NMB-R and BB3-R[19]. The agonists AMBA and Pesin are not selective and bind to both GRPR and NMB-R[18, 20].

With regard to absolute tumour uptake of radioactivity, the present study showed lower tissue uptakes than is described for these analogues in the literature. The variation in radionuclides, amounts of peptide, mouse strain (species, sex, weight), PC-3 tumour cells (passage number, culture conditions) used, tumour size and vascularisation of the tumour may all be factors that determine uptake of radioactivity resulting in variable outcomes. This further underlines the necessity to evaluate and compare such analogues under standardised conditions.

The present study showed that all analogues were able to target subcutaneous PC-3 tumours as visualized by SPECT-CT. Ex vivo autoradiography of PC-3 tumours further revealed that in all analogues there was considerable heterogeneity in the distribution of radioactivity between individual tumours. The heterogenic pattern could be histologically related to areas with reduced cell density, predominantly in the centre of the tumours. This was further supported by the SPECT-CT images showing a rim-like tumour image. When considering using radiolabelled BN for PRRT, it should be taken into account that the area that will be irradiated contains all vital PC cells. Mouse models are not ideal while central necrosis results in underestimation of uptake (%ID/g).

This underlines the necessity to perform biodistribution on mice with small tumours only. Furthermore it should be investigated whether central necrosis occurs in patient tumours as well.

In conclusion, the comparison of five analogues performed in the present study provides a realistic reflection of the potential of a number of peptides, in spite of the differences between tumour uptake and normal organ values found in our experiments and those of previous studies. The BN antagonist Demobesin-1 showed the highest tumour uptake 1 and 4 hours pi, with a particularly good tumour to pancreas ratios and a significantly higher in vivo analogue stability compared with the other analogues. This good performance, in combination with the advantages of the use of antagonists over agonists and its ^{99m}Tc label, make Demobesin-1 a very attractive analogue for PC imaging. Based on high tumour uptake, retention profiles and good tumour to blood ratios combined with sufficient stability in vivo, Pesin and AMBA outperformed the other two agonists. As they have a DOTA chelator, both analogues are currently more easily applicable for PRRT than the N_4–functionalized Demobesin-1 analogue. Our results indicate that all three BN analogues deserve further evaluation for clinical nuclear imaging, and possibly PRRT, of human PC. Demobesin-1 has been selected for a first proof of concept clinical study.

ACKNOWLEDGEMENTS

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CHAPTER 4

ANDROGEN-REGULATED GASTRIN-RELEASING PEPTIDE RECEPTOR EXPRESSION IN ANDROGEN-DEPENDENT HUMAN PROSTATE TUMOUR XENOGRAFTS

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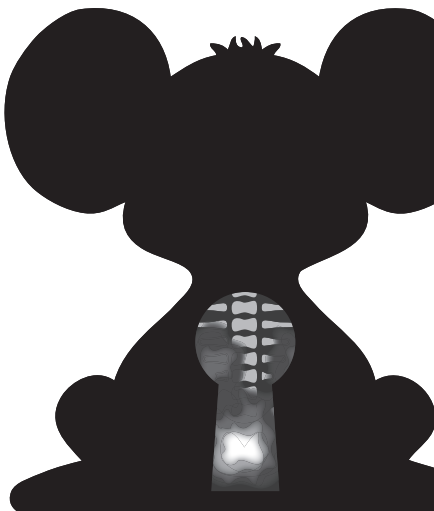

ABSTRACT

Human prostate cancer (PC) overexpresses the Gastrin-Releasing Peptide Receptor (GRPR). Radiolabelled GRPR-targeting analogues of bombesin (BN) have successfully been introduced as potential tracers for visualization and treatment of GRPR-overexpressing tumours. A previous study showed GRPR-mediated binding of radiolabelled BN analogues in androgen-dependent but not in androgen-independent xenografts representing the more advanced stages of PC.

We have further investigated the effect of androgen modulation on GRPR-expression in three androgen-dependent human PC-bearing xenografts: PC295, PC310 and PC82 using the androgen-independent PC3-model as a reference.

Effects of androgen regulation on GRPR expression were initially studied on tumours obtained from our biorepository of xenograft tissues performing RT-PCR and autoradiography (^{125}I-universal-BN). A prospective biodistribution study (^{111}In-MP2653) and subsequent autoradiography (^{125}I-GRP and ^{111}In-MP2248) was than performed in castrated and testosterone resupplemented tumour-bearing mice.

For all androgen-dependent xenografts tumour uptake and binding decreased drastically after 7 days of castration. Resupplementation of testosterone to castrated animals restored GRPR expression extensively. Similar findings were concluded from the initial autoradiography and RT-PCR studies. Results from RT-PCR, for which human specific primers are used, indicate that variations in GRPR expression can be ascribed to mRNA downregulation and not to castration-induced reduction in the epithelial fraction of the xenograft tumour tissue.

In conclusion, expression of human GRPR in androgen-dependent PC xenografts is reduced by androgen ablation and is reversed by restoring the hormonal status of the animals. This knowledge suggests that hormonal therapy may affect GRPR expression in PC tissue making GRPR-based imaging and therapy especially suitable for non-hormonally treated PC patients.

INTRODUCTION

Prostate cancer (PC) is the third leading cause of cancer-related deaths and the most frequently diagnosed cancer in men in Western countries [1]. PC will increase to be a major health problem due to the aging of people in the Western world and currently developed screening strategies that result in diagnosis at more early stage of disease. In case of metastasised late stage PC standard therapy exists of hormone deprivation which is initially effective in the majority of patients [2]. Eventually however PC patients will relapse due to progression of PC towards hormone-independent growth of the tumour. Unfortunately, advanced PC responds poorly to chemotherapeutic agents of which taxane-based treatments are nowadays considered the standard second-line treatment of hormone-resistant PC [3, 4]. Finally, most patients with metastasised PC will die of progressive, hormone-independent disease. Treatment and imaging of PC demands for new concepts, a potential strategy lies in targeting tumour-specific receptors. It could improve staging of PC determining locality of disease at time of diagnosis which is required to define treatment decisions.

It has been shown that prostate tumours overexpress the Gastrin-Releasing Peptide Receptor (GRPR) [5-9]. Overexpression of these receptors in PC is restricted to the malignant cells, as normal and hyperplastic prostate tissue were shown to be GRPR-negative [5]. The GRPR is a member of the bombesin (BN) receptor family consisting of four known receptor subtypes. Three of them the neuromedin B (NMB) receptor (BB_1), GRPR (BB_2) and BN receptor subtype 3 (BRS-3 or BB_3), are mammalian receptors, whereas the fourth subtype (BB_4) is found only in amphibians [9-13]. BN-like receptors, such as NMB, BB_2 and BB_3 are involved in the regulation of a large number of biological processes in the gut and central nervous system [14] and mediate their action by binding to their BN receptor subtypes. BN analogues are attractive peptides to be used to target the GRPR. Its use has been proposed for chemotherapy using cytotoxic BN analogues and for nuclear interventions employing radiolabelled BN analogues to perform peptide-receptor scintigraphy and radionuclide therapy (PRRT) [5].

In a previous study we evaluated the expression of GRPRs in different stages of tumour development using a panel of 12 xenograft models from 9 different patients representing the various stages of human PC ranging from androgen-dependent to androgen-independent disease [15, 16]. We found GRPR-mediated binding of radiolabelled BN analogues only in androgen-dependent, but not in androgen-independent xenografts representing the more advanced stages of prostate tumour development [17]. Also, in a pilot study, GRPR-expression of an androgen-dependent xenograft showed reduced uptake after androgen ablation treatment, suggesting that GRPR-expression may be influenced by androgens and may be related to the progressive state of PC.

Since the majority of patients are treated with hormonal therapy during the course of their disease, it is highly relevant to study the impact of hormonal ablation treatment on GRPR expression in order to reveal whether such patients could still be eligible to GRPR targeted modalities. Therefore, the purpose of the present study was to further investigate androgen regulation of the GRPR in three androgen-dependent human PC xenograft models PC82, PC295 and PC310 that represent the early androgen-responsive state of human PC [18]. We hypothesized that if GRPR is androgen regulated, the expression is reduced by androgen ablation in androgen-dependent xenograft-bearing mice, and should be reversed by restoring the hormonal status of the animals. This experimental design allows to elucidate the direct effects of hormonal changes on GRPR expression in relevant models for human PC. Since re-supplementation of patients with testosterone is not likely to be applicable in PC patients, the present study set-up is merely experimental in nature and less intended to mimic the clinical setting. Knowledge about the background of hormonal regulation of the GRPR will provide crucial information for the clinical use of GRPR-based technologies. It enables to define patient groups that may benefit from GRPR targeted modalities. As a reference the androgen-independent xenograft model PC3 was used. Although PC3 is androgen-independent, GRPR is constitutively overexpressed and, therefore, this model is frequently used in studies on GRPR expression in PC.

MATERIALS AND METHODS

PC Xenografts

For this study we made use of intact male nude NMRI (Naval Medical Research Institute) mice (Taconic M&B, Ry, Denmark) [15-17]. The experiment was approved by the Dutch National Animal Experimental Committee and performed in agreement with The Netherlands Experiments on Animals Act (1977) and the European Convention for protection of Vertebrate Animals used for Experimental Purposes (Strasbourg, 18 March 1986).

Mice were implanted subcutaneously with small fragments of the androgen-dependent PC82, PC295 and PC310 human prostate tumour [15]. Mice were supplemented with testosterone to obtain optimal tumour take (80-85%) and tumour growth by implanting silastic tubings filled with crystalline steroid (6mg/tubing; AppliChem, Darmstadt, Germany). These implants result in constant delivery of testosterone exceeding the low physiological levels in intact male nude mice for at least 75 days [19]. For the androgen-independent PC3 xenografts, cell suspensions (5 million cells per mouse) were injected subcutaneously without additional testosterone supplementation.

Androgen ablation and testosterone resupplementation

Androgen withdrawal was performed by surgical castration under Ketamin (Alfasan, Woerden, The Netherlands) and Rompun (Bayer AG, Leverkussen,

Germany) anaesthesia (mix of 1:1), and subsequent removal of testosterone implants. At 2, 4 and 7 days after castration mice were used for biodistribution studies. Control mice were used when tumour volumes were between 600 and 800mm^3, which was reached approximately 90 days after tumour implantation. Castration of tumour-bearing mice was planned in such a way that tumour volumes at time of inclusion were in the same range as those of the control mice.

Resupplementation of testosterone to mice was accomplished under Ketamin: Rompun anaesthesia by subcutaneous re-implantation of testosterone implants at 7 days after castration. Biodistribution was then performed at a predefined number of days after resupplementation (2, 4 or 14 days).

Radiolabelled Peptides

Bombesin analogue [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]BN(6-14), referred to as universal-BN, was provided by Biosynthema Inc. (St. Louis, USA) and radiolabelled with ^{125}I as described previously (radiolabelled compound as from now referred to as ^{125}I-universal-BN) [20]. ^{125}I labelled GRP was obtained commercially from Amersham Biosciences (Buckinghamshire, United Kingdom).

Bombesin analogues (MP2248) and (MP2653) were provided by Mallinckrodt Inc. (St. Louis, USA), and radiolabelled with ^{111}In ($^{111}InCl_3$, Tyco Healthcare, Petten, the Netherlands, DRN 4901, 370 MBq/ml in HCl, pH 1.5-1.9) as described earlier [21-24].

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR an independent set of PC82, PC295 and PC310 tumour tissue was obtained from our biorepository of xenograft tissues (snapfrozen material stored at minus 80°C) and not from tissues from the biodistribution studies as snapfrozen tissues are essential to obtain high quality RNA. Tumours from the biodistribution studies could only be frozen after counting in the gammacounter, which time delay strongly affects RNA quality.

Total RNA was isolated using RNAzol B kit (Bio-connect, Huissen, The Netherlands) according to the manufacturer's protocol. The reverse transcriptase reaction was performed with 1 µg RNA and 100 ng/µl oligo(dT)$_{12-18}$ primer (Invitrogen, Carlsbad, CA) for 10 min at 70°C in 11 µl volume and chilled on ice. cDNA was reverse transcripted in first-strand buffer (4 µl, 5x; Invitrogen) plus 2 µl 100 mM DTT, 1 µl 10 mM dNTP mix, 1µL RNasin ribonuclease inhibitor (Invitrogen) and 200 U MMLV-RT (Invitrogen) followed by 1 hr incubation at 37°C. The process was inactivated by heating to 95°C for 10 min samples were than stored at minus 20°C.

Gene expression of GRPR and the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was performed with human-specific primers and probes purchased as a FAM dye-labelled TaqMan MGB primer probe kit (Applied Biosystems, Foster City, USA). GAPDH was used as the

endogenous reference gene in order to relate GRPR mRNA levels to the total epithelial (human) cell content of each sample. The real time PCR reaction was performed in a volume of 25 ul containing cDNA. PCR mixture contained a final concentration of 360 nM primer, 100 nM probe, commercially available PCR mastermix (TaqMan Universal PCR Mastermix; Applied Biosystems). The PCR mastermix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U of AmpliTaq Gold DNA polymerase and 0.25 U AmpErase UNG per reaction. Samples were amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System; Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. The amount of target gene expressed was related to a calibrator composed of a mixture of cDNAs from different PC xenografts. Results were calculated for each cell line as the average relative quantity of the 2 tissue samples analysed in duplicate.

Statistical analysis was performed using the unpaired 2-tailed t-test using SPSS 15.0 (SPSS Inc., Chicago, United States). A probability of less than 0.05 was considered significant.

Biodistribution Studies

For biodistribution studies, mice were injected intravenously with 4 MBq / 60 pmol (0.1 µg) / 200 µl of the radiolabelled BN analogue ^{111}In- MP2653 at predefined time points, and sacrificed 4 hours after peptide injection. Tumour tissue and the GRPR-positive pancreas as well as kidneys and intestines (relevant organs for clearing) were collected for counting of radioactivity in a LKB-1282-compugamma system (Perkin Elmer, Oosterhout, the Netherlands). Radioactive uptake was calculated as % injected dose per gram of tissue (%ID/g) as a percentage of the control group (100%). Immediately after counting, the tumours were snapfrozen in liquid nitrogen for autoradiography.

Statistical analysis was performed using the Mann Whitney and unpaired t-test. A probability of less than 0.05 was considered significant.

Autoradiographic Analyses

PC xenograft tumour tissues derived from hormonally manipulated mice were evaluated for GRPR expression by in vitro autoradiography as described previously [17]. In short, frozen xenograft sections (10 µm) were incubated for 1 hour at room temperature with 0.1 nM radiolabeled compound. To discriminate between GRPR-mediated binding and non-receptor-mediated binding, sections were incubated in the presence of an excess (1 µM) of unlabelled GRP or [Tyr^4] BN (Sigma Aldrich, Zwijndrecht, The Netherlands) as a blocking agent. Sections were exposed to phosphor imaging screens (Perkin Elmer, Boston U.S.A.) for 16-72 hours. Screens were read using a Cyclone Storage Phosphor System (Packard, Meriden, USA) and

autoradiograms were quantified using Optiquant Software (Packard, Meriden, USA). Results are indicated as average GRPR-mediated radiolabelled peptide binding (net Density Light Units/ mm^2 of total binding minus non-specific binding) as a percentage of the control group (100%).

Statistical analysis was performed using the Mann Whitney and unpaired t-test. A probability of less than 0.05 was considered significant.

RESULTS

Effect of androgens on GRPR mRNA levels: Quantitative RT-PCR

To evaluate changes in mRNA expression of GRPR during androgen manipulation, RT-PCR was performed on RNA from an independent set of PC82, PC295 and PC310 tumour tissue obtained from our biorepository of xenograft tissues. In order to correct for variability of stromal (murine) content of the otherwise human (epithelial) tumours, human-specific probes were used and GRPR mRNA levels were related to GAPDH. Relative GRPR mRNA levels under control conditions were high for PC295 and PC310 with a mean GRPR/GAPDH ratio of 27.2 and 21.5, respectively. PC82 showed much lower GRPR mRNA levels with a mean GRPR/GAPDH ratio of 4.1. After androgen ablation, the relative GRPR expression dropped considerably for PC295, PC310 and PC82 (Figure 1). Re-supplementation with testosterone resulted in recovery of GRPR mRNA levels for all xenografts although recovery levels were quite variable (Figure 1).

Effect of Androgens on GRPR-Mediated Tumour Uptake: Biodistribution Studies

Subsequent to the RT-PCR and initial autoradiography a prospective biodistribution study was performed to evaluate potential androgen regulation of GRPR and thus the effect of androgen status on GRPR-mediated tumour uptake in vivo using the radiolabelled BN analogue ^{111}In-MP2653 in PC tumour-bearing mice under hormonal manipulation. Table 1 summarizes the results of 3 independent biodistribution studies. The first experiment was performed with the androgen dependent PC295 xenograft. The outcome of this study was further substantiated in a subsequent biodistribution study using the androgen sensitive xenograft PC82 with a more extended time point of testosterone re-supplementation. Finally, to validate the outcome of these 2 experiments, a third biodistribution study was performed with the androgen sensitive xenograft models PC295, PC82 and PC310, comparing GRPR expression in intact mice (control), castrated mice (cas 7), and castrated and re-supplemented mice (cas 7 T 14) with the androgen-independent PC3 as reference model.

The uptake of the ^{111}In-labeled peptide at 4 hours after injection in the GRPR-positive tumour and GRPR-expressing pancreas as well as in the clearance organs (kidneys and intestines) were found to be in agreement

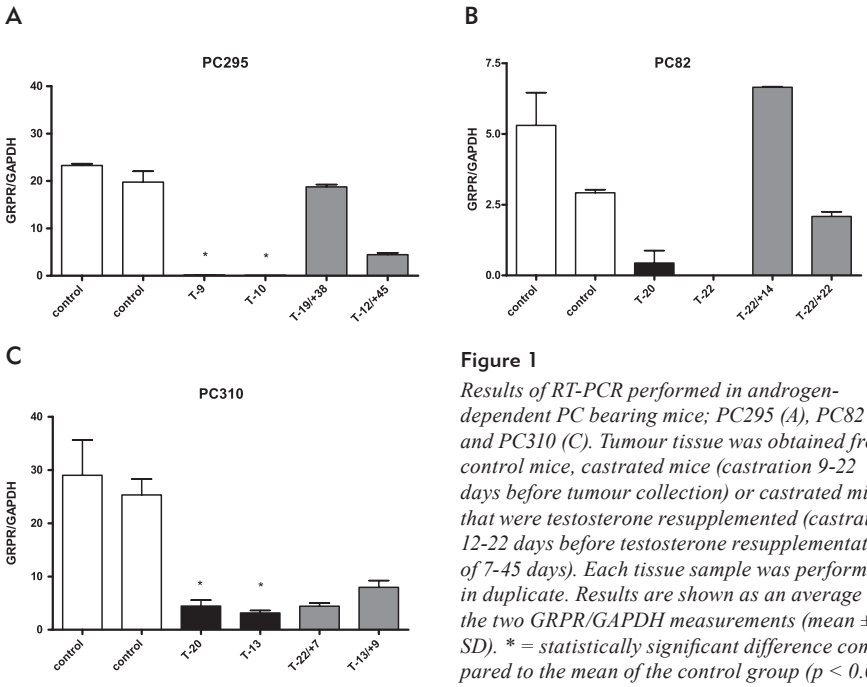

Figure 1

*Results of RT-PCR performed in androgen-dependent PC bearing mice; PC295 (A), PC82 (B) and PC310 (C). Tumour tissue was obtained from control mice, castrated mice (castration 9-22 days before tumour collection) or castrated mice that were testosterone resupplemented (castration 12-22 days before testosterone resupplementation of 7-45 days). Each tissue sample was performed in duplicate. Results are shown as an average of the two GRPR/GAPDH measurements (mean ± SD). * = statistically significant difference compared to the mean of the control group ($p < 0.05$).*

with our previous results obtained with ^{111}In-MP2653 in mice bearing PC3 tumours [25]. Tumour and pancreas uptake in all PC xenografts are summarized in Table 1A. The androgen-dependent PC tumours demonstrated a significant inhibition of growth after androgen ablation (data not shown). The androgen-dependent xenografts also show a reduction in tumour uptake of ^{111}In-MP2653 after 4 and 7 days of castration. This decline was strongest in PC295 showing a decrease in tumour uptake of 73% ($p = 0.0002$) after 7 days of castration. The reduction of peptide uptake in androgen depleted PC82 and PC310 tumours was less pronounced than in PC295, although still significant (54 % and 41% respectively after 7 days of castration, $p < 0.017$).

Re-supplementation of testosterone to castrated mice for 14 days resulted in near complete recovery of tumour uptake in PC82 and PC310 mice to control levels (86% and 99%, respectively). For PC295, a partial restoration (40%) of peptide uptake by the tumours was observed after 4 days of testosterone resupplementation.

Tumour growth of the androgen-independent PC3 model was not affected by ablation treatment nor by testosterone suppelmentation (data not shown). In contrast to the androgen sensitive xenografts, tumour uptake of ^{111}In-MP2653 was significantly increased up to 141% at 7 days post-castration, which was normalized after testosterone resupplementation.

Table 1

Group	study	PC295		PC82		PC310		PC3		Pancreas#	
		% control	(n)	% control	(n)	% control	(n)	% control	(n)	% control	(n)
control	A uptake ^{111}In-MP2653	100 ± 31	(11)	100 ± 31	(14)	100 ± 19	(4)	100 ± 33	(12)	100 ± 21	42
	B binding ^{125}I-GRP	100 ± 19		100 ± 37		100 ± 67		100 ± 53			
	C binding ^{111}In-MP2248	100 ± 36		100 ± 54		100 ± 25		100 ± 42			
cas 2	A uptake ^{111}In-MP2653	124 ± 29	(5)	109 ± 37	(3)						
	B binding ^{125}I-GRP	72 ± 6.9*		67 ± 26		nd		nd		nd	
	C binding ^{111}In-MP2248	55 ± 17*		67 ± 28							
cas 4	A uptake ^{111}In-MP2653	22 ± 7.3*	(5)	56 ± 30	(3)						
	B binding ^{125}I-GRP	11 ± 8.1*		29 ± 27*		nd		nd		nd	
	C binding ^{111}In-MP2248	6.5 ± 6.4*		27 ± 30							
cas 7	A uptake ^{111}In-MP2653	27 ± 22*	(12)	46 ± 17*	(8)	59 ± 4.0*	(5)	141 ± 24*	(5)	101 ± 32	33
	B binding ^{125}I-GRP	3.4 ± 1.9*		12 ± 5.3*		55 ± 14		123 ± 54			
	C binding ^{111}In-MP2248	1.5 ± 0.8*		7.7 ± 3.8*		52 ± 28		120 ± 19			
cas 7 T 2	A uptake ^{111}In-MP2653	35,8 ± 11	(5)	62 ± 10	(3)						
	B binding ^{125}I-GRP	20 ± 6.0**		33 ± 4.6**		nd		nd		nd	
	C binding ^{111}In-MP2248	14 ± 6.5**		28 ± 6.4**							
cas 7 T 4	A uptake ^{111}In-MP2653	40 ± 9.4	(4)	107 ± 12**	(3)						
	B binding ^{125}I-GRP	30 ± 9.7**		60 ± 23**		nd		nd		nd	
	C binding ^{111}In-MP2248	17 ± 6.3**		56 ± 10**							
cas 7 T 14	A uptake ^{111}In-MP2653			86 ± 46	(8)	99 ± 30**	(5)	95 ± 47	(5)	99 ± 17	20
	B binding ^{125}I-GRP	nd		91 ± 30**		50 ± 19		112 ± 76			
	C binding ^{111}In-MP2248			87 ± 40**		83 ± 21		125 ± 49			

Results of biodistribution and in vitro autoradiographic studies.

A Biodistribution results are average tumour and pancreas uptake of ^{111}In-MP2653 (% injected dose / gram tissue) as percentage of controls (100%) per hormonally manipulated group, data are expressed as mean ± SD. Numbers of animals used per group are stated under (n).

B + C In vitro autoradiography results are average GRPR-mediated binding (net Density Light Units / mm² of total binding minus non-specific binding) as percentage of controls (100%) of ^{125}I-GRP (B) and ^{111}In-MP2248 (C) data are expressed as mean ± SD. Numbers of animals used per group are stated under (n).

*cas = castration (for 2, 4 or 7 days); T = testosterone resupplementation for 2, 4, or 14 days; nd= not determined. * = significantly different from control group ($p < 0.05$); ** = significantly different from castration for 7 days group ($p < 0.05$). # Groups consist of pancreas from non tumour-bearing and tumour bearing androgen supplemented, castrated and castrated subsequently resupplemented intact mice.*

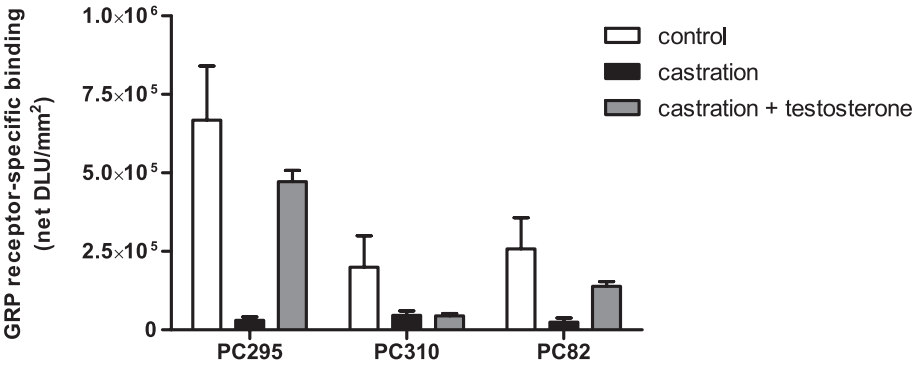

Figure 2

GRPR-specific binding of ^{125}I-universal-BN (= total binding ^{125}I-universal-BN 10^{-10}M) minus non-specific binding (^{125}I-universal-BN 10^{-10}M + GRP 10^{-6}M) to androgen-dependent PC xenograft sections. Tumour tissue was obtained from control mice, castrated mice (castration 7-10 days before tumour collection) or castrated mice that were testosterone resupplemented (castration + testosterone= 57 + 38 days, 29 + 13 days and 20 + 22 days respectively for PC295, PC310 and PC82). Results are shown as average net Density Light Units per mm² (net DLU/mm²). Results are average from 3 independent samples (except for the castration + testosterone group of which we had 1 sample) ± SD. We used 2-4 sections per independent sample.

^{111}In-MP2653 uptake in the GRPR expressing pancreas was similar for all mice under the various hormonal conditions (Table 1).

Effect of Androgens on GRPR-mediated binding: Autoradiographic Analyses

The effect of androgen status on GRPR-mediated binding in human PC was evaluated in vitro by autoradiography. Initially, autoradiographic analysis was performed on tumour sections derived from our biorepository of xenograft tissues (snapfrozen material stored at minus 80°C) using ^{125}I-universal-BN (74 TBq/mmol). This tumour collection contained tissues from control mice, castrated mice (castrated 8-10 days prior to sacrifice) and castrated mice that were resupplemented with testosterone for indicated days (see Figure 2). Binding of ^{125}I-universal-BN was drastically reduced after androgen ablation in all 3 androgen-dependent PC xenografts resulting in 95%, 77% and 90% reduced binding in PC295, PC310, and PC82, respectively. Testosterone re-supplementation partially restored binding to 70% and 54% of control level as observed for PC295 and PC82, respectively, while such restoration of peptide binding was not observed for PC310 after 13 days of testosterone administration (Figure 2).

Additional autoradiographic evaluations were performed on tumour sections derived from the prospective biodistribution studies using ^{125}I-GRP (74 TBq/mmol) or ^{111}In- MP2248 (200 MBq/nmol) as ligands. In compliance

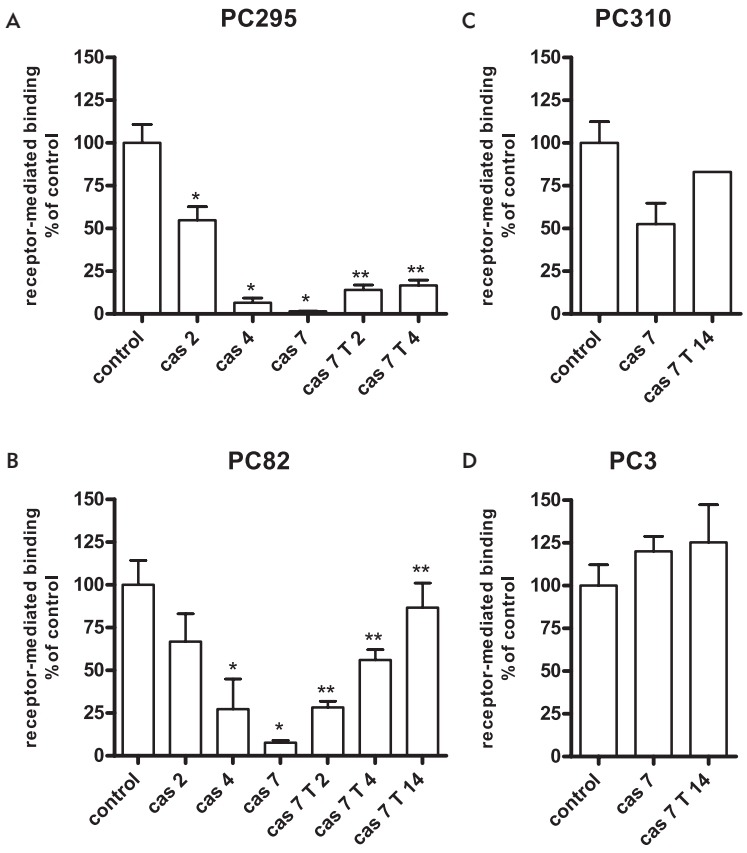

Figure 3

*Receptor-mediated binding of ^{111}In-MP2248 to androgen-dependent PC295 (A), PC82 (B), PC310 (C), and androgen-independent PC3 (D) xenograft sections. Tumour tissue was derived from the tumour-bearing mice from the biodistribution study. Autoradiography results are average receptor-mediated binding (net Density Light Units/ mm^2 of total binding minus non-specific binding) as percentage of the control group (100%) ± SEM. N = 4-14 xenografts / group, 2 sections per xenograft tumour were used. cas = castration for 2, 4 or 7 days; T = testosterone resupplementation for 2, 4, or 14 days; * = significantly different from control group ($p < 0.05$); ** = significantly different from cas 7 group ($p < 0.05$).*

with the biodistribution results, PC295 and PC82 showed strong and significant responses to androgen ablation. After 7 days of castration GRPR-binding was reduced by more than 96% ($p < 0.0001$) 88% ($p = 0.0003$) for PC295 and PC82, respectively (Figure 3A and B). A partial, though significant recovery of GRPR-binding was observed for PC295 and PC82 (17% $p = 0.004$ and 56% $p = 0.0002$, respectively) at 4 days of testosterone re-supplementation with near-complete restoration of receptor-binding at 14 days of testosterone resupplementation for PC82 (to 91%). For PC310, the reduction in

peptide binding after castration as was less pronounced (45%) and recovery after testosterone resupplementation was only moderate (Figure 3C). Consistent with the biodistribution data, the androgen-independent PC3 model demonstrated a rise in GRPR-binding after testosterone ablation, which was restored to control levels after testosterone resupplementation (Figure 3D).

metastatic stage of the tumour is often difficult to determine. The metastatic stage of PC greatly influences prognosis and determines therapeutic options of the disease. Therefore, reliable and sensitive diagnostic tools are highly wanted to improve staging at time of diagnosis. Radiolabelled BN analogues may be promising new radiopharmaceuticals for imaging and therapy for

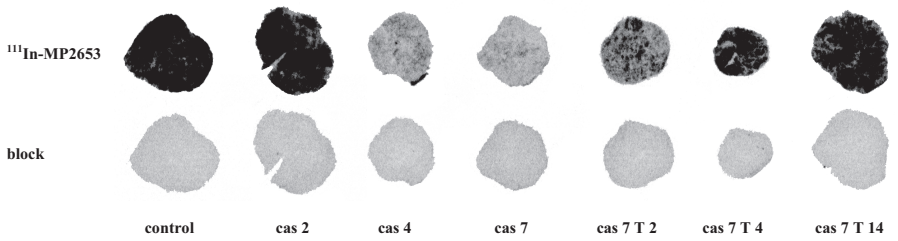

Figure 4

In vitro autoradiograms of PC82 tumour sections incubated with 10^{-10}M ^{111}In-MP2248 with or without an additional blocking agent Tyr^4-BN (10^{-6}M). Xenografts were derived from castrated and testosterone resupplemented mice used in the biodistribution.

Autoradiograms of PC82 tumour sections incubated with 10^{-10}M ^{111}In-MP2248 with or without blocking agent Tyr^4-BN (10^{-6}M) revealed a heterogeneous distribution pattern of the BN-tracer within these samples (Figure 4).

DISCUSSION

Opportunistic screening for PC has led to diagnosis of more PC tumours still confined to the prostate in which curative surgical intervention is still an option. However, due to lack of accurate imaging modalities for PC, the (micro)

patients with GRPR-expressing tumours, such as PC [26-31].

Evaluation of GRPR expression throughout the different stages of human prostate tumour development using a panel of 12 well established human PC xenograft models has shown that GRPRs were predominantly expressed in androgen-dependent xenografts and were very low in androgen-independent xenografts [17]. This observation stimulated further research into the potential effects of androgen status on the expression of the GRPR in PC patients and, consequently, the relevance of their clinical history (therapy sequence). To further study andro-

gen regulation of the GRPR we used three androgen-responsive prostate cancer models. We hypothesized that if GRPR-expression was indeed regulated by androgens, androgen ablation of androgen-dependent xenograft bearing mice would reduce GRPR expression and recovery of androgen status would induce re-appearance of GRPR expression. An initial in vitro autoradiographic binding study using ^{125}I-universal-BN as ligand supported our hypothesis. Complete inhibition of GRPR binding was observed after androgen withdrawal for all xenografts, which could be recovered after supplementation with testosterone for PC295 and PC82. The reason for the lack of recovery of GRPR binding in the PC310 tumour is not completely clear. It might be related to the shorter resupplementation period indicating that restoration of GRPR mRNA expression is a relatively slow process. (Figure 2).

RT-PCR study than showed that after ablation of androgens GRPR mRNA expression was significantly reduced in all androgen-dependent tumour types while restoration of relative GRPR mRNA levels was achieved after androgen re-supplementation. Variability in recovery of GRPR may be related to differences in the kinetics of the process of GRPR translation from mRNA to protein and reappearance. These results supported our hypothesis that in androgen-dependent xenografts representing early stage of PC, transcription of GRPR mRNA is androgen regulated.

Subsequent in vivo biodistribution studies were performed with the previously evaluated ^{111}In-MP2653 analogue [25]. Consistent with the autoradiographic results, ^{111}In-MP2653 tumour uptake was significantly reduced in all three androgen-dependent xenografts upon castration of tumour-bearing mice, which could be largely restored by resupplementation with testosterone. GRPR-expression of the androgen independent PC3 model was not down-regulated by androgen withdrawal. Likewise, uptake of peptide by the GRPR expressing pancreas was not affected indicating that GRPR expression in the pancreas was not androgen regulated. These observations were further substantiated by additional in vitro autoradiographies showing a similar trend in which castration induced reduction of GRPR binding and testosterone resupplementation induced restoration of GRPR expression in all 3 models of androgen-regulated human PC. Although results between biodistribution and autoradiography did show the same trend, the changes in GRPR expression were larger in autoradiography studies. This may be explained by the more complex physiology of the in vivo biodistribution process that involves interfering aspects such as in vivo stability of the peptide (affecting retention of the peptide/label), tumour vascularisation (influencing uptake), and clearance of the peptide from the blood [32-34].

We wanted to define to which bombesin receptor(s) variations in binding of ^{125}I-universal-BN were related and especially if they were exclusively GRPR-related. Therefore we examined if binding of ^{125}I-universal-BN to the androgen-dependent PC295, PC310

and PC82 tumour tissues could be blocked by 10^{-6} M of unlabelled GRP which was indeed the case (data not shown). Results are consistent with our previous findings [17]. Since ^{125}I-universal-BN binding showed to be exclusively GRPR related the natural GRPR ligand, ^{125}I-GRP, was employed for a subsequent autoradiography study.

GRP is however a larger peptide than bombesin and therefore it is more vulnerable to enzymatic degradation, making it not a very suitable peptide for in vivo experiments. For this reason the shorter and more stable bombesin analogue ^{111}In- MP2248 was also used. Similarities between the autoradiographies of both peptides confirmed that the bombesin analogue In-111-MP2248 indeed targets the GRPR like the natural GRP peptide. The relatively small variations in absolute values can be explained by the small differences in GRPR affinities of GRP and MP2248. The present study showed that peptide uptakes by the tumours are comparable. These data indicate that for future studies only one peptide, preferably a BN-analogue, may be chosen as the preferred ligand since this ligand can be employed for both in vitro and in vivo studies.

PC3 is a GRPR expressing androgen-independent model of human PC that is extensively used in literature. It has no androgen receptor and lacks the ability to produce PSA. Although GRPR is constitutively active in PC3 and the model is not suitable for studying androgen regulation of GRPR, its use in the field of bombesin-targeted imaging of PC obliged its use as reference model in this study. Our results confirm that GRPR expression in PC3 xenografts is not downregulated by castration and upregulated by resupplementation of androgens.

Xenograft tumour tissue consists of human epithelial tumour cells and supportive stromal cells originating from the mouse. Androgen-dependent PC xenografts will have regression of epithelial tumour fraction upon androgen ablation. Consequently tumours in such xenografts will contain less (human) epithelial cells as compared to the (non androgen responsive) stromal compartment. It was essential to verify whether the observed effects were caused by regulation of GRPR expression itself or by changes in the total epithelial (human) content of the xenograft. RT-PCR enables to correct for these changes while expression of GRPR mRNA was related to GAPDH mRNA expression levels using human-specific primer probe sets for both GRPR and GAPDH hereby excluding the non human stromal compartment.

Although none of our xenograft models representing late stage disease expressed GRPR [35], it cannot be excluded that GRPR may be re-expressed in progressive, late stage disease as has been suggested from patient samples [5] and few patients in clinical studies [31].

CONCLUSION

GRPR-binding of androgen-dependent human PC xenograft models is androgen-regulated. Androgen ablation results in down-regulation of GRPR expression. This expression can be (partially) restored by subsequent resupplementation with testosterone. Data further showed that the decline in GRPR-binding is the result of GRPR mRNA down-regulation rather than the consequence of the castration-induced reduction in the epithelial fraction of the xenograft. This suggests that hormonal therapy may affect GRPR expression in early stage, hormonally-treated patients with PC. Our observations do not exclude, however, that GRPR expression may reappear in progressive late stage patients.

Our data indicate that GRPR is a promising target for imaging of early PC in patients that have not been treated by hormonal therapy. GRPR-based imaging modalities may thus be used to improve staging of hormone naïve patients with PC. Its potential as a therapeutic modality for PC may be limited to non-hormonally treated patients, although reports suggest that GRPR may be reexpressed at later stages of PC. Studies are underway to further validate and compare GRPR expression in tumour tissue derived from both hormonally treated and untreated patients at different stages of PC.

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CHAPTER 5

GASTRIN-RELEASING PEPTIDE RECEPTOR EXPRESSION IN HUMAN TISSUE SAMPLES AND THE ANDROGEN-DEPENDENT HUMAN VCAP PROSTATE CANCER CELL LINE: REGULATION BY ANDROGENS?

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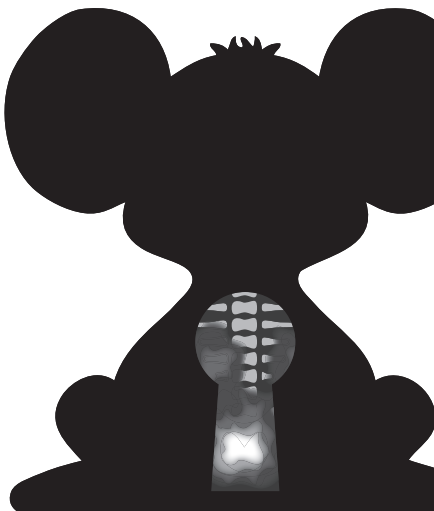


ABSTRACT

Prostate cancer (PC) is a major health problem in the Western world due to its high incidence and significant overall mortality. A crucial step in the management of PC is to discriminate between localised and metastasised disease. Current imaging techniques are not accurate enough to make this differentiation. Radiolabelled bombesin (BN) analogues with high affinity for the Gastrin-Releasing Peptide Receptor (GRPR), overexpressed in PC, form promising radiopharmaceuticals for targeted imaging and therapy of PC. Metastasised PC is initially treated by hormonal ablation therapy resulting in tumour regression, but eventually develops into castration resistant PC (CRPC). Previously, we have demonstrated androgen regulation of GRPR in androgen-responsive xenografts of human PC. In this study we further investigated the dynamics of GRPR expression and BN analogue binding under various hormonal conditions using the androgen-responsive VCaP cell line and evaluated GRPR expression in human PC samples of various disease stage.

GRPR mRNA expression levels as determined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and GRPR-specific uptake of the BN analogues ^{111}In-DOTA-AMBA and ^{99m}Tc-N_4-Demobesin-1 were performed with VCaP cells under various hormonal conditions in vitro and in vivo. In addition, GRPR mRNA expression (by RT-PCR) and binding of the BN analogue ^{111}In-DOTA-Pesin and ^{111}In-MP2346 (by autoradiography) to PC cell lines and xenografts and to clinical PC samples.

In contrast to our earlier findings in early androgen-responsive PC xenografts, GRPR mRNA expression in VCaP cells was not significantly affected by androgen manipulation both in vivo and in vitro. GRPR-specific uptake of the BN antagonist ^{99m}Tc-Demobesin-1 was similar between VCaP cells cultured in the presence of androgens (27.3±9.7%D/mg protein) or depleted from androgens for 6 days (30.3±7.0%D/mg protein). Human PC samples were predominantly GRPR positive, although mRNA expression levels were highly variable. In contrast to PC samples from patients not previously treated by hormonal therapy, only samples of (hormonally treated) CRPC showed consistent reduced GRPR mRNA expression, suggesting therapy-induced down-regulation of GRPR. Median GRPR-specific uptake of the BN-analogue ^{111}In-DOTA-Pesin was higher in PC samples as compared to benign prostate tissue. The overlap of GRPR-specific binding between PC and benign prostate tissue negatively affects BN-specificity.

In conclusion, the present study could not proof androgen regulation of GRPR in androgen-responsive VCaP cells. We hypothesize that GRPR expression

is androgen-regulated in early PC, and that the overexpression of GRPR observed in more advanced PC3 and VCaP is caused by an escape from 'normal' androgen control resulting in constitutively activated GRPR in these cell lines. While the majority of (hormonally untreated) PC was GRPR positive, the relatively low GRPR mRNA expression levels observed in CRPC suggest that hormonal therapy generally results in down-regulation of GRPR expression. The above described escape phenomenon may thus be a rather uncommon clinical event. Overall, these data suggest that BN-based GRPR targeted imaging and therapy of PC seems feasible in the early diagnosis of PC in men that have not previously been treated by hormonal therapy. Furthermore, outcomes reveal that pre-selection of GRPR expressing patients is required when opting for GRPR-targeted BN-based response monitoring and therapy of PC.

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among men in the Western world and the third cause of cancer related death [1, 2]. Aging of the general population, increased public awareness as well as the strong rise in the use of prostate specific antigen (PSA) to screen for PC have all contributed to the dramatic increase in its early diagnosis [3].

PC presents as a heterogeneous and multi-stage disease [4]. As long as the tumour remains confined to the prostate capsule, treatment may be curative either by surgical or radiotherapeutic options. However, when the tumour has invaded surrounding tissue or when tumour cells have escaped the primary tumour, only systemic palliative treatment strategies are available. Like the normal prostate, early PC generally depends on androgens for growth and survival [5]. Initially, advanced (disseminated) PC is treated with androgen ablation therapy leading to PC regression [6, 7]. However, eventually all patients will develop castration resistant PC (CRPC), rendering androgen ablation therapy ineffective [8, 9]. As opposed to androgen-responsive PC, CRPC does not require androgens to proliferate. When facing metastatic CRPC, treatment options are scarce leaving a median survival of 10 months [10-12]. Clearly, discrimination between organ-confined disease and PC beyond the borders of the gland is a crucial step in the optimal management of PC. Unfortunately, current imaging techniques are not sufficiently accurate to determine extent of PC at diagnosis and to visualise micrometastases [3-5]. More sensitive imaging techniques are therefore strongly needed.

Nuclear imaging using single photon emission computed tomography (SPECT) and positron emission tomography (PET), may offer applications

to improve accuracy of PC imaging [13]. The Gastrin-Releasing Peptide Receptor (GRPR) constitutes an interesting target for peptide-receptor radionuclide imaging and therapy as this receptor is reported to be overexpressed on the cell membrane of PC while it is not detectable in normal prostate tissue [14, 15]. Bombesin (BN) and its mammalian counterpart, the Gastrin-Releasing Peptide (GRP), are natural ligands for the GRPR to which they bind with high affinity [14]. BN analogues are applicable for SPECT when labelled with gamma emitting radionuclides while labelling of the BN analogue with positron emitting radionuclides enables utilisation for PET imaging. Moreover, labelling of BN analogues with cytotoxic radionuclides, emitting beta or alpha rays, may provide a therapeutic agent for PC.

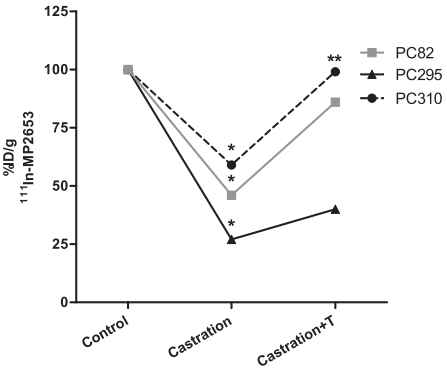


Figure 1

*GRPR-mediated average tumour uptake of ^{111}In-MP2653 in androgen-responsive PC82, PC295 and PC310 human prostate cancer xenografts. Tumours were obtained from male mice supplemented with testosterone (T) (control), tumour-bearing mice castrated for 7 days (Castration) and from 7 days castrated mice resupplemented with T for 14 days (Castration+T) in case of PC82 and PC310, and for 4 days in case of PC295. Results are presented as percentage of the injected dose per gram of tissue (%ID/g) per xenograft sample. Per treatment group 4 to m14 animals were used. * significantly different ($p<0.05$) from control, ** significantly different from Castration ($p<0.05$).*

Markwalder et al. reported a generally high density of GRPR in primary PC and high-grade PIN against a generally low density of GRPR in benign hyperplasia of prostate (BPH) and non-neoplastic prostatic stroma [15]. Previously, we have shown high GRPR expression predominantly in androgen-responsive, early PC xenografts with low expression in xenografts representing late stage disease [16]. Moreover, we described reduced GRPR-mediated tumour uptake of BN after androgen ablation and restoration of uptake after resupplementation of testosterone in androgen-responsive xenografts. These studies suggested that expression of GRPR is androgen regulated [16, 17]. These observations (see Figure 1) and the dominant role of androgen ablation therapy in the management of PC called for a more detailed characterisation of the androgen regulation of the GRPR in order to define the potential role of BN-based imaging and therapy of PC.

Androgens and the androgen receptor (AR) are strongly involved in PC growth and play a key role in the transformation from androgen-responsive PC towards progressive CRPC [8, 18, 19]. To better define the role of androgens and the AR in GRPR regulation, we studied the dynamics of GRPR expres-

sion and BN analogue binding under various hormonal conditions in an experimental human PC model system. To allow direct comparison of both in vitro and in vivo data, we selected a human GRPR expressing, androgen-responsive VCaP cell line that can be applied in vitro and has tumourigenic capacity to grow as a xenograft. The PC-3 cell line is the most often selected PC cell line in BN-related studies because of its high GRPR expression and easy use [13]. PC-3 cells, however, do not express AR and lack PSA, important characteristics of clinical CRPC, and therefore this cell line may not accurately represent late stage disease. We selected the androgen-responsive and GRPR expressing VCaP cell line based on its high GRPR expression and general PC characteristics such as expression of wild type, non-mutated AR, PSA and prostatic acid phosphatase (PAP) [16]. The cell line belongs to the acinar adenocarcinomas accounting for more than 95% of the prostate tumours [20]. VCaP tumours in athymic nude mice show androgen-responsive growth [21, 22]. Clearly, these characteristics make VCaP a very relevant cell line to study the mechanisms of androgen regulated GRPR expression.

In this study we investigated the dynamics of GRPR gene expression and of BN analogue binding under various hormonal conditions in vivo and in vitro using VCaP. PC-3 was used as reference cell line. To further define the potential role for GRPR-targeted BN-based imaging and therapy of PC, tissue samples from PC patients from various clinical stages were assessed for GRPR expression and BN binding. We show that, in contrast to our earlier observations in 3 different androgen-responsive PC xenograft models [17], GRPR expression in the VCaP cell line was not androgen regulated and that BN analogue binding was not different under different hormonal conditions. These results may suggest that constitutively active GRPR, like in VCaP and PC-3 cells, is provoked by an escape of 'normal' androgen regulation mechanisms that is present in early, androgen-responsive, non-hormonally treated PC. Patient samples from early PC were predominantly GRPR positive, although with variable expression. GRPR expression in samples from CRPC patients was absent or low. Despite low numbers, this observation suggest that GRPR expression, and consequently BN binding, is (at least initially) androgen regulated. GRPR expression may become abrogated during tumour progression in a subset of patients resulting in constitutively high expression levels.

MATERIALS AND METHODS

Cell culture

VCaP and PC-3 cells were cultured in RPMI (LONZA, Verviers, Belgium) supplemented with 10% or 5% fetal calf serum (FCS) (GIBCO Invitrogen Co, Grand Island, NY, USA), respectively. 5mL/500mL penicillin/streptomycin antibiotic solution (LONZA, Verviers, Belgium; 10.000Units/mL penicillin, 10.000Units/mL streptomycin) was added to medium of both cell lines. Cells were grown in Cellstar

tissue culture flasks (Greiner Bio-one GmBH, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5%CO2 at 37°C.

Cells were grown to near confluency, harvested using Trypsin/EDTA (LONZA, Verviers, Belgium), 170000U/L Trypsine-Versene and 200mg/L EDTA and counted for further experimental use.

Hormonal manipulation of VCaP

For RT-PCR analysis of GRPR and AR mRNA levels, VCaP cells were cultured in T25 flasks in RPMI with 10% FCS (containing an undefined amount of androgen) or 10% of DCC (steroid-deprived serum) with or without supplementation of 10^{-10}M of the synthetic androgen R1881 (T). Control VCaP cells were cultured with DCC and R1881 for 72h (DCC72+T). Separate cultures of VCaP cells were incubated with DCC and R1881 for 24h and were then depleted of R1881for 8, 16 or 24h respectively (DCC24+T>8,16,24-T). Finally, VCaP cells were cultured with DCC and R1881 for 24h, depleted of R1881 for 24h and subsequently resubstituted with R1881 for 8, 16 or 24h (DCC24+T>24-T>8,16,24+T). Reference groups received RPMI 10%FCS (FCS72) or DCC without addition of R1881 for 72h (DCC72-T). All culture conditions were performed in duplicate.

To study uptake of radiolabelled BN analogues, VCaP and PC-3 cells were cultured in RPMI with FCS and seeded into 6-well plates at a density of $1x10^6$ cells per well and cultured in the presence or absence of R1881 for several days. Control VCaP cells were cultured with 10% of DCC in the presence of R1881 for 3 days (DCC3d+T). In addition, VCaP cells were cultured with 10% of DCC without R1881 for 3 and 6 days (DCC6d-T and DCC6d-T). The reference group using PC-3 cells was seeded at a density of $2x10^6$ cells per well with RPMI 5% FCS for 3 days (FCS3d).

PC xenografts

VCaP and PC-3 bearing mice were established as described earlier [23]. NMRI nu/nu mice were inoculated subcutaneously with PC-3 or VCaP cells (approximately $5x10^6$/100µl PBS) on the right shoulder of athymic male NMRI nu/nu mice (Taconic, Ry, Danmark). The experiment was approved by the Dutch Animal Experimentation Committee (DEC Consult) and performed in agreement with the Dutch Animal Experimentation Act (1977) and the European Convention for Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18 March 1986).

Hormonal manipulation of tumour-bearing mice

Hormonal manipulation of VCaP xenografts was performed as described earlier [17]. Tumour-bearing mice were surgically castrated at 2, 4 or 7 days (7d-T) prior to biodistribution studies. Other mice were castrated and after 7 days resupplemented with testosterone by subcutaneous implantation of testosterone-containing silastic tubings for 2, 4 or 14 days (7d-T/2,4,14d+T).

Radiolabeling and Quality Control

Analogues and Radiolabelling

BN-agonists DOTA-AMBA and DOTA-Pesin were kindly provided by Prof. Dr. Mäcke, University Hospital Basel, Switzerland [24, 25]. MP2346 is an agonistic BN-peptide supplied by BioSynthema (St Louis, MO, USA) [26]. Radiolabelling of MP2346, Pesin and AMBA with Indium-111 was performed using 111InCl3 (Covidien, Petten, the Netherlands), as described earlier [26-29].

Demobesin-1 an antagonistic BN-analogue was a gift from Dr. T. Maina and Dr. B. Nock, Demokritos, Athens, Greece. This peptide was radiolabelled with Technetium-99m (^{99m}Tc) as previously described by Nock et al. [30].

In order to prevent oxidation and radiolysis quenchers were added to all radiolabelled analogues. Quenchers used were ascorbic acid (Bufa BV, Uitgeest, the Netherlands), gentisic acid (Covidien, Petten, the Netherlands) and methionine (Fluka Biochemika, Buchs, Switzerland) [29].

Quality Control and HPLC purification

Incorporation of radiolabelling was assessed by instant thin layer chromatography (ITLC) and radiochemical purity (RCP) of the labelled analogues was performed by *high performance liquid chromatography (HPLC)*. ITLC and HPLC analysis of ^{99m}Tc-Demobesin-1 and ^{111}In labelled peptides was performed as described earlier [31].

For HPLC-purified ^{111}In-AMBA solution the HPLC derivative of ^{111}In-AMBA was quantified and diluted for injection (0.1mL saline and 0.1MBq per animal). The peptide amount of ^{111}In-AMBA from the main peak was considered negligible, since 37MBq ^{111}In ≈ 2.15x10-11gram atoms. For this reason, to obtain an HPLC-purified ^{111}In-AMBA solution containing a total AMBA amount of 10pmol per injective, 10pmol of non-labelled AMBA was added.

Biodistribution studies

Biodistribution studies were performed as described earlier [31]. VCaP or PC3 tumour bearing mice were injected with HPLC-purified ^{111}In-AMBA (0.1MBq in 10 pmol) into the lateral tail vein. Animals were sacrificed by cervical dislocation at 1h post injection (pi). Each study group consisted of 4 mice. Selected tissues were excised, weighed, and radioactivity was determined in a 1480 Wizard 3'' γ-counter (Perkin Elmer, Groningen, the Netherlands). Radioactivity data were shown as percentage of the injected dose per gram of tissue (%ID/g).

In vitro BN uptake studies

GRPR-mediated uptake of VCaP was compared with PC-3 under standard culture conditions (RPMI with 5 or 10% FCS) using the BN analogue ^{111}In-MP2346, diluted from 50MBq/nmol stock. BN analogue uptake by VCaP cells that were cultured under different hormonal conditions (see subheading cell culture) was studied using the BN analogue ^{99}mTc-Demobesin-1.

BN analogue uptake by the cells (total peptide uptake into the membrane fraction plus cellular internalisation of the peptide) was studied essentially as described earlier[32]. Cell cultures were seeded into 6-well plates and all conditions were performed in triplicate. Wells were washed twice with 2mL warm (37°C) PBS (pH7.4). One ml of internalisation media (sterilised RPMI1640 plus GlutaMAX, 20mM HEPES, 1%BSA fraction V purity≥96% pH7.4) containing 10^{-10}M labelled BN analogue was added. To determine specific versus non-specific uptake control wells were incubated with an excess of blocking agent, 10^{-6}M Tyr^4-BN (Sigma Chemical Co., St. Louis, MO) in addition to the 10^{-10}M of labelled BN analogue. Each plate (one per time point) was incubated on a rotating wheel at 37°C for 1h. VCaP cells were cultured in the presence or absence of R1881 for 1h. To verify whether incubation without R1881 had effect on BN analogue uptake, an additional 6-well plate was incubated with 10^{-10}M R1881. After 1h, cellular uptake of BN was stopped by removal of the incubation medium. Cells were rinsed twice with 2ml ice-cold PBS (4°C). 1ml of 0.1M sodium hydroxide was added for lysis of cells to determine radioactivity in a gamma counter; 1480 Wizard 3'' (Perkin Elmer, Groningen, the Netherlands). Protein content was determined by Protein Assay Kit (Bio-Rad, Veenendaal, the Netherlands). Receptor-specific uptake in the membrane fraction and internalisation of BN analogue in the cells was combined to obtain (total) peptide uptake. BN analogue uptake is expressed as percentage of dose per mg of cellular protein (%D/mg protein). Results are indicated as mean±standard deviation (SD) of 2-3 wells with experiments being performed in triplicate.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to evaluate GRPR gene expression in PC cell lines and xenografts and in a set of human patient samples. Patient samples were obtained from the Erasmus MC biorepository and consisted of 12 specimens of radical prostatectomy, 13 lymph node metastases, 19 samples of transurethral resection of prostate, 8 CRPC samples and 11 benign prostate hyperplasia tissues. Procedures for RNA isolation and RT-PCR were performed as described earlier[17]. Total RNA was isolated using RNAzol B kit according to the manufacturer's protocol and stored at -20°C.

Gene expression of GRPR, AR, PSA and the reference housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was performed with human-specific FAM dye-labelled TaqMan probes. Gene expression of the second reference gene porphobilinogen deaminase (PBGD) was determined in Power SYBR Green PCR Master Mix (25µl), containing 0.33µmol/L forward and reverse primer in an ABI Prism 7700 sequence Detection System (Applied Biosystems, Foster City, USA) by the quantification of fluorescence signals of site-specific hybridization probes. Relative mRNA expression

of GRPR is indicated for each sample relative to the average of GAPDH plus PGBD expression.

In vitro autoradiography

From the Erasmus MC bio-repository of human tissue samples, 27 independent patient specimens were collected: 10 radical prostatectomy specimens, 7 samples of transurethral resection of prostate, 3 samples of benign prostate hyperplasia (BPH) and 7 samples of stromal tissue of 'normal' prostate. Frozen tumours were cut into sections of 10 µm using the HM 560 Cryo-Star (Microm, Walldorf, Germany) and mounted on Superfrost plus slides (Menzelgläser, Braunschweig, Germany). In vitro autoradiography was performed as described earlier[16, 17]. Frozen xenograft sections (10 µm) were incubated with ^{111}In-Pesin, exposed to Super Resolution phosphor imaging screens and read using a Cyclone (Perkin Elmer, etc.). Radioactivity was quantified using Optiquant software. Results are depicted in digital light units per millimeter square (DLU/mm^2).

Statistics

Statistical analyses were performed using SPSS 11.01 (SPSS Inc., IL, USA). For this study the unpaired t-test was applied. Statistical significance was assumed if p-values were <0.05.

RESULTS

RT-PCR of PC cell lines and xenografts

Relative mRNA levels of GRPR were detectable (higher than 0.05) in the majority of androgen-responsive xenografts (PC82, PC295, PC310, PC374) and cell lines (VCaP, DuCaP, LNCaP, PC346mAR) (Figure 2A). This was not the case for androgen-unresponsive PC cell lines and xenografts in which mRNA levels of GRPR were only detectable in PC-3 and PC374F. GRPR mRNA expression was 8.4 times higher in VCaP cells than in PC-3 cells. DuCaP cells showed the highest mRNA GRPR expression. In line with this observation, GRPR-mediated uptake of the BN agonist ^{111}In-MP2346, was significantly higher in VCaP compared to PC-3 cells ($p<0.01$). ^{111}In-MP2346 uptake was 91.1±2.3%D/mg protein for VCaP and 63.0±2.7%D/mg protein for PC-3 (Figure 2B). In vivo studies showed that both PC-3 and VCaP xenografts showed substantial uptake of the BN agonist ^{111}In-AMBA; 4.9±1.7%ID/g and 4.6±1.3%ID/g, respectively, which was not significantly different (Figure 2C). As was expected, BN analogue uptake in normal pancreas was not different between mice bearing different tumour types. Co-injection of an excess of unlabelled [Tyr4]BN resulted in >90% blockade, confirming BN-specificity of ^{111}In-AMBA tumour and pancreatic uptake (data not shown).

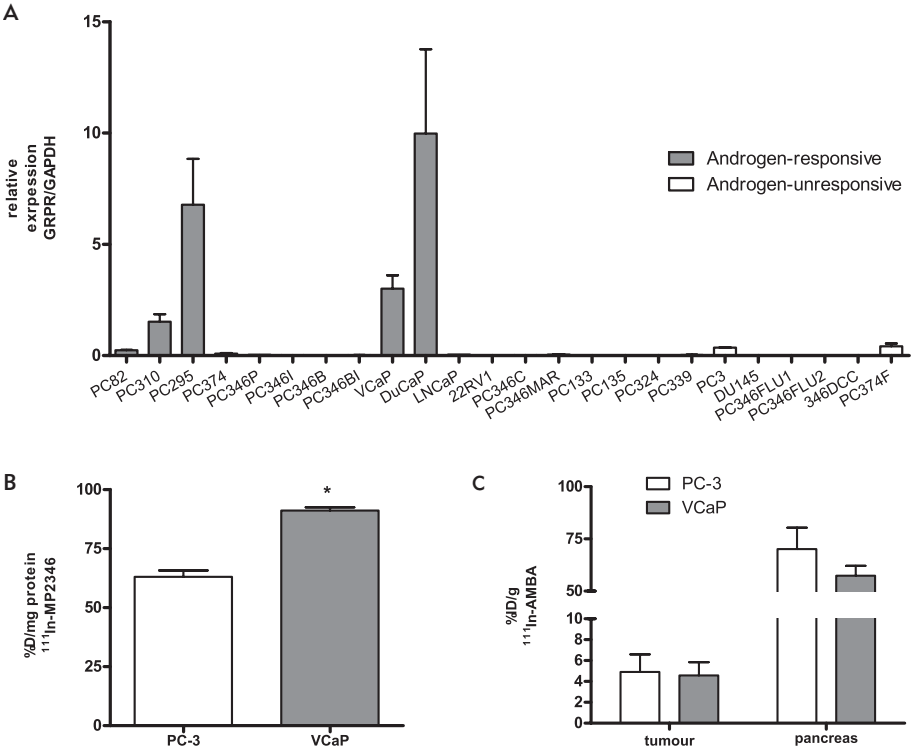


Figure 2A

Quantitative RT-PCR analysis of GRPR in PC cell lines and xenografts. Results are presented as relative mRNA expression to the housekeeping gene GAPDH and are shown as mean±SD of two independent samples.

Figure 2B

In vitro uptake of 10^{-10}M BN-analogue ^{111}In-MP2346 in PC-3 and VCaP cells after incubation for 1h. Results are shown as percentage of dose per mg protein (%D/mg protein) and represent mean±SD of 3 independent cultures.

** significantly different between VCaP and PC-3 ($p<0.01$)*

Figure 2C

Receptor-mediated binding of the BN analogue ^{111}In-AMBA in VCaP and PC-3 bearing mice. An injection-volume of 0.1 mL containing 0.1 MBq and 10 pmol was administered into a lateral tail vein. Mice were sacrificed at 1h pi. Data are presented as percentage of the injected dose per gram of tissue (%ID/g) and are shown as mean±SD of 4 mice per analogue per time point.

Hormonal manipulation of VCaP cells and xenografts

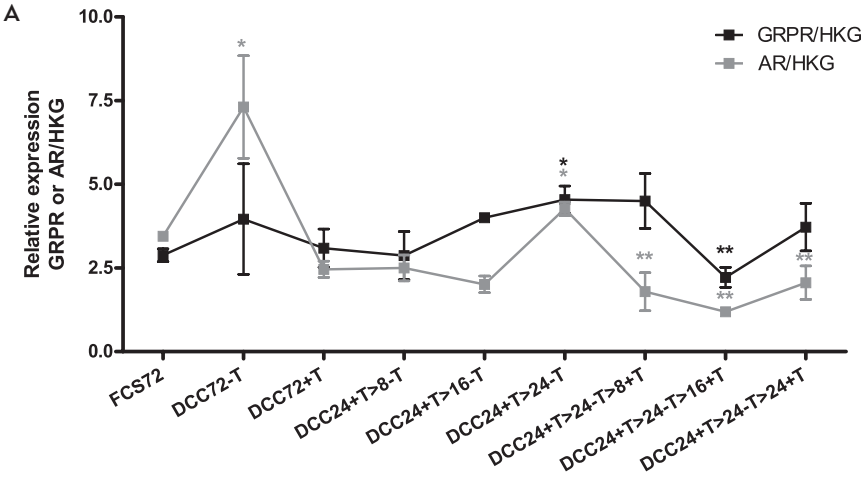
Relative mRNA expression of GRPR and AR as determined by RT-PCR in VCaP cultures under various hormonal conditions are shown in Figure 3A. Withdrawal of androgen from the culture medium for 72h led to a significant increase ($p<0.01$) of relative AR mRNA levels over control (DCC72+T or FCS72), but not of GRPR mRNA levels. When cells were cultured for

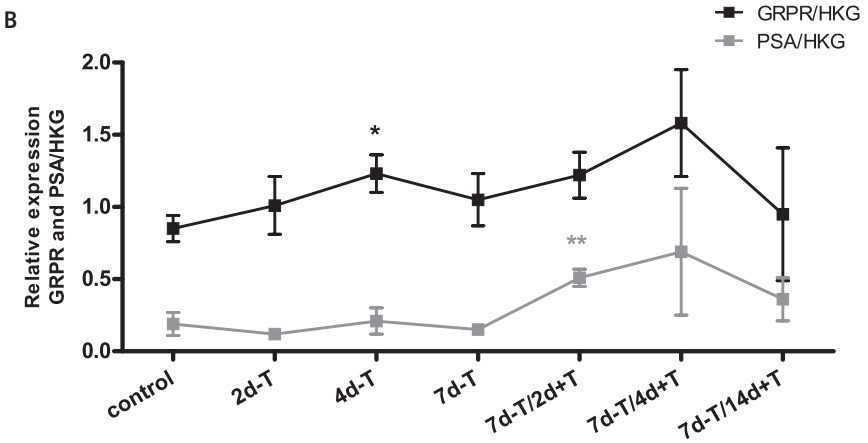
24h in DCC + R1881 (DCC24+T) and subsequently incubated in media without R1881 for various time periods, relative GRPR and AR mRNA were not affected until 24h of depletion (DCC24+T>24-T) at which time point relative mRNA expression of both genes was significantly increased as compared to control. Subsequent resupplementation with R1881 (DCC24+T>24-T>X+T) resulted in a rapid and significant decrease of expression of AR mRNA, but not of GRPR, at 8h, which was restored to control levels at 24h of resupplementation (DCC24+T>24-T>24+T). RT-PCR of GRPR in hormonally manipulated VCaP bearing mice revealed a similar trend (Figure 3B). Castration of VCaP bearing mice for 4 days (4d-T) induced a small, but significant ($P<0.03$) increase in relative mRNA levels of GRPR as compared to the levels detected in intact control mice, which was not observed at 7 days post-castration (7d-T). In contrast to the in vitro data, subsequent re-supplementation of animals for up to 14 days (7d-T/14d+T) did not result in substantial downregulation of GRPR and PSA mRNA to control values.

Also, GRPR-mediated uptake of the BN analogue ^{99m}Tc-Demobesin-1 in VCaP cultures under various hormonal conditions was not affected by androgens (Figure 3C). VCaP cells grown in the absence of R1881 for 3 or 6 days (DCC3d-T or DCC6d-T) showed no significant difference in BN uptake as compared to control (androgen containing) cultures (FCS3d or DCC3d+T). Mean uptake of ^{99m}Tc-Demobesin-1 at 6 days of R1881 depletion was 30.3±7.0%D/mg protein and 27.3±9.7%D/mg protein in control cells. ^{99m}Tc-Demobesin-1 uptake in VCaP cells was comparable to that of the reference androgen independent PC-3 cell line (30.7±8.3%D/mg protein). Furthermore, uptake of ^{99m}Tc-Demobesin-1 in VCaP cells grown without R1881 for 1h was not different from that of cells to which 10^{-10}M R1881 was added (data not shown).

GRPR expression in human PC samples

Relative mRNA levels of GRPR determined in a set of human PC samples are summarised in Figure 4A. Radical prostatectomy specimens, lymph node metastases, transurethral resection of prostate (TURP), benign prostate hyperplasia (BPH) material all showed a comparable mean of relative mRNA expression of the GRPR. Interestingly, a lower median, although not statistically significant, was observed only in the CRPC group as compared to the other tumour groups. No correlation of GRPR mRNA levels could be found when stratifying for percentage of glandular tissue per sample or Gleason score (data not shown). 45 out of 52 PC samples were GRPR positive (relative mRNA GRPR levels higher than 0.05). From the 7 GRPR negative PC samples, 3 were obtained from patients that were treated with hormone deprivation therapy. From all PC samples (including CRPC) known to be treated by androgen deprivation therapy 19 out of 21 tumour samples were GRPR positive (relative mRNA GRPR higher than 0.05).


A
Relative expression
GRPR or AR/HKG
10.0
7.5
5.0
2.5
0.0
GRPR/HKG
AR/HKG
FCS72
DCC72-T
DCC72+T
DCC24+T>8-T
DCC24+T>16-T
DCC24+T>24-T
DCC24+T>24-T>8+T
DCC24+T>24-T>16+T
DCC24+T>24-T>24+T


B
Relative expression
GRPR and PSA/HKG
2.0
1.5
1.0
0.5
0.0
GRPR/HKG
PSA/HKG
control
2d-T
4d-T
7d-T
7d-T/2d+T
7d-T/4d+T
7d-T/14d+T

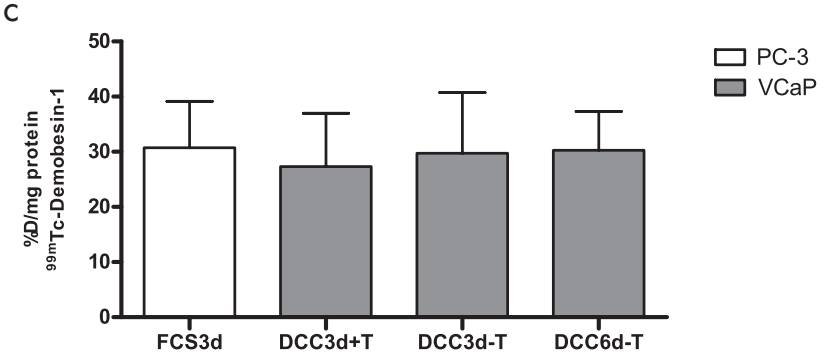

C
%D/mg protein
99mTc-Demobesin-1
50
40
30
20
10
0
PC-3
VCaP
FCS3d
DCC3d+T
DCC3d-T
DCC6d-T

Figure 3A

Quantitative RT-PCR analysis of GRPR and AR in VCaP cell lines cultured under various hormonal incubations. In vitro androgen manipulation was performed by culturing in DCC containing medium (steroid-deprived serum) in the presence or absence of the synthetic androgen R1881 (referred to as +T). FCS72= incubation for 72h in full 10% serum-containing medium serving as control. GRPR and AR mRNA expression is indicated relative to housekeeping gene (HKG = the average of the housekeeping genes GAPDH and PBGD). RT-PCR analysis was performed in duplicate. Data are expressed as mean±SD of two independent cultures per condition.

** significantly different to control (DCC72+T) ($p<0.01$), ** significantly different to DCC24+T>24-T ($P<0.01$)*

Figure 3B

Quantitative RT-PCR analysis of GRPR and PSA in VCaP xenografts that were grown under various hormonal conditions. In vivo hormonal manipulation was performed by surgical castration of tumour-bearing mice and subsequent androgen (re-)substitution with testosterone containing pellets (indicated as +T). Tumours were sampled at various days of treatment (d). GRPR and PSA mRNA expression is indicated relative to housekeeping gene (HKG = the average of the housekeeping genes GAPDH and PBGD). RT-PCR analysis was performed in duplicate. Data are expressed as mean±SD of 3 mice per condition.

** significantly different to control ($P<0.03$); ** significantly different to mice castrated for 7 ($P<0.00$).*

Figure 3C

In vitro uptake (membrane plus internalisation) of 10^{-10}M BN-analogue ^{99m}Tc-Demobesin-1 in PC-3 and VCaP cells. VCaP was incubated in DCC containing medium (steroid-deprived serum) in the presence or absence of the synthetic androgen R1881 (referred to as T) for 3 or 6 days. PC-3 incubated in FCS for 3 days served as reference. Results are shown as percentage of dose per mg protein (%D/mg protein). VCaP cells were cultured under variable testosterone conditions. PC-3 cells were used as reference. Results are indicated as mean±SD of 2 independent cell cultures per condition.

In vitro autoradiography performed on a small set of frozen samples from a different patient group, showed that GRPR-specific uptake of ^{111}In-Pesin was higher in cancer (radical prostatectomy and TURP) than in the benign tissue groups (BPH and stroma), although again with substantial overlap (Figure 4B). BN uptake in radical prostatectomy samples and stromal tissues were significantly different ($p<0.05$). Also now, no relation was found between BN-uptake and Gleason score in tissue from radical prostatectomies (data not shown).

DISCUSSION

In this study we aimed at elucidating the putative regulation of GRPR by androgens and its relationship to the GRPR expression in PC tumours of patients in different stages of PC progression. We analysed GRPR expression in clinical samples from our biorepository of human PC tissue samples and attempted to relate clinical PC characteristics with GRPR expression levels. For studying androgen regulation of GRPR we selected the VCaP cell line as model system. We did not select the highly GRPR-expressing DuCap cell line while GRPR expression in this cell line may be affected by stromal contamination (of murine origin), which can be as high as 50% [4]. To focus on androgen regulation rather than the characteristics of a specific BN analogue, we deliberately varied in BN analogues between independent studies. Amongst a large panel of cell lines and xenografts VCaP (and DuCaP)

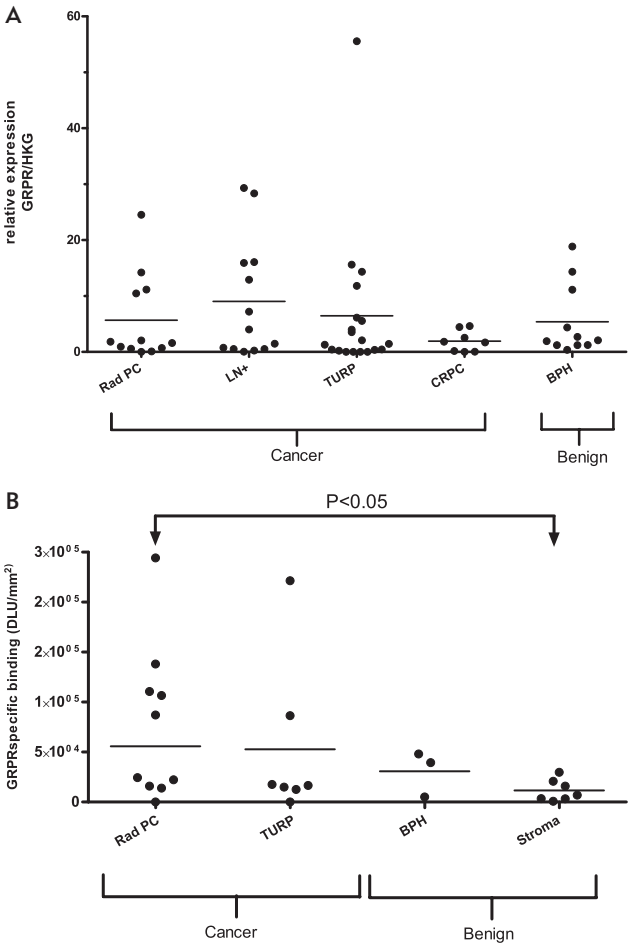


Figure 4A

Quantitative RT-PCR analysis of GRPR of human PC samples at different stages of disease from the Erasmus MC tissue bank. The sample set consisted of 12 specimens of radical prostatectomy (Rad PC), 13 lymph node-metastases (LN+), 19 transurethral resection of prostate (TURP), 8 castration-resistant prostate cancer = TURP samples shown to be hormone refractory (CRPC) and 11 benign prostate hyperplasia (BPH). RT-PCR analysis was performed in duplicate. GRPR mRNA expression is indicated relative to housekeeping gene (HKG = the average of the housekeeping genes GAPDH and PBGD). Median expression per tissue group is indicated.

Figure 4B

GRPR-specific binding of the BN-analogue ^{111}In-Pesin to frozen sections of clinical prostate cancer tissue from PC patients at different stages of disease. Tissues were obtained from the Erasmus MC biorepository of human tissue samples. Autoradiography was performed on frozen sections of 10 radical prostatectomy specimens (Rad PC), 7 transurethral resection of prostate (TURP), 3 benign prostate hyperplasia (BPH) and 7 samples of stroma from prostate origin. GRPR specific binding is depicted in digital light units per millimeter square (DLU/mm^2). Median expression per tissue group is indicated. GRPR-specific binding was significantly higher in the radical prostatectomy group than in the stroma group ($p<0.05$).

showed strong mRNA expression of GRPR. Also, GRPR-specific uptake of ^{99m}Tc-Demobesin-1 was high in VCaP and both mRNA GRPR levels and BN-analogue uptake were similar from those of PC-3, a cell line often used for GRPR-related research. This finding was in line with our earlier PET study in which we used the BN-tracer AMBA labelled with Galium-68 [23].

In previous studies we have shown GRPR expression of early stage androgen-responsive xenograft models to be strongly dependent on androgen levels [16, 17]. GRPR-specific binding of BN analogues was reduced after castration of tumour-bearing mice while resubstitution with androgen restored BN-uptake. These data suggested that GRPR expression is androgen regulated. Our present study does not confirm this as both in vitro and in vivo androgen manipulation did not result in significant changes in GRPR mRNA expression and BN analogue uptake in the andro-

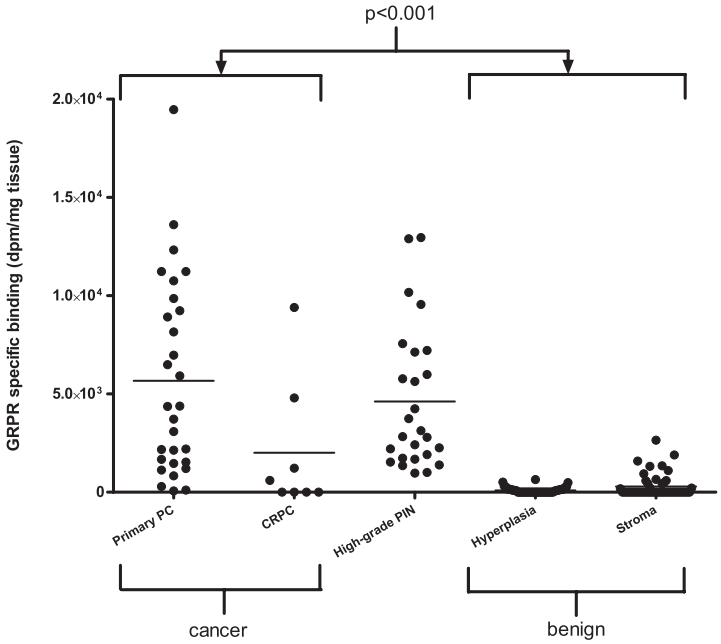


Figure 5

GRPR-specific binding of the BN-analogue ^{125}I-Tyr^4-bombesin in human non-neoplastic prostate and prostate cancer tissue sections as obtained by autoradiography. Groups were subordinated in 5 independent prostate tissue groups consisting of the following samples: 29 primary PC, 8 CRPR, 26 High-grade prostatic intraepithelial neoplasia (PIN), 50 hyperplasia (known as BPH), and 50 stroma from prostate origin. Results are depicted in disintegrations per minute per mg tissue (dpm/mg tissue). The median of each tissue group is indicated. ^{125}I-Tyr4-bombesin binding was significantly higher in combined cancer groups as compared to benign tissues (hyperplasia and stroma) (P<0.001).

This scatter plot was modified from original data from; 'Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation' by R. Markwalder and J.C. Reubi in Cancer Research 1999 (PMID: 10070977).

gen-responsive VCaP cells. In vitro and in vivo androgen withdrawal resulted in slightly higher GRPR mRNA levels, only statistically significant at one time point. GRPR expression was reduced to its control levels upon androgen re-supplementation. The AR expression on the other hand did show androgen regulation. The androgen-responsive behaviour of VCaP cells was endorsed by reduced cell proliferation after androgen depletion (data not shown). It appears that androgen depletion of VCaP drives compensatory mechanisms that stimulate GRPR mRNA transcription. However, at the protein level, GRPR-specific binding of ^{99m}Tc-Demobesin-1 was not affected after 3 and 6 days of androgen ablation. Overall, our data do not support a strong androgen regulation of GRPR expression. It may be hypothesised that VCaP and PC3 cell lines, representing a more advanced stage of PC, have a constitutively activated GRPR expression that has escaped 'normal' androgen regulation control mechanisms, while, in the case of VCaP, growth control is still (partially) retained by androgens. It remains to be determined if this is a physiological relevant phenomenon that may occur in a subset of tumours as represented by these two cell line models.

GRPR protein expression in late stage metastatic PC has been reported in the literature with varying results. Markwalder et al.[15] described either absent (43%) or low (57%) GRPR binding in 7 human samples of androgen-unresponsive PC bone metastasis as determined by autoradiography. In an autoradiography study by Thomas et al. [33] AMBA labelled with ^{177}-Lutetium showed specific GRPR-binding in 14 out of 17 PC patients with no difference in uptake between 3 primary androgen-unresponsive PC tissues and their secondary lymph nodes metastates. In an immunohistochemical study by Ananias et al. [34] GRPR density was studied in 15 early stage PC lymph node samples derived from radical surgical treatment (of a total of 21 samples since some samples were ipsilateral lymph nodes from the same patient) and 17 samples of human PC bone metastasis obtained either by biopsy or by resection as part of orthopaedic surgery. Bone metastases were GRPR positive in 53% of cases while PC lymph nodes were GRPR positive in 86%. Interestingly, only 2 of 7 CRPC samples showed positive GRPR staining as opposed to 5 of 8 positive samples in the group that received no hormone therapy. The drawback of this study was, however, that the GRPR antibody used resulted in poor contrast of staining between tumour and background. As a consequence, the GRPR expression scores were very weak and not very convincing. From 27 positive samples weak staining was reported in 24 samples and moderate staining in only 3 samples. None of the positive samples showed strong staining. We also attempted to use immunohistochemistry for evaluation of GRPR expression in clinical PC samples. Several antibodies that claimed GRPR specificity as well as different immunohistochemical protocols were tested, but we were unable to reach acceptable contrast between tumour and background required for reliable scoring. Acceptable staining contrast was only

observed in pancreatic Islets of Langerhans that were used as positive control. In our hands the antibody and protocol used by Ananias et al. [34] was neither successful.

As an alternative for the evaluation of GRPR in human samples of PC, we used RT-PCR to determine GRPR mRNA expression. Also, we performed autoradiography for GRPR density in a subset of patient samples. Tumour samples of early PC were predominantly GRPR positive, although with considerable variability in mRNA levels. PC and non-cancerous tissues showed considerable overlap in expression levels resulting in comparable mean expression levels of relative mRNA GRPR. A lower average uptake was only observed in the CRPC group, although difference was not statistically significant. Autoradiography on frozen sections of human prostate tissue revealed higher average GRPR-specific uptake of ^{111}In-Pesin in cancer (radical prostatectomy and TURP) than in the benign tissue groups (BPH and stroma), but also with considerable overlap. Only between radical prostatectomy and stromal tissues BN peptide uptake was significantly different ($p<0.05$), confirming PC specificity. The data reveal that GRPR expression is present in nearly all PC tissues. Although the sample numbers are too low for a definite conclusion, the data also indicate that expression of GRPR, and GRP-specific binding, is variable and overlaps with non-cancerous tissue.

In order to be able to compare our present findings with those obtained by Markwalder et al.[15] we composed a scatter plot of their data (Figure 5) similar to the one composed for our data (Figure 4B). We interpreted primary PC similar to our specimens of radical PC, bone metastases of androgen-unresponsive prostatic cancers similar to our CRPC group and hyperplasia similar to our BPH samples. In addition, Markwalder et al. also included high-grade PIN samples. The comparison of studies reveals that differences in GRPR expression between primary PC and benign tissue is more pronounced in the study of Markwalder et al., which may be caused by the larger sample set analysed in their study. Also in this study GRPR specific binding showed substantial overlap between PC and benign tissue. The observed overlap in relative GRPR mRNA levels as well as in GRPR-specific binding in benign and malignant prostate tissue negatively affects specificity of BN-based GRPR targeting for diagnosis of PC, leading to false-positive cases. This finding may limit the use of BN as a reliable diagnostic tool for PC. The reduced mean levels of GRPR-specific binding in CRPC samples described by Markwalder et al. corresponds with our data suggesting that hormonal therapy generally results in down-regulation of GRPR expression. The above described escape phenomenon of VCaP and PC-3 may thus be a rather uncommon clinical event. Despite this reduced expression, we were able to detect (at least some) expression of GRPR mRNA in 19 out of 21 PC samples (including CRPC) that underwent hormone treatment. This is supported by a few studies that report accurate visualisation of androgen-un-

responsive PC in 6 out of 10 patients [35, 36]. It appears that pre-selection of GRPR expressing patients is required when opting for GRPR-targeted BN-based response monitoring and therapy of PC in previously hormonally treated patients. The use of GRPR-based imaging and therapy in the hormone-naïve PC population, however, continues to be a promising strategy.

ACKNOWLEDGEMENTS

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CHAPTER 6

IMPROVING RADIOPEPTIDE PHARMACOKINETICS BY ADJUSTING EXPERIMENTAL CONDITIONS FOR BOMBESIN RECEPTOR-TARGETED IMAGING OF PROSTATE CANCER

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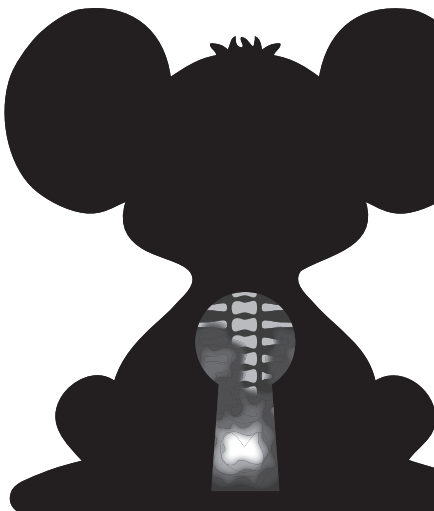

ABSTRACT

Prostate cancer (PC) is a major health problem. The Gastrin-Releasing Peptide Receptor (GRPR) offers a promising target for staging and monitoring of PC since it is overexpressed in PC and not in normal prostatic tissue. To improve receptor-mediated imaging we investigated the impact of various experimental conditions on pharmacokinetics using the Indium-111 labelled bombesin (BN) analogue AMBA. Besides frequently used androgen-resistant PC-3 also the clinically more relevant androgen sensitive VCaP celline was used as human PC xenograft in nude mice.

Non-purified ^{111}In-AMBA was compared with HPLC-purified ^{111}In-AMBA. Effect of specific activity was studied administrating 0.1MBq ^{111}In-AMBA supplemented with different amounts of AMBA (1-3000pmol). GRPR was saturated with Tyr^4-BN 1 and 4h prior to injection of ^{111}In-AMBA.

GRPR-positive tissue showed a significant 2 to 3-fold increase in absolute uptake after HPLC-purification while keeping a stable tumour-to-pancreas ratio. Lowering specific activity resulted in decline in uptake to 43% in tumour, 49% in kidney and 92% in pancreas between 10 and 3000pmol. Tumour to pancreas ratio improved six-fold from 0.1±0.0 after 10pmol up to 0.6±0.2 after 3000pmol ($p<0.01$). When saturating GRPR 4h prior to ^{111}In-AMBA injection tumour to pancreas ratio improved from 0.10±0.3 to 0.22±0.2 ($p<0.01$) and tumour to kidney ratio increased from 0.92±0.16 to 3.45±0.5 ($p<0.01$).

Besides specific peptide characteristics also the experimental conditions, such as HPLC-purification, variations in specific activity and saturation of the GRPR prior to ^{111}In-AMBA administration essentially affect radiopeptide pharmacokinetics. Experimental conditions therefore need to be carefully selected in order to compose ideal standardised protocols for optimal targeting.

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among men in the Western world and the second cause of cancer related death [1]. PSA-based screening of PC has dramatically increased early diagnosis [2], but for optimal management of disease it is essential to discriminate between organ-confined disease and PC beyond the borders of the gland. Current imaging techniques are not sufficiently accurate to determine extent of PC at diagnosis and to visualise micrometastases [3-5]. Therefore more sensitive imaging techniques are required.

Nuclear imaging - using single photon emission computed tomography (SPECT) and positron emission tomography (PET) - might offer applications for desired accurate PC imaging. The Gastrin-Releasing Peptide Receptor (GRPR) constitutes an interesting target for peptide-receptor radionuclide imaging and therapy. GRPR is a G-protein coupled receptor overexpressed on cell-membranes of PC while not on normal prostate [6]. Bombesin (BN) and its mammalian counterpart, the Gastrin-Releasing Peptide (GRP), are natural ligands for the GRPR and can bind to it with high affinity. The 14 amino acid COOH-terminus of the 27 amino acid GRP is the biologically active end and corresponds to that of BN [6].

Several radiolabelled BN-analogues have been developed and tested in preclinical and clinical settings as diagnostic and therapeutic tools for management of PC in patients. Our group has performed a standardised study on PC targeting efficacy of five radiolabelled BN analogues [7]. In this study the agonist DOTA-*CH2CO-Gly*-[*4-aminobenzoyl*]-*Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2 (AMBA*) was demonstrated to be one of the best BN-analogues with relatively high in vivo stability, tumour uptake and retention [8]. Also, AMBA is one of the few BN-based analogues that have been used for PET imaging in patients [9-10].

The focus for improvement of BN-based imaging (by increasing tumour to background ratio) has predominantly relied on development of new BN peptides with enhanced affinity for the GRPR [8, 10-12]. Instead, in the present study we investigated the impact of various experimental factors in order to improve peptide receptor-mediated imaging.

To ensure purity of tracer peptide, many study groups perform purification of BN-analogues by high-performance liquid chromatography (HPLC) between labelling and administration. In order to determine the impact of this purification step, we studied the effect of HPLC-purification methods on BN biodistribution.

An essential parameter to evaluate the performance of targeted imaging probes is the ratio between radioactivity in tumour and that in background organs. Contrast between the two is dependent on the amount of peptide administered. BN analogues have pharmacological effects in the nanomolar range. Capacity of the GRPR in tumour and other tissues is relatively low and varies between tissues [8, 13-14]. Therefore, in the present study we investigated receptor-saturation of target versus non-target organs using different peptide amounts [15]. We studied the impact of specific activity on tumour to background ratio by varying injected peptide amounts while remaining a constant radioactivity level.

GRPR-binding of BN-agonists like AMBA causes internalisation of the receptor [12, 16] and over time gradually re-expression of receptors on the cell membrane occurs indicating receptor turn-over. Tumour cells are assumed to have a higher metabolic rate than nor-

mal tissue [17-18]. We therefore assumed that GRPR turnover is faster in PC than in normal tissue resulting in a higher tumour to background ratio. We performed a biodistribution study to evaluate the impact of GRPR saturation with unlabelled compound (GRP or BN) 4h prior to labelled tracer injection.

In this study we investigated the effects of HPLC-purification, specific activity and GRPR saturation prior to labelled tracer on BN tumour uptake. Biodistribution studies were performed administrating Indium-111 labelled BN-agonist AMBA (^{111}In-AMBA) as a reference peptide intravenously to athymic nude mice bearing GRPR-expressing PC-3 or VCaP xenografts. The data show that all factors affect peptide receptor-mediated biodistribution and thereby influence PC imaging.

MATERIALS AND METHODS

Cell culture

Human PC cells were cultured in RPMI (LONZA, Verviers, Belgium) supplemented with fetal calf serum (GIBCO Invitrogen Co, Grand Island, NY, USA), 5% for PC-3 and 10% for VCaP cells. An additional 5mL/500mL penicillin/streptomycin antibiotic solution (LONZA, Verviers, Belgium; 10.000Units/mL penicillin, 10.000Units/mL streptomycin) was added to medium of both cell lines. Cells were grown in T175 Cellstar tissue culture flasks (Greiner Bio-one GmBH, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5%CO_2. Cells were passaged using a Trypsin/EDTA solution (LONZA, Verviers, Belgium), 170000U/L Trypsine-Versene and 200mg/L EDTA. Cells were grown to near confluency after which they were harvested and counted. Suspensions of approximately 5x10^{6}cells were yield and dissolved in 100µL PBS for subcutaneous injection in xenografts.

Prostate cancer xenografts

Six to seven-week old male Swiss nu/nu mice (Charles River, Kißlegg, Germany) were inoculated subcutaneously with PC-3 or VCaP cells (5 x 10^{6}cells/100µL PBS) on the right shoulder. A maximum of 4 mice per cage were kept in 14x13x33cm^3 individually ventilated cages (Techniplast BV, Wognum, The Netherlands), on Woody-Clean sawdust type BK8/15 (Technilab-BMI, Someren, The Netherlands) under a 12-h light-dark cycle, at 50±5% relative humidity and controlled temperature (~22°C). Mice had access to irradiated chow and acidified drinking water ad libitum. Experiments were initiated when tumours were established and clearly visible ranging from 200-500mm^3 (3-5 weeks after inoculation).

The experiment was approved by the Animal Experimental Committee of the Erasmus MC and performed in agreement with the Dutch Animal Experimentation Act *(*1977) and the European Convention for Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18 March 1986).

Radiolabeling of AMBA and Quality Control

AMBA (MW: 1503g/mol) was kindly provided by Prof. Dr. H.R. Mäcke (University Hospital Basel, Switzerland) and was dissolved in Milli-Q water, final peptide concentration 10^{-3}M. ^{111}In-Cl_3 (DRN 4901, 370MBq/mL in HCl, pH1.5–1.9) was obtained from Covidien (Petten, The Netherlands). All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) at the highest available grade. Radioactivity was measured in a VDC-405 dose calibrator (Veenstra Instruments BV, Joure, The Netherlands).

AMBA, ^{111}In-Cl_3 and quenchers (methionin, ascorbic acid and gentisic acid) were heated for 20min 80°C. Sodium acetate was used to control pH (3.5-4) [19]. Small volume reactions were performed in typical vials as described [20]. Vials were placed in a temperature-controlled heating block. After cooling down DTPA was added to bind free ^{111}In-. Instant thin-layer chromatography Silica Gel (ITLC-SG) was performed with mobile phase: both sodium citrate 0.1M and ammonium acetate 1M / methanol (1:1 v/v) [21-22]. Activity was subsequently detected using a Phosphor Imaging system, Packard Cyclone, with OptiQuant software (PerkinElmer, Groningen, The Netherlands). HPLC quality control and purification were performed using a Breeze system with a 1525 binary HPLC-pump (Waters, Etten-Leur, The Netherlands). Radioactivity was detected with a Unispec MCA γ-detector (Canberra, Zelik, Belgium). For separation a Symmetry 5µm, 4.6mmx250mm C_{18} column (Waters, Etten-Leur, The Netherlands) was used. HPLC-gradient was applied as described [20]. Injections of 200µL were performed with a 717plus autosampler (Waters, Etten-Leur, The Netherlands).

HPLC-Purification

To study the effects of specific activity on imaging we needed to obtain a fixed and well-characterized amount of ^{111}In-AMBA. Hereto we performed HPLC-purification of a labelled AMBA solution.

HPLC-purified ^{111}In-AMBA was quantified and diluted with saline for injection (0.1MBq per animal). The peptide amount of HPLC-purified ^{111}In-AMBA from the main peak was considered negligible, since 37MBq ^{111}In- ≈ $2.15x10^{-11}$gram atoms. Eventually non-labelled AMBA (AMBA) was added to obtain a HPLC-purified ^{111}In-AMBA solution containing a determined amount of AMBA (range 1 to 3000pmol). For stabilisation and reduction of radiolysis methionin, ascorbic acid and gentisic acid were added as quenchers directly after HPLC-purification.

Biodistribution studies

Mice (either non-tumour bearing, PC-3 or VCaP tumour-bearing) were injected with ^{111}In-AMBA solution into the lateral tail vein. Animals were sacrificed by cervical dislocation at fixed time points post injection (pi). Study groups consisted of 3-5 mice. Tumour and other selected tissues were excised, weighed, and radioactivity was determined in a LKB-1282-compugamma γ-counter

(Perkin Elmer, Groningen, The Netherlands). Data were expressed as percentage of the injected dose per gram of tissue (%ID/g) using 10µL of the original injectate as reference (100%).

Effect of HPLC-purification

A biodistribution study comparing tissue distribution of non-purified ^{111}In-AMBA and HPLC-purified ^{111}In-AMBA was performed. An equal peptide amount (10pmol) and radioactivity (0.1MBq) of purified or non-purified ^{111}In-AMBA was injected in PC-3 and VCaP bearing mice (n=4/group). Mice were sacrificed 1h pi.

Effect of specific activity

In PC-3 bearing mice a biodistribution study was performed comparing tissue distribution of injections with different specific activity: a fixed radioactivity level of HPLC-purified ^{111}In-AMBA (0.1MBq per mouse) supplemented with different amounts of AMBA (1-3000pmol). Mice (n=4 per condition) were sacrificed 1h pi.

Effect of receptor saturation

The effect of pre-saturation of GRPR prior to the administration of ^{111}In-AMBA was studied in PC-3 bearing mice. At different time points after GRPR saturation with Tyr^4-BN (60 nmol), HPLC-purified ^{111}In-AMBA (0.1MBq≈10pmol) was administered (n=4/time point). Mice received ^{111}In-AMBA without pre-saturation (t=0) or ^{111}In-AMBA at 1 (t=1) or 4 (t=4) hours after Tyr^4-BN injection. Mice were sacrificed 1h after injection of labelled peptide.

Statistics

Statistical analyses were performed using the unpaired 2-tailed t-test in SPSS 11.01 (SPSS Inc., Chicago, United States). Statistical significance was assumed if p-values were <0.05.

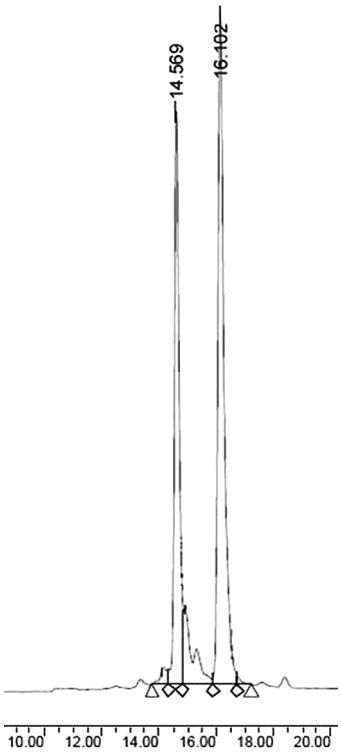

Figure 1

HPLC-chromatogram with UV at 278 nm and base to base separation. The graph shows HPLC of an ^{111}In-AMBA solution. Difference in retention time between AMBA and ^{111}In-AMBA is 1.5 min.

RESULTS

Biodistribution studies

Effect of HPLC-purification

HPLC-purification of ^{111}In-AMBA solution resulted in carrier free end product, as shown in Figure 1. The main peak containing carrier free ^{111}In-AMBA had a retention time (RT) of 16.1min while for unlabelled AMBA this was 14.6min. Radiochemical purity, measured directly after labelling, was always >90%.

When administrating non-purified ^{111}In-AMBA in PC-3 bearing mice tumour uptake was 3.3±0.7%ID/g and pancreas uptake was 28.6±2.2%ID/g 1h pi. Significantly higher uptakes ($p<0.01$) for both tissues were seen after injection of HPLC-purified ^{111}In-AMBA: tumour uptake was 6.8±1.1%ID/g and pancreas uptake was 60.5±14.0%ID/g at 1h pi. However, uptake was significantly higher in all gastro-intestinal organs after HPLC-purified ^{111}In-AMBA as compared to ^{111}In-AMBA as well (Figure 2).

In VCaP-tumour-bearing mice the same tendency was seen. Uptake in tumour increased significantly from 1.6±0.2%ID/g for non-purified ^{111}In-AMBA to 4.3±1.3%ID/g after injection of HPLC-purified ^{111}In-AMBA at 1h pi. Pancreatic uptake values were also higher: 23.4±2.9%ID/g and 70.0±20.3%ID/g, respectively.

Tumour to pancreas ratio remained the same in both experiments.

Effect of specific activity

Injecting ^{111}In-AMBA at a fixed amount of radioactivity while varying in peptide amount resulted in significant differences in PC-3 tumour uptake when injecting over 1000pmol of peptide. Lower specific activity resulted in a 43% decline ($p=0.09$) in tumour uptake between 10pmol (5.7±2.5%ID/g) and 3000pmol (2.9±1.0%ID/g), as shown in Figure 3.

Significant differences in pancreas uptake were observed already at peptide amounts beyond 100pmol. Compared to tumour uptake, a stronger decline in uptake (92%) was observed for pancreas uptake when lowering specific activity. Injection of 10pmol ^{111}In-AMBA resulted in a pancreas uptake of 63.2±14.9%ID/g. For 3000pmol uptake was 5.1±0.4%ID/g (Figure 3). Due to a rather stable tumour uptake and decreasing pancreas uptake, tumour to pancreas ratios improved six-fold from 0.1±0.0 at 10pmol up to 0.6±0.2 at 3000pmol.

Uptake in kidneys showed a gradual decrease at lower specific activities. Uptake decreased 49% from 4.3±1.0%ID/g (10pmol) to 2.0±0.3%ID/g (3000pmol). Tumour to kidney ratio remained stable with an average ratio of 2.1±0.6.

Effect of receptor saturation

Tumour uptake of ^{111}In-AMBA when no receptor saturation was applied was 3.9±0.6%ID/g at 1h pi. When ^{111}In-AMBA was injected 1h after receptor saturation with Tyr^4-BN pre-dosing, tumour uptake decreased significantly to 1.3±0.5%ID/g ($p<0.01$). When tracer

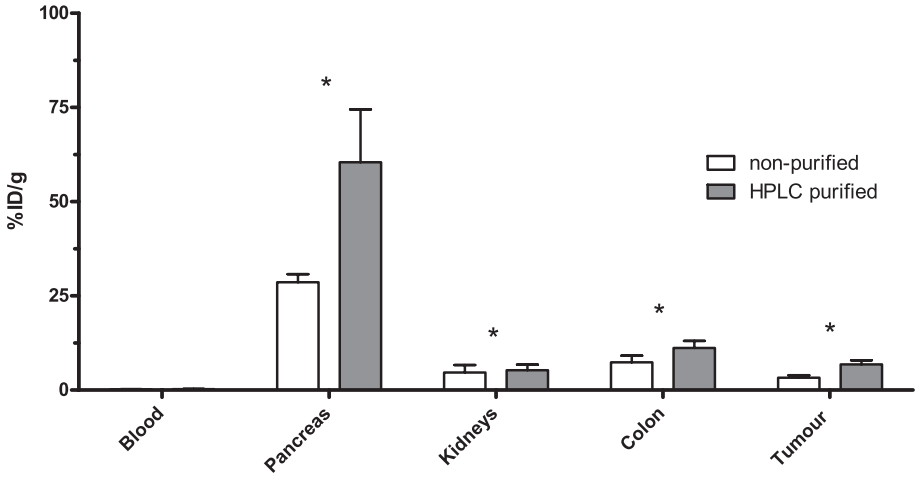

Figure 2

Biodistribution results of non purified versus purified ^{111}In-AMBA in PC-3 bearing mice, 1h post injection.

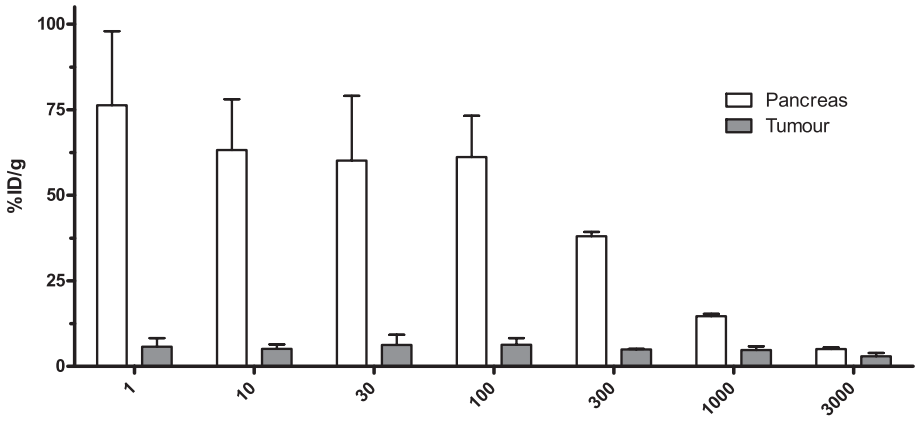

Figure 3

Uptake in pancreas and PC-3 tumour after administration of ^{111}In-AMBA with variable specific activity. X-axis depicts amount in pmol.

was injected 4h after receptor saturation with Tyr^4-BN, ^{111}In-AMBA tumour uptake was 2.4±0.7%ID/g at 1h pi.

Pancreas uptake of ^{111}In-AMBA without receptor saturation was 29.8±1.7%ID/g 1h pi. Uptake was significantly lower ($p<0.001$) when receptor saturation was applied 1h and 4h prior to tracer application (12.2±0.6%ID/g and 10.8±3.0%ID/g, respectively). As a result tumour to pancreas ratio improved

significantly ($p<0.01$) from 0.10±0.3 to 0.22±0.2 (Figure 4A).

Tumour to kidney ratios significantly increased after GRPR saturation by administration of Tyr^4-BN prior to ^{111}In-AMBA. Without pre-dosing kidney uptake was 4.4±1.5%ID/g 1h pi, while ^{111}In-AMBA uptake after pre-dosing for 1h and 4h was 0.8±0.1%ID/g and 0.7±0.1%ID/g. As a result also tumour to kidney ratio increased significantly ($p <0.01$) from 0.92±0.16 to 3.45±0.5 (Figure 4B).

DISCUSSION

Nuclear imaging offers new applications for desired accurate imaging of PC. Targeting of GRPR with radiolabelled BN has shown promising results in preclinical studies [7-8, 11-12]. A variety of BN analogues have been developed and tested for (in vivo) stability, receptor affinity and specificity. Since not only specific tracer characteristics but also experimental conditions like HPLC-purification of labelled peptide, specific activity (peptide amount) and saturation of GRPR (pre-dosing) may affect GRPR-targeting performance of BN analogues, we have focused on

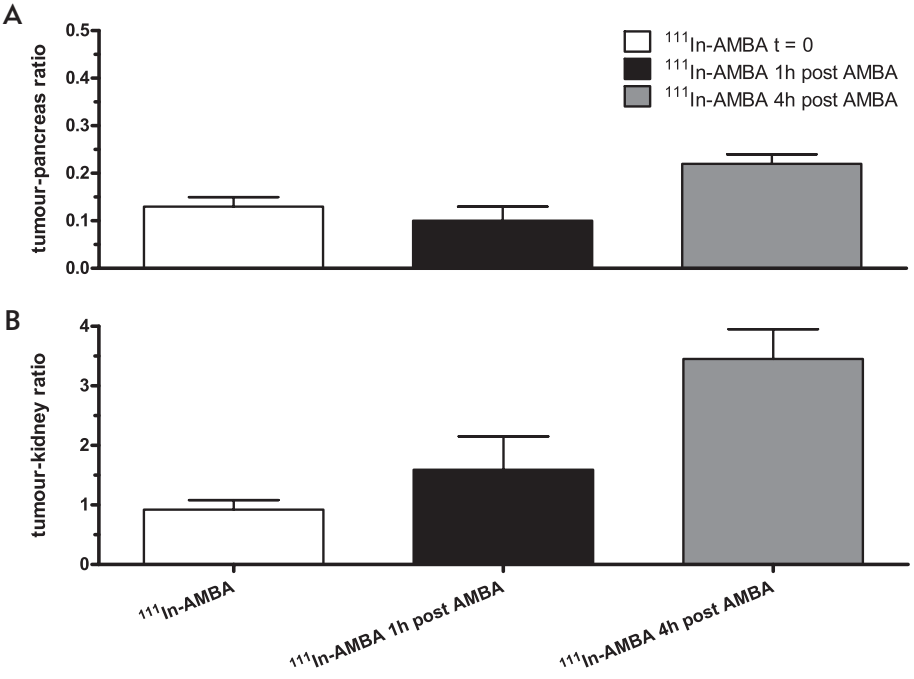

Figure 4

Tumour-pancreas (A) and tumour-kidney (B) ratio when administering purified ^{111}In-AMBA at different time points after injection of Tyr^4-BN in PC-3 bearing mice.

these aspects is this study. Hereto we compared biodistribution results of purified versus unpurified tracer, various peptide amounts, and saturation versus no saturation of GRPR before injection of labelled peptide injection, aiming at optimising GRPR targeting. Many factors showed to have an effect receptor-mediated uptake.

mental PC studies. In addition, in some experiments also the less applied VCaP celline was used. It has GRPR expression comparable with PC-3 while it is more clinically relevant because of its expression of the androgen receptor and ability to secrete PSA [23-24]. Therefore, it is a more realistic model for future peptide-based imaging as well as

Table 1

Uptake in %ID/g in pancreas (and tumour) after administration of variable ^{111}In-AMBA solutions in VCaP tumour-bearing mice.

Uptake ^{111}In-AMBA	**mean**	**SD**	**P-value**	**mean**	**SD**	**P-value**
	Tumour			Pancreas		
Sep-Pak C_{18}						
Non-purified	-	-	-	22.5	1.5	
Purified	-	-	-	27.1	7.5	0.30
Eluents						
None	5.4	0.9		42.9	5.1	
Methanol 14%	5.5	1.4	0.73	48.0	6.9	0.49
Ethanol 14%	7.5	2.5	0.25	55.0	1.8	*0.02
Acetonitril 14%	7.4	0.6	*0.03	62.0	5.9	*0.01
HPLC purification						
Non-purified	-	-	-	19.4	4.1	
Purified before labelling	-	-	-	29.3	17.4	0.39
Purified after labelling	-	-	-	41.8	6.0	*0.01

** indicates significance compared to normal (non-purified / eluents: none)*

To determine the impact of the different experimental conditions on peptide biodistribution and resulting target to background ratio, we chose the BN agonist ^{111}In-AMBA as reference agonist peptide. AMBA was selected as a result of good performance in preclinical and clinical studies [7-8]. We used the GRPR-expressing PC-3 and VCaP xenografts as model systems. The androgen-resistant PC-3 cell line was chosen because of its use in the vast majority of experi-

therapeutic PET/SPECT studies. In this study VCaP indeed showed reasonable tumour uptake indicating expression of the GRPR, nevertheless absolute tumour-uptake of PC-3 was significantly higher.

HPLC enables separation of radiolabelled analogue from non-incorporated (free) radionuclides and from unlabelled intact or oxidised peptide to achieve radiolabelled carrier-free

peptide (Figure 1). We showed that compared to non-purified ^{111}In-AMBA HPLC-purified ^{111}In-AMBA showed a significant two-to threefold higher uptake in GRPR positive tissues. We investigated a few possible explanations for these differences in biodistribution, results are summarized in Table 1.

HPLC-purification requires aqueous solutions containing various organic eluents such as methanol, ethanol or acetonitril to separate labelled and non-labelled peptides. When supplementing these commonly used organic solvents to non HPLC-purified ^{111}In-AMBA before administration a tendency towards higher uptake in GRPR-expressing organs was seen. These differences, however, could not fully explain the significant differences seen with HPLC-purified ^{111}In-AMBA. We also performed a biodistribution study in order to investigate whether the HPLC-procedure itself caused differences in uptake. In this study we used an ^{111}In-AMBA solution in which the unlabelled AMBA peptide was purified by HPLC prior to labelling and compared that to HPLC-purified labelled AMBA. No increase in uptake of GRPR-positive tissues was seen though. Only HPLC-purification after peptide labelling showed a significant increase in uptake. In contrast to HPLC-purification, separation procedures using Sep-Pak C_{18} do not separate ^{111}In-AMBA from non-labelled AMBA. Consequently, after purification of labelled peptide, in the injection solution no additional non-purified AMBA amount was needed. As a result the potential contamination of ^{111}In-AMBA solution is higher. In non-tumour-bearing mice no difference in pancreas uptake was observed when comparing Sep-Pak C_{18} purified ^{111}In-AMBA with ^{111}In-AMBA. Also, a biodistribution study was performed in which we added 10pmol of HPLC-purified ^{115}In-AMBA to HPLC-purified ^{111}In-AMBA (neglectable amount) in order to study the differences in affinity and uptake between labelled and unlabelled AMBA. As compared to HPLC-purified ^{111}In-AMBA the solution of $^{111/115}$In-AMBA showed no significant differences in GRPR uptake (data not shown). Clearly, substitution of ^{111}In-AMBA by ^{115}In-AMBA did not affect GRPR affinity.

There is no consensus in literature about the optimal BN peptide amount for optimal targeting in mice. We previously reported an optimal peptide amount for GRPR targeting between 10pmol and 50pmol for analogue [^{111}In-DTPA-Pro^{1},Tyr^{4}]BN in non-tumour-bearing Lewis rats [15]. Basso et al. reported that in man a BN infusion of 15ng/kg per minute resulted in the maximal gastrin response [25]. In experimental studies using mice often 10pmol of radiolabelled BN is injected [11-12]. Our current study clearly showed that differences in peptide amount have a significant influence on BN biodistribution. The best tumour to background ratios were reached when injecting 3000pmol of purified ^{111}In-AMBA (Figure 3). This was caused by rapid decrease in tracer uptake in most background organs, especially pancreas, while tumour uptake remained fairly stable. This implies that the GRPR saturation is faster in background than in tumour tissue.

Similar results - although saturation of the tumour seemed not reached - were seen in a study by Lantry et al. in which ^{177}Lu-AMBA with various specific activities (between 1.7 and 426pmol) was administered to PC-3 bearing mice [8].

In this study we assumed that the main peak from HPLC of an ^{111}In-labelled AMBA solution contains carrier-free ^{111}In-AMBA only. It needs to be mentioned that the ^{111}In-AMBA peak might also contain AMBA labelled with other metals like Fe and Zn. Total peptide amount collected from HPLC might therefore be higher than foreseen. Since we neglected the amount of ^{111}In-AMBA from HPLC we have added 10pmol AMBA to achieve a final injection solution of 0.1MBq (10pmol). In theory, if the total amount of peptide injected to HPLC would be completely incorporated with ^{111}In- and trace metals, this would result in a maximum peptide amount of 10pmol. Therefore, after addition of 10pmol AMBA, the maximum peptide amount of ^{111}In-AMBA solution is 20pmol. As concluded earlier this would not affect tumour uptake since receptor-mediated uptake remained stable up to 300pmol.

Biodistribution of BN was also affected when GRPR was saturated using unlabelled Tyr^4-BN prior to injection of radiolabelled ^{111}In-AMBA. It resulted in significantly lower uptakes in tumour, pancreas and kidney. At four hours after injection of the pre-dose, a recovery in HPLC-purified ^{111}In-AMBA uptake in tumour was seen while restoration of uptake in pancreas and kidney was not observed. Our data support the hypothesis that malignant PC cells regenerate the GRPR faster than normal tissues, probably caused by a higher metabolic rate [17-18]. In agreement with Lantry et al. pre-dosing with unlabelled AMBA resulted in just a small reduction of ^{111}In-AMBA uptake in tumour whereas a stronger reduction of ^{111}In-AMBA uptake in pancreatis was seen. This resulted in an optimised tumour-to-pancreas ratio [8].

Noticeably, receptor saturation goes with a reduction in absolute tumour uptake and therefore higher amounts of peptide will be required to equal uptake when pre-dosing with unlabelled peptide. The need for higher peptide amount may result in more side-effects especially when receptor agonists are being used. The use of BN antagonists instead will however likely circumvent this problem.

CONCLUSIONS

In the present study we investigated on experimental factors that interfere with GRPR-targeted biodistribution using the BN analogue AMBA as a test tracer. We demonstrated that HPLC-purification of ^{111}In-AMBA, variations in specific activity and saturation of the GRPR prior to the administration of ^{111}In-AMBA essentially affect radiopeptide pharmacokinetics. All influence absolute tumour uptake and often tumour to background ratio is affected as well. Experimental conditions therefore need to be carefully selected in order to compose ideal standardised protocols for optimal targeting.

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CHAPTER 7

GASTRIN-RELEASING PEPTIDE RECEPTOR-BASED TARGETING USING BOMBESIN ANALOGUES IS SUPERIOR TO METABOLISM-BASED TARGETING USING CHOLINE FOR IN VIVO IMAGING OF HUMAN PROSTATE CANCER XENOGRAFTS

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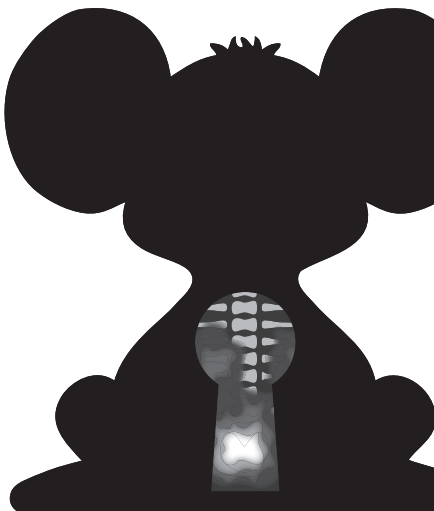


ABSTRACT

Prostate cancer (PC) is a major health problem. For staging and monitoring of PC the Gastrin-Releasing Peptide Receptor (GRPR) offers a promising target since this receptor is overexpressed in PC, but not in hyperplastic prostate. As cancer cells show increased metabolic activity also metabolism-based tracers are being used for PC imaging. We compared GRPR-targeted imaging using the ^{68}Ga-labelled bombesin (BN) analogue AMBA with metabolism-based imaging using ^{18}F-methylcholine (^{18}F-CH) in nude mice bearing human PC xenografts (VCaP and as reference PC-3).

PET and biodistribution studies were performed with both tracers in all mice. Scanning started immediately post-injection (pi). Dynamic PET was reconstructed and analyzed quantitatively. Biodistribution uptake was expressed as % of injected dose per gram tissue (%ID/g).

All tumours were clearly visualised using ^{68}Ga-AMBA. ^{18}F-CH showed significantly less tumour/background contrast. Quantitative PET analyses showed fast tumour uptake and high retention for both tracers. VCaP tumour uptake determined from PET at steady state was 6.7±1.4 %ID/g (20-30 min pi, N = 8) for ^{68}Ga-AMBA and 1.6±0.5 %ID/g (10-20 min pi, N = 8) for ^{18}F-CH which was significantly different ($p < 0.001$). PC-3 outcome was comparable. Biodistribution results were in accordance with PET showing a VCaP tumour uptake of 9.5±4.8 %ID/g for ^{68}Ga-AMBA and 2.1±0.4 %ID/g for ^{18}F-CH. Apart from the GRPR-expressing organs, uptake in all organs was significantly lower after ^{68}Ga-AMBA than ^{18}F-CH.

Tumour uptake of ^{68}Ga-AMBA was higher while overall background activity was lower than observed for ^{18}F-CH in the same PC-bearing mice. These results suggest that peptide-receptor mediated PET using BN analogue is superior to metabolism-based PET using choline for scintigraphy of PC.

INTRODUCTION

Prostate cancer (PC) is the third leading cause of cancer-related deaths and the most frequently diagnosed cancer in men in Western countries[1-2]. Measurement of serum prostate-specific antigen (PSA) is widely used for the detection of early PC[3-4] and PSA-based screening has resulted in a sharp increase of PC detection. As long as PC is organ-confined, prostate surgery or radiation therapy with curative intent is the first choice of treatment. However,

when facing metastasised PC curative treatment is no longer available and palliative hormone ablation therapy is indicated. Therefore, accurate staging of early PC at time of diagnosis as well as monitoring of patients following local or systemic treatment are crucial steps in management of the disease.

Accuracy of conventional imaging techniques – such as transrectal ultrasound (TRUS), CT, MRI, and bone scintigraphy - is not sufficiently adequate to determine extent of PC at diagnosis and to visualise micrometastases[5-7]. New and more sensitive, preferably non-invasive, imaging strategies are required. Molecular imaging by nuclear scintigraphy using positron emission tomography (PET) or single photon emission computer tomography (SPECT) may provide alternative technologies for detection. It enables biochemical cellular targets, such as cell-specific receptors, and more general metabolic processes to be targeted with tracers coupled to radionuclides for sensitive imaging.

In peptide-receptor based scintigraphy radiolabelled peptides are being used to target specific cell membrane receptors. For PC imaging the Gastrin-Releasing Peptide Receptor (GRPR) offers a promising target since this receptor is overexpressed in malignant cells originating from the prostate while normal and hyperplastic prostate cells show low or no expression of GRPR[8]. Gastrin releasing peptide (GRP), consisting of 27-amino acids, is the mammalian homologue of the linear tetradecapeptide bombesin (BN) found in amphibians. Both peptides are natural ligands with a very high affinity for the GRPR. Several analogues - predominantly BN based - with the ability to be labelled with radionuclides have been developed and tested for their potential to treat and image PC using SPECT and PET modalities; for review see Schroeder et al.[9]. AMBA is a BN analogue which has shown good targeting performance in (pre-)clinical studies[10-11]. It is coupled to the DOTA chelator which enables labelling with Galium-68 (^{68}Ga) - a positron emitting radionuclide suitable for PET - resulting in ^{68}Ga-DOTA-AMBA (^{68}Ga-AMBA).

Apart from peptide-receptor-based targeting, also metabolism-based tracers are being used to image cancer cells. Metabolic targeting is based on the assumption that cancer cells show increased activities of several metabolic processes; for review see Jager et al.[12]. Malignant transformation of cells has been found to be associated with increased metabolic activity[13]. High cell activity and cell turnover in cancer is assumed to be directly related to high activity of a variety of biological processes such as glycolysis, proliferation and membrane synthesis. Although metabolic activity of PC is considered to be rather low because of its relatively low proliferative activity[14-15], results obtained with metabolism-based tracers have been promising. For imaging of PC radiolabelled choline and acetate have shown to be the most promising tracers[6, 16, 17-20]. We selected choline as metabolic reference tracer in this study to compare its imaging ability with that of the peptide-receptor-based BN analogue AMBA.

Choline is an essential nutrient that serves as a precursor for the synthesis of phosphatidylcholine, a major constituent of the cell membrane[21]. Nuclear magnetic resonance (NMR) spectroscopy has demonstrated higher concentrations of phosphocholine in human tumour tissues and in normal cells when stimulated by (mitogenic) growth factors[22-23]. In prostate cancer the cellular uptake and phosphorylation of choline is often increased compared to normal prostate epithelial and stromal cells[24-25]. Most PC imaging studies perform PET using choline labelled with Carbon-11 (^{11}C)[26, 16, 6, 27]. Since ^{11}C has a relatively short half-life of 20 minutes (min), use of this radionuclide is limited to centres with on-site cyclotrons. This drawback has led to the development of choline derivatives like N-[18]fluoromethyl-N-(2-hydroxyethyl)-N,N-dimethylammonium (^{18}F-CH) with a radionuclide half life of 110 min. Biodistribution of ^{18}F-CH is comparable to ^{11}C-choline although ^{18}F-CH shows higher renal activity[28]. Structures of bombesin, ^{68}Ga-AMBA and ^{18}F-CH are shown in Figure 1.

To compare the potential of both radioactive tracers we made use of PC-tumour-bearing male NMRI nu/nu mice. The GRPR-expressing PC-3 cell line, originating from a PC bone metastasis, is the most widely used model for the study of radioactive BN-analogues[29, 9, 27]. PC-3 is an androgen independent cell line[34] that does not express the androgen receptor or secretes PSA while most prostate tumours in patients, except for late stage disease, do. The VCaP cell line on the other hand is androgen responsive. It not only expresses the androgen receptor and secretes PSA, it also expresses the GRP receptor[30-31]. While it is a more representative PC model than PC-3 in this study we used the VCaP cell line. GRPR expression of both is comparable (data not shown). For comparison with literature we included the PC-3 xenograft as reference model.

To our knowledge, this is the first study comparing BN-analogue-based GRPR targeting to metabolic targeting for PC imaging. From PET and biodistribution in VCaP-bearing nude mice it became clear that peptide-receptor based imaging using ^{68}Ga-AMBA for targeting of the GRPR is superior to metabolic imaging using ^{18}F-CH for detection of PC in nude mice.

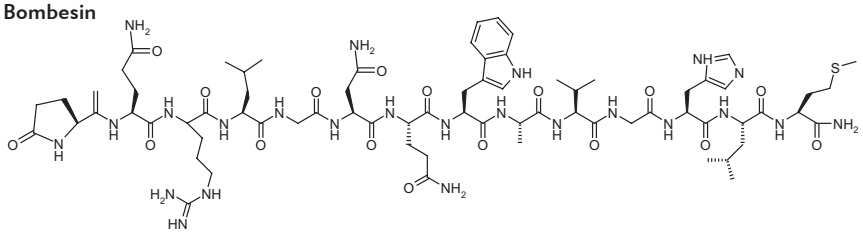
MATERIALS AND METHODS

Cell culture

Human PC-3 and VCaP cells were cultured in RPMI-1640 (LONZA, Verviers, Belgium) supplemented with penicillin/streptomycin antibiotics (10.000 Units/mL penicillin, 10.000 Units/mL streptomycin; LONZA, Verviers, Belgium) with addition of 5% fetal calf serum (GIBCO Invitrogen Co, Grand Island, NY, USA) for PC-3 or 10% for VCaP. Cells were grown in T175 Cellstar tissue culture flasks (Greiner Bio-one GmBH, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged using a *Trypsin - EDTA* solution (LONZA, Verviers, Belgium) 170000

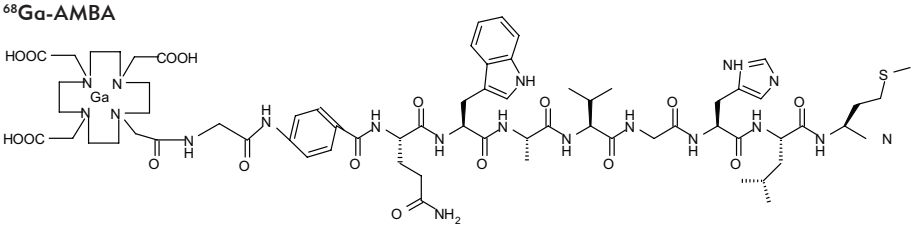
Figure 1
Structure formulas of bombesin, ^{68}Ga-AMBA and ^{18}F-CH.

Bombesin



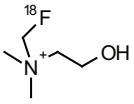
pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH

^{68}Ga-AMBA



68Ga-DOTA-CHCO-Gly-4-aminobenzyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH

^{18}F-CH



N-[18F]fluormethyl-N-(2-hydroxyethyl)-N,N-dimethylammonium

U/L Trypsine-Versene and 200 mg/L EDTA. For the present study, cells were grown to near confluency, harvested and counted. Cells were resuspended in PBS to yield approximately 5 x 10^6 cells / 100 µL for subcutaneous injection into nude mice.

PC xenografts

Eight male NMRI nu/nu mice (Taconic, Ry, Denmark) aged six to seven weeks were inoculated subcutaneously with VCaP cells on the right shoulder. For reference, 3 mice were injected with PC-3 cells in the same way. A maximum of 4 mice were kept in 14 x 13 x 33.2 cm^3 individually ventilated cages (Techniplast) on sawdust (Woody-Clean, type BK8/15; BMI) under a 12-h light-dark cycle, at 50 ± 5% relative humidity and controlled temperature (~22 °C). Mice received irradiated chow and acidified drinking water ad libitum. Experiments were initiated when tumours

reached a volume of 200-600 mm^3 (2-5 weeks after inoculation).

This study was approved by the Animal Experimental Committee (DEC) of the Erasmus MC and performed in agreement with The Netherlands Experiments on Animals Act (1977) and the European Convention for Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18 March 1986).

Radiolabelling & quality control *68Ga-DOTA-AMBA*

Physical characteristics and radiochemistry of the $^{68}Ge/^{68}Ga$ generator: $^{68}Ge/^{68}Ga$ 370MBq generator (obtained from IDB Holland BV, Baarle Nassau, the Netherlands and originating from iThemba Labs, Somerset West, South Africa), $t_{1/2}$ ^{68}Ge: 280 days, $t_{1/2}$ ^{68}Ga: 68 min was used. The carrier of this generator is SnO_2. The generator was eluted with 1 M Ultrapure HCl 30% (J.T. Baker, Deventer, the Netherlands). All chemicals were of the highest available grade. Fractionated elution of the generator was performed in the following fractions: 1.5 mL (void volume), 2.0 mL (80% of total activity) and 2.5 mL (waste), the fractions were collected and measured in a VDC-405 dose calibrator (Veenstra Instruments BV, Joure, the Netherlands). ^{68}Ga was quantified as described previously[32]. Anion purification was performed with an Oasis® WAX 1 cc column (Waters, Etten-Leur, the Netherlands). Before use the anion column was pretreated with 2 mL ethanol followed by 2 mL HCl 5 M. Total peak fraction (2 mL ≈ 300 MBq) was added to a 4 mL HCl solution (final concentration HCl 5 M). This solution was eluted over the anion column and subsequently washed with 2 mL of HCl 5 M containing ^{68}Ge, which was quantified as described[32]. Approximately 0.4 mL of Milli-Q was used to desorp ^{68}Ga (recovery ±80%).

Radiolabeling:

DOTA-AMBA (MW: 1503 g/mol) was kindly provided by Prof. Dr. H.R. Maecke (University Hospital Basel, Switzerland). Before application of the peptide, it was dissolved in Milli-Q water (final concentration 10^{-3} M). Peptide, desorped ^{68}Ga (200µL in Milli-Q) and HEPES 1 M (200µL) were heated for 10 min at 80°C. Radiolabelling was performed in reaction volumes of 1.5 mL in polypropylene or glass vials (Waters, Etten-Leur, the Netherlands). Final pH of radiolabelled product was between 3-3.5. The vials were placed in a temperature-controlled heating block. Instant thin-layer chromatography Silica Gel (ITLC-SG) was performed with mobile phase sodium-citrate 0.1 M and ammoniumacetate 1 M / methanol (1:1 v/v)[32-33]. Activity was subsequently detected using a Phosphor Imaging system, Packard Cyclone, with OptiQuant software (PerkinElmer, Groningen, the Netherlands). HPLC quality control and purification were performed using a Waters breeze system with a 1525 binary HPLC pump (Waters, Etten-Leur, the Netherlands). Radioactivity was detected with a Unispec MCA γ-detector (Canberra, Zelik, Belgium). For separation a Symmetry 5 µm, 4.6mm x 250mm C_{18} column (Wa-

ters, Etten-Leur, the Netherlands) was used.

HPLC mobile phase: 0.1% TFA (A) and methanol (B). HPLC gradient: 0-2 min 100% A (Flow 1 mL / min), 2-3 min 55% B (Flow 0.5 mL / min), 3-20 min 60% B (Flow 0.5 mL/min), 20-20:01 min 100% B (Flow 1 mL/min), 20:01-25 min 100% A (Flow 1mL / min), 25:01- 30 min 100% A (Flow 1mL / min). Injections of 200 µL were performed with a 717 autosampler (Waters, Etten-Leur, the Netherlands).

After labeling, the main peak containing ^{68}Ga-AMBA was collected carrier free. Retention times were 12.4 min for DOTA-AMBA and 13.5 min for ^{68}Ga-DOTA-AMBA. After activity quantification of the purified main peak the solution was diluted for injection (0.5-1.5 MBq per animal). Non-labeled DOTA-AMBA was added to obtain a solution containing a fixed mass (300 pmol). The ^{68}Ga-DOTA-AMBA mass, collected with HPLC, was considered to be negligible (^{68}Ga ≈ 3,6x10^{-13} moles/ 37 MBq). After HPLC purification methionin, ascorbic acid and gentisic acid were added as quenchers to stabilize. Radiochemical purity was ±90%.

18F-fluoromethyl-dimethyl-2-hydroxyethylammonium (18F-CH)

Radiosynthesis and control of radiochemical purity was adapted from the methods described by Iwata et al.[34]. The synthesis of ^{18}F-CH was performed at the VU University Medical Centre (Amsterdam, the Netherlands). An in-house developed remotely operated radiosynthesis system was used. The ^{18}F was isolated from ^{18}O-enriched water through a PS-HCO_3 ion-exchange column and was subsequently eluted into the reaction vial with 1 mL of a Kryptofix 2.2.2 / K_2CO_3 solution (12.5 mg K2.2.2, 2 mg K_2CO_3 in aconitril/water 9:1 v/v). Under reduced pressure and a flow of helium (50 mL/min) the solvents were evaporated at 100°C. The residue was azeotropically dried by addition of 500 µL dry acetonitril followed by its evaporation as before. After cooling the reaction vial to room temperature followed by removal of the vacuum and helium, a dry solution of 50% dibromomethane in acetonitril was added. The temperature was raised to 100°C, and the dibromomethane was allowed to react with the ^{18}F for 5 min after which the vial was again cooled to 35°C. ^{18}F-bromofluoromethane was than distilled from the vial using a stream of helium (50 mL/min) and passed through 4 connected Sep-Pak Plus Silica cartridges and consecutively through a ‘on column reaction’ setup, consisting of 1) a Sep-Pak Plus C_{18} cartridges loaded with 700 µL of dimethylethanolamine, 2) a second Sep-Pak Plus C_{18} cartridge and 3) an activated Sep-Pak Light Accell Plus CM ion-exchange cartridge connected in series. Activity on these cartridges was monitored and after it reached a maximum (8-12 min) distillation was terminated. The ‘on column reaction’ setup was rinsed with 10 mL ethanol followed by 10 mL of water and subsequently the ^{18}F-CH was eluted with 5 mL of 0.9% NaCl (aq) into a flask containing 10 mL of a 0.9% NaCl/7.09 mM NaH_2PO_4 (aq) solution yielding the final product.

PET scanning

Mice were anaesthetized by a mixture of isoflurane / oxygen and were placed in prone position and kept under anaesthesia in a MicroPET scanner (Inveon©, Siemens / CTI, Knoxville, TN). Tumour-bearing mice were injected intravenously using a tail vein cannula with 300 pmol / 100 µl ^{68}Ga-AMBA (^{68}Ga-AMBA: 1.5-0.5 MBq) or 100 µl ^{18}F-CH (8.1-1.2 MBq). Based on their unique pharmacokinetics an ideal scanning schedule for each tracer was constructed. So, dynamic full body acquisition started at time of injection for a continuous period of 30 min using ^{68}Ga-AMBA or 20 min using ^{18}F-CH. During scanning mice were kept warm by an external heating mat.

Each mouse was scanned after injection of the tracers ^{68}Ga-AMBA and ^{18}F-CH on two consecutive days. In order to be able to correct for potential interference between the tracers, the first group of mice was scanned first with ^{68}Ga-AMBA and one day later with ^{18}F-CH and the remaining animals were scanned first with ^{18}F-CH and one day later with ^{68}Ga-AMBA (see Table 1).

List mode data were stored on IAW 1.2.2.2 (Inveon© Acquisition Workplace; Siemens). From there they were histogrammed and framed: 25 x 60 sec frames for ^{68}Ga-AMBA and 15 x 60 sec frames for ^{18}F-CH. Attenuation correction on sinograms was subsequently performed. Images were reconstructed using filtered back projection (2DFBP) with a 50% rampfilter for analyses and quantification. For visualisation the ordered-subsets expectation maximization / maximum a priori (OSEM3D/MAP) algorithm was used. To achieve steady state for both quantification and visualisation a sum of the last 10 frames was displayed.

Quantification was performed by manually drawing volumes of interest (VOIs) over preselected organs (kidneys and bladder) and tumours in all directions with a VOI diameter not exceeding the total volume of selected tissue to avoid interfering signals from other tissues[35]. Average outcome from two independent skilled analysts were used. The percentage of injected dose per gram tissue (%ID/g) was calculated as VOI activity (MBq/ml) / total injected dose (MBq) * 100%. To decrease interference of background we allowed this uptake to wash out and quantified mean total uptake at steady state from the last 10 min of scanning in %ID/g. The median and interquartile range (IQR) in %ID/g were determined for the timepoints 0.5-30.0 min post-injection (pi). Statistical analysis was performed using the Mann-Whitney U test. A probability of less than 0.05 was considered significant.

Biodistribution Studies

After PET scanning, VCaP tumour-bearing mice were sacrificed for biodistribution at ideal time points for each tracer. Biodistribution of ^{68}Ga-AMBA was performed 1 hour (h) pi and for ^{18}F-CH this was 30 min pi following the previously mentioned schedule (Table 1).

Table 1
Time chart of the set up for PET and biodistribution studies.

Day	^{68}Ga–DOTA-AMBA PET	^{18}F-CH PET	^{68}Ga–DOTA-AMBA biodistribution	^{18}F-CH biodistribution
1	Mouse I – IV			
2		Mouse I - VIII		Mouse I – IV
3	Mouse V - VIII		Mouse V - VIII	

Tumour, liver, heart, blood, muscle, tail and kidneys as well as the GRPR-expressing organs pancreas and colon[36], were collected for counting of radioactivity in the LKB-1282-compugamma system (Perkin Elmer, Oosterhout, the Netherlands). Radioactive uptake was calculated as %ID/g after correction for remaining activity in the tail. Mean uptakes of each group of mice (n = 4) were then calculated.

Statistical analysis was performed using the unpaired t-test. A probability of less than 0.05 was considered significant.

RESULTS

PET scanning

Using ^{68}Ga-AMBA, all VCaP and PC-3 tumours were clearly visualised by PET. High uptake was seen in tumour tissue as well as in GRPR-positive pancreas tissue and in organs responsible for clearance (kidneys and bladder) while uptake in background organs was low (Figure 2 row A). When performing PET scans using ^{18}F-CH it was more difficult to distinguish VCaP and PC-3 tumours from the relatively high uptake present in surrounding background organs (Figure 2 row B). In 20% of all ^{18}F-CH scans it was not possible to distinguish the tumour-related contrast from background.

Dynamic tracer uptake in VCaP tumour, bladder and kidney over time is

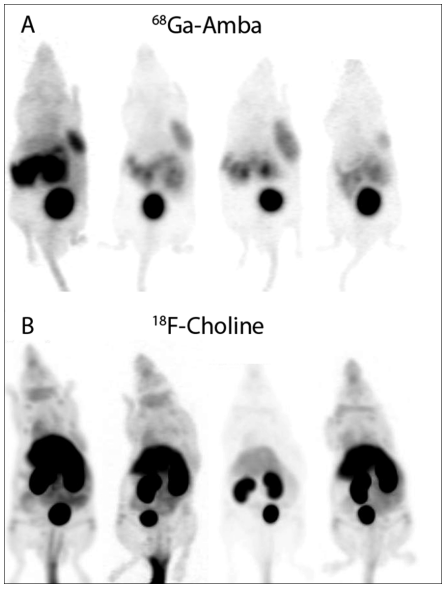


Figure 2
PET scans from four out of eight corresponding VCap bearing mice. Row A shows scans after tail vein injection of ^{68}Ga-AMBA (300 pmol, 1.5-0.5 MBq). Row B shows scans after tail vein injection of ^{18}F-Choline (100 µl, 8.1-1.2 MBq). Using the OSEM3D/MAP algorithm the last 10 frames of each scan were summed up for image reconstruction. Arrows indicate tumour location.

shown in Figure 3. Tumour uptake for both tracers was fast, reaching peak values within 3-5 min. ^{68}Ga-AMBA uptake reached a plateau phase approximately 20 min, with ^{18}F-CH this took less then 10 min (Figure 3A). This plateau phase - occurring at the end of scanning - made us decide to calculate total tumour uptakes derived from PET as the mean of data from the last 10 min of scanning. Renal clearance of ^{68}Ga-AMBA was gradually progressing resulting in accumulation of bladder radioactivity after 10 min pi (Figure 3B). Renal clearance of ^{18}F-CH started earlier resulting in an increase in bladder radioactivity immediately after injection (figure 3C).

The average tumour uptake as calculated from the PET data over the last 10 min of scanning in VCaP-bearing mice was 6.7±1.4 %ID/g (n = 8) using ^{68}Ga-AMBA, while for ^{18}F-CH this was only 1.6±0.5 %ID/g (n = 8). This difference was highly significant ($p < 0.001$). For PC-3 bearing mice average outcomes were 9.2±1.1%ID/g (n = 3) for ^{68}Ga-AMBA and 1.2±0.3%ID/g (n = 3) for ^{18}F-CH.

Biodistribution Studies

Biodistribution results are summarized in Figure 4. Average VCaP tumour uptake for ^{68}Ga-AMBA (n = 4) 60 min p.i was 9.5±4.8 %ID/g. For ^{18}F-CH tumour uptake was 2.1±0.4 %ID/g at 30 min pi (n = 4). These differences in tumour uptake were highly significant ($p < 0.03$).

As was expected using GRPR-based tracers, ^{68}Ga-AMBA uptake was high in the GRPR positive pancreas (57.5±7.1 %ID/g). This was much higher than the pancreas uptake of ^{18}F-CH (3.6±1.0 %ID/g). Also uptake of ^{68}Ga-AMBA in colon (8.5±2.9 %ID/g) was significantly higher as compared to that of ^{18}F-CH.

On the other hand, uptake of ^{18}F-CH was much higher in kidneys (35.7±4.1 %ID/g) as compared to ^{68}Ga-AMBA (2.2±0.2 %ID/g). Like the kidneys, most other (background) organs had significantly higher uptakes for ^{18}F-CH as compared to ^{68}Ga-AMBA: blood (0.8±0.1 vs 0.5±0.1 %ID/g tissue), heart (4.7±0.5 vs 0.2±0.0 %ID/g tissue) and liver (13.0±1.7 vs 0.9±0.5 %ID/g tissue). Only the uptake of choline in muscle was not significantly different from that observed with ^{68}Ga-AMBA (0.9±0.2 vs 0.7±0.2 %ID/g tissue). Both radiolabelled tracers showed low activity levels in blood.

DISCUSSION

Accurate imaging of PC in patients is crucial for decision making as it strongly determines management of the disease. Accuracy of conventional imaging techniques is not sufficient[5-7]. Nuclear scintigraphy provides promising applications for sensitive imaging of PC. In this study we have compared a peptide-receptor-based tracer, the BN-analogue AMBA, with a metabolism-based tracer, the choline derivative ^{18}F-CH, for targeting of PC.

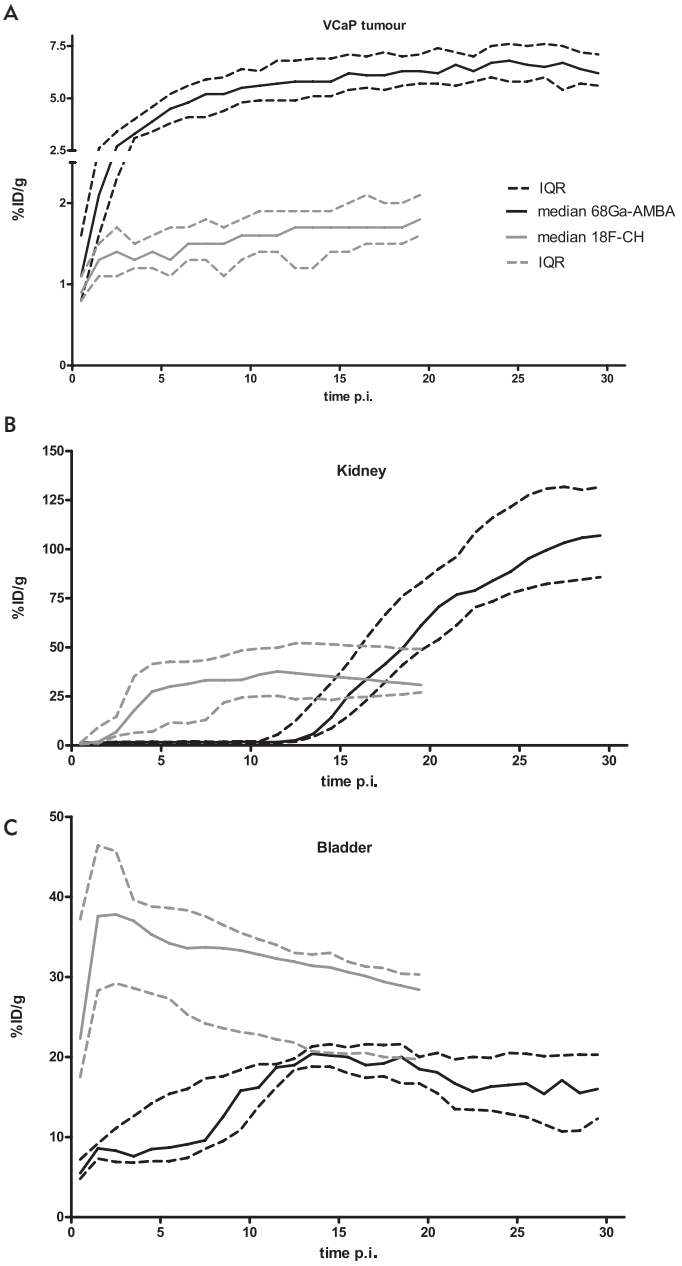


Figure 3

Uptakes in different tissues over time: A. VCaP tumour (Y axis in two segments), B. kidney, C. bladder. Black solid lines show median uptake and black dashed lines IQR after tail vein injection of ^{68}Ga-AMBA (300 pmol, 1.5-0.5 MBq, n = 8). Gray lines show median uptake and IQR after tail vein injection of ^{18}F-CH (100 µl, 8.1-1.2 MBq, n = 8).

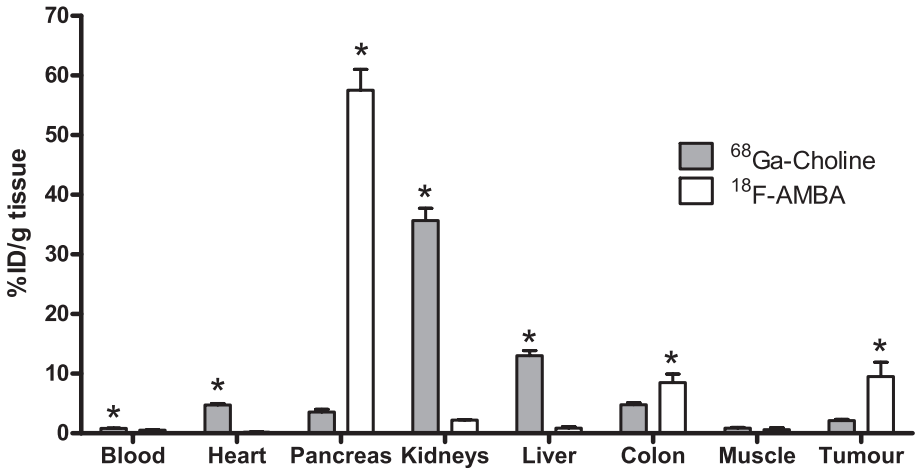


Figure 4

Uptake in preselected organs after tail vein injection of both ^{68}Ga-AMBA (300 pmol, 1.5-0.5 MBq) and ^{18}F-CH (100 µl, 8.1-1.2 MBq) in VCaP bearing mice. Ex vivo biodistribution was performed 60 min after injection of ^{68}Ga-AMBA and 30 min after injection of ^{18}F-CH. Results are indicated as mean±standard deviation of 4 mice per tracer per time point.

Since only few imaging studies have been performed with radiolabelled choline in PC-bearing mice, no consensus has been made about the optimal scanning protocol for it in PET. Zeng et al. performed a PET study using ^{11}C-choline in PC-3 bearing mice starting a 60 min scan immediately after injection[27]. In another group of PC-3 bearing mice Zheng et al. performed biodistribution at 30 min after i.v. injection. Belloli et al. decided to start a 30 min ^{11}C-choline PET acquisition in TRAMP mice immediately after tail vein injection[37]. Ebenhan et al. decided to start ^{18}F-CH acquisition in xenograft-bearing mice, including DU145 prostate xenografts, only at 15 min pi[38]. Although sparse, dynamic data and reconstructions in all three reports implied that tumour uptake of choline is rapid and that choline uptake and ratios to background do not improve when scanning is prolonged. Based on these data and our own pilot experiments (data not shown), we performed PET scans for 20 min starting immediately after injection of ^{18}F-CH. For biodistribution purposes, mice were sacrificed at 30 min pi, in accordance with the time point used for biodistribution by Zheng et al.[16].

Indeed there has been a change of paradigm in the field of bombesin radiopharmaceuticals from agonists towards antagonists as possibly preferable for tumour targeting. In fact in a previous study from our group - comparing prostate cancer targeting efficacy of five radiolabelled bombesin analogues – the BN antagonist Demobesin-1 was the best performing analogue. Nonetheless

in this study also AMBA showed to be an analogue with good, roughly comparable, performance.

PET imaging requires analogues labeled with positron-emitting radionuclides. AMBA is coupled to a DOTA chelator which enables labelling with the positron-emitting radionuclide ^{68}Ga. Demobesin-1 has a N_4 chelator which does not allow labelling with radionuclides suitable for PET. ^{68}Ga-AMBA was therefore selected in our study.

Despite a recent change of paradigm in the field of bombesin radiopharmaceuticals from agonists towards antagonists as possibly preferable for tumour targeting, we made use of the agonistic BN analogue AMBA in this study[2]. Indeed in a previous study from our group - comparing prostate cancer targeting efficacy of four agonistic and one antagonistic radiolabelled bombesin analogues – antagonist Demobesin-1 performed best[11]. With its N4 chelator it does not allow for labeling with positron-emitting radionuclides required for PET. The DOTA chelator of AMBA does enable labelling with PET radionuclides like ^{68}Ga. It has shown to be a BN analogue with high potential for imaging of Pc, a roughly comparable performance to Demobesin-1 and good targeting performance in one of the few clinical BN studies[10-11]. For future PET studies it would be interesting to use a BN antagonist with a chelator suitable for a positron-emitting radionuclide. They have shown to washout from the pancreas more rapidly than agonists and seem to have a higher uptake and retention in PC[2]. Another advantage of antagonist is their lower expected toxicity as compared to the pharmacologically active agonists.

For ^{68}Ga-AMBA a slightly different protocol than for ^{18}F-CH was followed. The biological processes targeted by the two tracers are quite different and therefore require different protocol details. Although AMBA can be labelled with positron emitting radionuclides it has not been published in (animal) PET studies nor has it been published labelled with ^{68}Ga yet. Therefore, apart from our own experience with ^{68}Ga-AMBA, the only available information to base an optimal PET protocol on came from studies using AMBA labelled with Lutetium-177 (^{177}Lu) or Indium-111 (^{111}In). In a biodistribution study using ^{177}Lu-DOTA-AMBA in PC-3-bearing mice, animals were sacrificed at time points 1h and 24h pi[10]. Absolute uptake was almost twice as high at the 1h time point and overall tumour to background ratio was also favourable. Another study performing biodistribution in PC bearing mice using ^{111}In-DOTA-AMBA chose to sacrifice mice at 1h pi only, showing sufficient tumour uptake[39]. In our standardised comparative study between different BN-analogues ^{111}In-AMBA also showed high tumour uptake and promising tumour-to-background ratios at 1h pi[11]. Based on these data, and to be able to compare our data with those from literature, we decided to perform biodistribution 1h pi of ^{68}Ga-AMBA. To get insight in the process of biodistribution with ^{68}Ga-AMBA we decided to start imaging immediately after injection.

Comparison of tumour uptake in VCaP and PC-3-bearing xenografts revealed that peptide-receptor targeting was superior to metabolic targeting for both tumour types. This may be explained by the fact that GRP receptor expression in early PC is generally high[8], while metabolic activity is predominantly low[14-15]. Although choline uptake has been reported to increase with PC aggressiveness and rising PSA levels[5], targeted tumour imaging using AMBA performed better in both model systems.

Besides the favourably higher tumour uptake of AMBA another important aspect that selected AMBA against choline was its lower uptake in most non-targeted (GRPR-negative) organs. This is particularly visible when comparing PET images of the two types of tracers (Figure 2). The high background signal found with choline relates to the relative high metabolic activity in these organs which originates from general cellular processes not specifically related to cancer. Clearly, although is does show high background activity in GRPR-expressing tissues peptide-receptor targeting is more tissue-specific than metabolism based targeting.

In order to make use of choline feasible for a large number of clinical centres that do not have a cyclotron, derivatives with a longer-lived radionuclide than ^{11}C, like ^{18}F-CH, were introduced by DeGrado et al.[28]. Experiments with this derivative have shown to approach natural choline in the rate of phosphorylation by yeast choline kinase, as well as the rate of uptake by cancer cells (PC-3). ^{18}F-CH can therefore be considered as a prototypical choline tracer. The renal uptake and excretion of this derivative is, however, known to be higher compared to natural choline. High activity in the bladder ensuing from this excretion could cause diagnostic limitations for the prostatic region[40, 25]. Zheng et al. describe kidney uptake of 5.1 ± 1.8 %ID/g in PC-3 bearing athymic mice 30 min after injecting ^{11}C-choline[27] while in our study using ^{18}F-CH this uptake was 35.7 ± 4.11 %ID/g with comparable protocols. More importantly in our PET scans activity in the bladder was also very high. The use of ^{11}C-choline instead of ^{18}F-CH may probably reduce the undesirable high uptake in kidneys and bladder to a great extent while maintaining a tumour uptake comparable with that of ^{18}F-CH.

Dedicated PET for imaging of small animals is a very useful application in the preclinical research of nuclear medicine. Besides its use to establish and validate novel tools for detection and visualization of tumours, PET data are also being used for in vivo quantification. Quantification with PET allows biodistribution processes to be followed dynamically without using separate groups of laboratory animals for each time point. Quantification of our own in vivo PET data was reliable and could be supported by ex vivo biodistribution results. The graphs showing tumour, kidneys and bladder uptake over time are good examples showing the benefits of PET. Since spatial resolution of PET remains inferior to that of other imaging techniques like CT, MRI and ultrasound, dual-modality scanners

like PET/CT have been developed to maximise imaging accuracy[42, 13].

Time activity curves derived from PET data showed that both imaging protocols used for scanning of AMBA and choline were well chosen. Especially for ^{18}F-CH immediate imaging after injection was required, since tumour uptake was fast. Tumour uptake reached a plateau at the endpoint of both scanning protocols - which was 20 min pi for ^{18}F-CH and 30 min pi for ^{68}Ga-AMBA - indicating that the dynamic process of tumour uptake did not require extended imaging.

In conclusion: targeted nuclear imaging of tumours based on receptor-specific radiolabelled analogues offers good applications for clinical diagnosis of (early) PC. PET scanning and biodistribution studies indicated that tumour uptake of ^{68}Ga–AMBA was higher while overall background activity was lower than observed for ^{18}F-CH in the same PC-bearing mice. These results suggest that peptide-receptor mediated PET using BN analogues is superior to metabolism-based PET using choline for scintigraphy of PC. The present study warrants further clinical evaluation of GRPR-targeted nuclear imaging of PC using BN-analogues.

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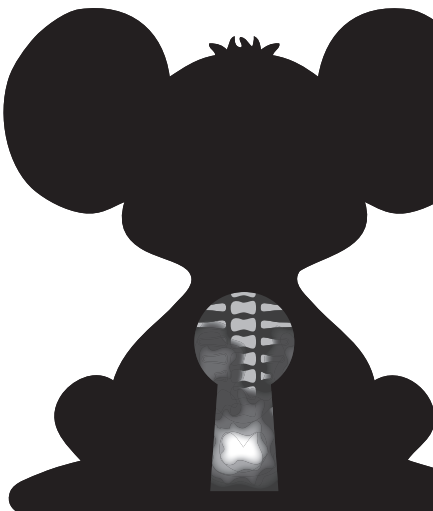
SUMMARY AND DISCUSSION



Prostate cancer (PC) is the third leading cause of cancer-related deaths and the most frequently diagnosed cancer in men in Western countries [1, 2]. Nowadays, suspicion on the presence of relevant disease mostly ensues from elevated blood levels of prostate specific antigen (PSA) [3-5]. Ultimately, PC is diagnosed by histopathology of transrectal ultrasound-guided prostate biopsies.

An important challenge in PC management is to differentiate patients that need curative treatment from those that do not need intervention. Clinical T-stage, that is determination of the extent of PC prior to treatment, is a crucial determinant for risk stratification and management of the disease. Unfortunately, conventional imaging techniques are not accurate for visualisation of locally advanced PC at time of diagnosis [6-9] and new imaging approaches are urgently needed to improve staging. Sensitive imaging options would also be welcomed for localisation of PC recurrence after initial therapy with curative intent.

Nuclear medicine provides a new PC-targeted imaging application by employing radiolabelled bombesin (BN) or Gastrin-Releasing Peptide (GRP) analogues to target the Gastrin-Releasing Peptide Receptor (GRPR), a membrane-receptor overexpressed on PC cells [10, 11]. BN-based radiopeptide analogues have shown high affinity to the GRPR, leading to excellent tumour-specific binding [12], and they can be visualised by imaging modalities such as single photon emission tomography (SPECT) and positron emission tomography (PET). Moreover BN analogues may also be applied for peptide receptor radionuclide therapy (PRRT) with the intention of treating focal PC or (occult) PC metastases.

In this thesis we describe preclinical investigations to explore and optimise the potential of BN-GRPR-mediated targeting of human PC. We analysed GRPR expression in various stages of clinical PC, and studied hormonal regulation of GRPR using experimental xenograft and cell line models of human PC. Also, we compared various BN analogues for their efficacy to visualise PC in mice. Furthermore, in order to optimise BN-targeting conditions, we carefully analysed the influence of experimental conditions on biodistribution of BN peptides. Metabolism-based imaging has been extensively studied for imaging of PC, while the potential of peptide-receptor-based imaging using BN is not well-studied yet. We evaluated a BN analogue against a metabolism-based imaging tracer using PC xenograft-bearing nude mice.

Chapter 1 of this thesis provides an introduction into the field of PC, its disease progression, management and the clinical issues that are related to that. It enunciates the need for more accurate PC imaging and introduces the promising and GRPR-specific BN peptide. The review article in **chapter 2** provides more extensive insight on the GRPR as a target for imaging and radionuclide therapy of PC using radiolabelled BN analogues. Besides a general introduction on BN and BN analogues, it summarises the preclinical and clini-

cal literature on BN analogues for PC targeting and imaging by SPECT and PET.

From a survey of the literature, it seems that consistency in study protocols that would allow for a valid comparison between radiolabelled BN analogues is lacking as different research groups have used different animal models, labelling techniques, radionuclides and evaluation protocols. In order to select the best analogue for clinical validation, we initiated a preclinical study to compare five radiolabelled analogues under identical experimental conditions using one standardised protocol. This study is described in **chapter 3** and shows that the receptor antagonist Demobesin-1 was the best performing BN analogue with superior in vivo stability, highest tumour-uptake and retention of radioactivity in the tumour, while pancreatic and renal clearance were fast. With sufficient in vivo stabilities as well as high uptake and retention in tumour, the Pesin and AMBA peptides were shown to be good performing GRPR-agonists. Receptor agonists have been reported to activate the receptor upon internalisation, stimulate cell growth [10, 13, 14] and induce side effects in other GRPR expressing organs such as pancreas and colon. Therefore the use of non-bioactive receptor antagonists is strongly preferable [15-19]. Moreover, in contrast to Pesin and AMBA [20, 21], the receptor antagonist Demobesin-1 is highly selective for GRPR and has no affinity to other BN receptor subtypes [22].

Since the majority of patients with disseminated PC will be offered hormonal therapy as first line therapy, it is essential to identify the impact of hormonal ablation treatment on GRPR expression in order to establish the appropriate position of GRPR-targeted imaging and therapy in the management of PC and the potential requirement for selection of patients eligible for GRPR-targeted modalities. **Chapter 4 and 5** both concentrate on this issue. Growth of prostate epithelial (tumour) cells depends on a functional androgen axis with physiological levels of androgens that stimulate the androgen receptor (AR) and drives (tumour) cell growth [23]. In the case of disseminated disease, the first and so far most effective choice of treatment is endocrine deprivation therapy aiming at reducing circulating testosterone levels and/or blocking the AR [24, 25]. Eventually, however, disease relapse occurs due to tumour progression towards so-called castration resistant PC (CRPC) in the majority of patients leaving limited palliative treatment options only [26, 27].

Previous studies from our group showed down-regulation of GRPR expression after androgen withdrawal in experimental xenograft models bearing PC tumours that represent early stage disease [28]. Low GRPR expression in CRPC was also reported in literature [11]. In **chapter 4** we explored androgen regulation of GRPR expression in three in vivo early stage human PC xenograft models (PC82, PC295 and PC310) in more detail. This study revealed expression of GRPR in these androgen-responsive xenografts to be significantly reduced by androgen ablation and to be reversed after restoring the hormon-

al status of the animals. These data suggested that hormonally ablated patients might not be eligible for BN-GRPR based imaging and therapy.

The vast majority of studies on GRPR-targeting of PC are based on the GRPR overexpressing, but androgen-unresponsive, AR-negative PC3 cell line. In order to unravel in more detail the effect of hormonal manipulation and AR status on GRPR expression in PC, we performed in vitro and in vivo studies as described in **chapter 5** using the GRPR-overexpressing, androgen-responsive and AR-positive human VCaP cell line derived from a human PC metastasis. Our data showed that GRPR-specific uptake was similar between the VCaP and PC-3 cell line. In contrast to our earlier observations in other experimental models described in chapter 4, however, GRPR mRNA expression as well as BN-specific binding of VCaP cells was not affected significantly by androgen manipulations in vitro and in vivo suggesting that GRPR expression was not androgen regulated in VCaP cells. One explanation for these contradictory results may be that, in contrast to the human PC xenograft models representing early androgen responsive disease with 'normal' upregulated GRPR expression, VCaP and PC3, representing a more advanced stage of PC, have a constitutively activated GRPR expression. This expression has escaped 'normal' androgen regulation control mechanisms, while in the case of VCaP, growth control is still (partially) retained by androgens.

In **chapter 5** we also assessed relative mRNA expression levels of the GRPR and GRPR-specific binding in human tissue samples derived from patient material. The Erasmus MC tissue bank contains PC from patients from various clinical stages. Human PC mRNA samples were predominantly GRPR positive, but the expression was variable. Only CRPC samples showed a tendency towards lower GRPR mRNA expression levels. Although the sample numbers in this group were low, this observation suggests that GRPR seems (at least initially) androgen regulated. Overall, higher median GRPR-specific uptake was observed in PC compared to benign tissue, but with a considerable overlap between the lower GRPR mRNA expression levels of PC and benign tissue samples. This outcome was supported by the original data from the influential study by Markwalder et al. [11]. A modified scatter plot based on these data showed a similar overlap in GRPR expression between PC and benign tissue samples. In conclusion, these data suggest that BN-based imaging of PC is especially indicated for imaging of early PC; in men that have not received hormonal therapy. Furthermore, our data indicate that selection of GRPR expressing patients may be required when opting for GRPR-targeted BN-based response monitoring and therapy of PC.

In **chapter 6,** the impact of various experimental conditions on the pharmacokinetics of BN-specific binding in nude mice bearing human PC xenografts is described with the aim to improve receptor-mediated targeting.

From this study it became clear that not only receptor affinity of BN analogues determines binding to GRPR in vivo, but that also experimental variations, including HPLC peptide purification of the BN analogue, variations in specific activity and saturation of the GRPR (by predosing with cold BN prior to administration of radiolabelled BN), significantly affect peptide-receptor binding in vivo. These findings underscore the need for standardised protocols for optimal targeting.

As metabolic activity in cancer cells is often increased, the use of metabolism-based targeting is being studied as a strategy for visualisation of cancer cells, including PC. In **chapter 7** we describe a comparative experimental animal PET-study between GRPR-mediated targeting using ^{68}Ga-AMBA and metabolism-based targeting by Flourine-18 labelled choline (^{18}F-CH) in VCaP tumour-bearing nude mice. VCaP tumour uptake of ^{68}Ga-AMBA was shown to be significantly higher than uptake of ^{18}F-CH while overall background activity was lower. In this study, peptide-receptor imaging was clearly superior to metabolism-based imaging of PC using PET.

WHAT HAS BEEN ACCOMPLISHED BY THE STUDIES IN THIS THESIS?

This thesis describes preclinical work to determine the potential options of BN-GRPR-based targeted imaging and therapy in the management of PC. The critical examination of various protocols that are being used in preclinical evaluation of peptide-receptor targeted imaging emphasises the importance of protocol standardisation to allow for valid selection of the most-promising peptides to be tested in a clinical setting. To our knowledge we were the first to perform such a standardised experiment allowing for a valid comparison of five BN analogues designed by and obtained from different research groups. In accordance with literature [29-31] we found the GRPR antagonist to be superior over the agonists tested; for our future clinical studies we will therefore focus on the use of GRPR antagonists. Also, original work was performed to elucidate the role of androgens in expression of the GRPR. It provided more insight in GRPR expression in human (PC) tissues at different stages of disease. Finally, as there is no gold standard for imaging of PC, we used metabolism based imaging to set against GRPR-based imaging in a preclinical mouse study in order to determine the potential value of this new imaging approach. The potential of GRPR based imaging was underscored by a more than 4-times higher PC uptake and a generally lower uptake in background organs in comparison to metabolism-based imaging.

The data presented in this thesis raised the interest in and were the basis for further clinical evaluation of a GRPR antagonist in PC detection. Since the developments in BN analogues are fast, the best performing peptide Demobesin-1 will not be the analogue of choice in the future clinical studies. It will

be replaced by a 'second generation' GRPR antagonist that is also useful for PET scanning and that was developed in a parallel research project.

FUTURE DIRECTIONS

Development of new BN analogues

The field of radiolabelled analogues for receptor-based targeting is rapidly evolving. New peptide analogues are constantly being designed in order to improve targeting characteristics such as specificity, sensitivity and in vivo stability. BN analogues ideally have a high affinity for the GRPR and have an in vivo stability that is high enough to allow for proper accumulation in the target (tumour) tissue. Besides this, the radiolabelled analogue should be cleared fast enough to prevent radiotoxicity when considering radionuclide therapy. The development of radiolabelled analogues is focused not only on amino acid sequence for ligand binding, it also studies development of appropriate chelators, the chemical structure attached to a biotracer that facilitates specific radiolabelling. The choice of the chelator determines to which radionuclide(s) the BN analogue can be complexed and herewith defines the potential application of the radiolabelled analogue. Moreover, the molecular charge of the BN analogue is an important characteristic as it has been reported to affect biodistribution. Introduction of a single negative charge to BN analogues has been shown to improve biodistribution by increasing tumour uptake and reducing uptake in background tissues [32]. In contrast to this Marsouvanidis et al. showed that an additional positive charge, by adding a single N-terminal lysine-residue to the peptide chain of a GRPR agonist, significantly improved GRPR-specific uptake in PC xenografts [33].

For long it has been assumed that receptor agonists are superior to receptor antagonist for peptide receptor based targeting [34], because of their efficient endocytosis-regulated [35] internalisation and subsequent higher in vivo accumulation and retention in target tissues. Recent findings including ours, however, indicate that peptide receptor antagonists with comparable affinities to the respective receptor as their agonistic counterparts, exhibit higher tumour uptake and at the same time lower retention of radioactivity in normal tissues, leading to superior target-to-background ratios [29-31]. It has been suggested that the numbers of binding sites for receptor agonists and antagonists differ, which may be caused by the putative existence of different types of receptors they can bind to [36]. Although the underlying mechanism for this differential effect is not yet fully understood, this finding paved the way for intensified development of new GRPR antagonists. From a pharmacological point of view the use of non-bioactive receptor antagonists is strongly preferable as it is expected to cause less side effects and to prevent non-desirable stimulation of tumour growth [15-19] known from GRPR agonists [10, 13, 14]. This will be particularly beneficial when using high therapeutic doses of peptide.

Standardised comparison of BN analogues

From current literature it is difficult to compare the potency of different BN analogues as study protocols vary considerably. We have shown that minor differences in study protocols may already have considerable effect on radiopeptide pharmacokinetics and strongly impair a proper and reliable comparison between studies. Examples of factors that often vary between studies and which may affect study outcome include mouse strain (species, sex, weight), tumour cell type (cell line, culture conditions, passage number) and xenograft tumour characteristics such as tumour size, inoculation site and tumour vascularisation.

Nuclear imaging modalities (SPECT and PET)

The current developments in scanning modalities resulting in increased spatial resolution, sensitivity and accuracy, are crucial. In order to improve the ability to discriminate target from background and to improve localisation and dosimetry issues, the field of nuclear scintigraphy for functional or metabolic imaging is dominated by multimodal SPECT and PET in combination with CT. Currently, new PET/MRI systems are entering the clinic, opening a whole new field of high resolution, high sensititvity imaging options.

Specificity of GRPR overexpression

When considering a receptor as candidate for tumour targeting it should ideally be highly expressed in the diseased tissue only. High expression in non-targeted tissue will cause non-specific radiopeptide binding resulting in high background signal during scanning and in toxicity to healthy tissue when radionuclide therapy is concerned. Both in the literature and in this thesis (chapter 5), PC has been shown to overexpress the GRPR while expression in benign prostatic tissue is low [11]. As expected, high uptake of BN analogues in the GRPR-expressing pancreas has been described in two patient studies [37, 38]. In case of PC staging, high pancreatic uptake might cause interference with uptake in the often affected para-aortic lymph nodes [39, 40]. For allowance of GRPR-targeted BN-based therapy of (local) PC, careful dosimetry studies are required in order to prevent toxicity due to pancreatic uptake of high amounts of BN-peptide. Clinical studies are required to optimise tumour-to-background ratios for the different radiolabelled BN analogues and explore whether these are acceptable for implementation for PC imaging and therapy.

Androgen regulation of GRPR

Hormonal deprivation therapy is standard treatment for disseminated PC. Understanding the role of androgens in the regulation of GRPR expression is essential for defining the potential use of BN-based imaging and therapy at different stages of disease. PC xe-

nograft models representing early, androgen-responsive disease showed androgen-regulated GRPR expression. On the other hand GRPR of VCaP and PC3 cell lines, reflecting a more progressed disease stage, lacked androgen regulation (chapters 4 and 5). We hypothesise that more advanced stage of PC, such as represented by the PC3 and VCaP cell lines, have a constitutively activated GRPR expression that has escaped 'normal' androgen regulation control mechanisms. In human samples the majority of (hormonally untreated) PC was GRPR-positive (see chapter 5). Relatively low GRPR mRNA expression levels were only observed in CRPC suggesting that hormonal therapy generally results in down-regulation of GRPR expression. The above described escape phenomenon suggested for VCaP cells, may thus be a rather uncommon clinical event. GRPR expression in human PC samples as deduced from BN binding data showed considerable overlap with those of benign tissue samples negatively affecting BN specificity. Overall, data suggest that BN-based GRPR-targeted imaging and therapy may be offered to hormone naive PC patients and may be applicable also in (a subset of) hormonally treated CRPC patients with constitutively high GRPR.

PC is a heterogeneous and multi-stage disease [41]. Also GRPR expression is variable between PC patients. In order to identify patients who will likely benefit from GRPR-targeted disease monitoring and/or therapeutic intervention, assessment of the individual GRPR status is essential to select patients for GRPR-targeted imaging and/or therapy of PC. Determination of GRPR expression at an individual basis may be performed on PC biopsies taken for diagnosis, on the premises that a good performing GRPR antibody is available or that frozen sections are available for autoradiogaphy. Alternatively, assessment of individual GRPR levels may be obtained by pre-scanning with a BN radio-analogue. The opportunity to have a tumour-targeted analogue for imaging with the option to use it as a therapeutic entity when labelled to a radiotoxic radionuclide (the theranostics approach [42]) offers personalised medicine tailored to individual patients improving disease management.

IN CONCLUSION

Novel biotracers for imaging and therapy of PC are required as currently PC staging is inaccurate and no curative treatment for disseminated PC exists. Molecular imaging using radiolabelled analogues for PET and SPECT enables non-invasive in vivo detection of targets at low nanomolar to picomolar concentrations [43]. Radiolabelled BN analogues have shown to be promising tools for imaging of PC since GRPR expression is predominantly high in PC. Furthermore, receptor antagonists appeared to be superior to agonists in our preclinical studies. This thesis provides the required knowledge to start a clinical study in early PC patients to determine the potential of GRPR based imaging (and therapy) of PC.

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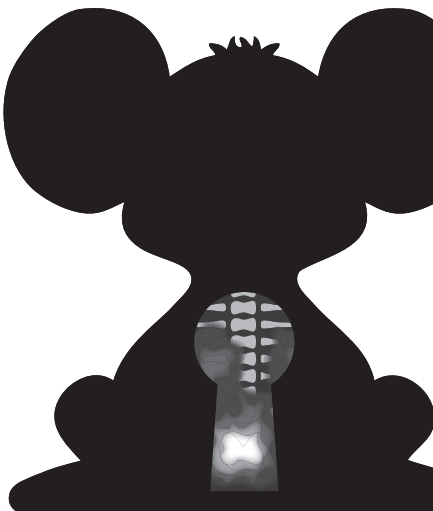
SAMENVATTING



Stadiëren van prostaatkanker (PC) en lokaliseren van tumor-recidief blijft een onopgelost klinisch probleem vanwege het gemis aan accurate beeldvorming. Uitgezaaide PC kent geen gerichte therapie en is tot op heden niet curatief te behandelen. De nucleaire geneeskunde biedt veelbelovende mogelijkheden om dit gemis aan accurate beeldvorming en tumor-selectieve therapie van PC te ondervangen door gebruik te maken van doelgerichte radioactief gemaakt peptiden. Een van deze peptiden is bombesine (BN). Het bindt met hoge affiniteit aan de Gastrin-Releasing Peptide Receptor (GRPR), een membraanreceptor, welke tot overexpressie komt op PC cellen.

SAMENVATTING

Gezien de hoge incidentie en het aanzienlijk aantal sterftegevallen ten gevolge van PC vormt de aandoening er een groot probleem voor de volksgezondheid [1, 2]. Verdenking op de ziekte volgt tegenwoordig meestal het na het vinden van een verhoogde waarde van het prostaat specifiek antigen (PSA) in het bloed [3-5]. De diagnose PC kan alleen worden gesteld op basis van histopathologisch onderzoek, meestal verkregen door middel van echogeleide transrectale biopsieën van de prostaat. Het ziektebeloop van PC is vaak indolent [6,7] en kan prognostisch zo gunstig zijn, dat behandeling achterwege kan blijven onder voorwaarde van een nauwgezette controle ('active surveillance') [8, 9]. Indien echter de kans op uitbreiding groot is, kan gelokaliseerde PC curatief worden behandeld door chirurgisch of radiotherapeutisch ingrijpen [10-18]. Patiënten met uitgezaaide PC zijn tot op heden niet te genezen en kunnen enkel palliatief behandeld worden.

Voor het opstellen van een behandelplan is het van cruciaal belang om zowel agressiviteit als het stadium van PC te kunnen bepalen. Agressiviteit van PC wordt bepaald door de histopathologische Gleason score. Een gradering tussen 0-5 wordt toegekend aan de twee meest voorkomende groeipatronen in prostaatbiopsieën. De optelsom van beide geeft de Gleason Score, waarbij lagere scores beter gedifferentieerde tumoren representeren [19]. Stadiëren van PC ofwel onderscheid maken tussen lokaal, lokaal uitgebreid en uitgezaaide ziekte is tot op heden een onopgelost probleem gebleken. Huidige c*onventionele beeldvormende technieken, zoals echo, C*omputerised Tomography (CT), Magnetic Resonance Imaging (MRI) en bot-scintigrafie, hebben beperkte waarde *vanwege hun matige accuratesse* [20-23].

Het gelokaliseerd geacht prostaatcarcinoom wordt ingedeeld in drie risicogroepen (laag, matig en hoog) aan de hand van de EAU/ESTRO indeling [24]. Deze indeling maakt gebruik van de 3 tumorparameters om de kans op uitbreiding van ziekte buiten de prostaat te voorspellen, te weten, initieel PSA (iPSA), de Gleason score en het T-stadium. Het iPSA is de PSA bloedwaarde bepaald vóór enige vorm van behandeling [3-5]. Het T-stadium is een benadering van uitgebreidheid van PC voor radicale chirurgie. Deze zogenaamde

klinische stadiëring wordt bepaald op basis van het rectaal toucher van de prostaat (digital rectal eximanation; DRE), PSA laboratoriumwaarden, bevindingen bij transrectale echografie (transrectal ultrasound: TRUS) en resultaten van PC gezien in gereseceerd prostaatweefsel (na transuretrale resectie; TUR). De Nederlandse richtlijn prostaatcarcinoom raadt aan om nomogrammen, welke gebruik maken van dezelfde parameters, toe te passen na in opzet curatief ingrijpen om een individuele uitspraak te kunnen doen over de kans op tumor-metastasering [25]. Met het Kattan of Partin nomogram wordt een accuratesse tot 79% bereikt [26, 27].

Nucleaire geneeskunde maakt gebruik van radioactieve biomarkers welke zich bij voorkeur in een bepaald orgaan of afwijking ophopen. Na injecteren van een radiofarmacon vindt dit zijn specifieke doel. Met een detector kan vervolgens in beeld gebracht worden waar de toegediende radioactiviteit zich concentreert. Naast planaire scintigrafie wordt meer en meer gebruik gemaakt van 3-dimensionale tomografie met behulp van "single photon emissie"-computertomografie (SPECT) en positron emissie tomografie (PET) [28, 29]. Een varieteit aan radiofarmaca gericht op PC wordt ontwikkeld en onderzocht. Het radioactief gemaakte BN ofwel Gastrin-Releasing Peptide (GRP) is een veelbelovend nieuw peptide vanwege de hoge affiniteit voor de membraanreceptor GRPR, welke tot overexpressie komt op PC cellen [30, 31]. In de literatuur zijn met BN uitstekende PC-specifieke opnames beschreven [32]. BN-GRPR gerichte radiofarmaca zouden van toegevoegde waarde kunnen zijn voor het stadiëren van PC. Ook kunnen ze mogelijk ingezet worden om PC recidieven te lokaliseren. Wellicht zou het zelfs mogelijk zijn om radioactieve BN analogen te gebruiken voor therapie (peptide receptor radionuclide therapy; PRRT) in de behandeling van locaal uitgebreid en (occult) gemetastaseerd PC.

In dit proefschrift worden pre-klinische onderzoeken beschreven, die gericht zijn op onderzoeken naar het potentieel en de optimalisatie van BN-GRPR gemedieerde beeldvorming en therapie van PC. Het expressie niveau van de GRPR in verschillende stadia van PC en onder hormonale manipulatie werd onderzocht in PC-dragende muizen, PC-cellijnen en patiëntenspecimen. Ook vergeleken we, onder gestandaardiseerde omstandigheden, verschillende BN analoga naar hun effectiviteit inzake beeldvorming van PC in muizen. Ten einde specifieke opname te optimaliseren onderzochten we verder de invloed van experimentele omstandigheden op BN-GRPR gerelateerde biodistributie. Metabolisme gerelateerde radiofarmaca zijn uitgebreid bestudeerd en veelbelovend gebleken als nucleaire toepassing voor de beeldvorming van PC [32]. PC scans met BN analoga, welke gebaseerd zijn op peptide-receptor binding, is een minder uitgekristaliseerde en bekende nucleaire toepassing voor beeldvorming van PC. In PC-dragende muizen vergeleken we het metabolisme gerelateerde radiofarmacon choline met het GRPR gerichte BN analoog AMBA.

De introductie van dit proefschrift, **hoofdstuk 1**, richt zich vooral op PC en de klinische vraagstukken, die met deze ziekte samenhangen. Het benadrukt het gemis van accurate beeldvorming voor PC en introduceert de GRPR-specifieke BN peptiden. Het review artikel in **hoofdstuk 2** gaat uitgebreid in op de toepassing van GRPR-gerichte beeldvorming en therapie van PC met behulp van radioactief BN. Naast een algemene beschrijving wordt een literatuuroverzicht gegeven van het preklinisch en klinisch onderzoek naar de potentie van BN in de beeldvorming van PC met SPECT en PET.

In onderzoeken naar radioactieve BN-analoga ontbreekt het in de literatuur aan gestandaardiseerde studie-protocollen. Studiegroepen maken gebruik van verschillende diermodellen, labelling technieken, radionucliden en experimenten. Het is derhalve onmogelijk om analoga uit verschillende studies op betrouwbare wijze met elkaar te vergelijken. Ten einde een selectie te maken van BN-analoga voor gebruik in klinische validatie-studies, hebben we 5 radioactief gelabelde BN-analoga met elkaar vergeleken onder identieke experimentele condities met een gestandaardiseerd protocol. Deze vergelijkende studie staat beschreven in **hoofdstuk 3** en toonde aan, dat de receptor antagonist Demobesin-1 het best presterende BN-analoog is vanwege een superieure tumor-opname en in vivo stabiliteit in PC. Ook bleek de retentie van radioactiviteit in PC bij gebruik van Demobesin-1 hoger dan bij de overige peptiden, terwijl de klaring uit de achtergrond-organen (met name pancreas en nier) het snelst verliep. Met een behoorlijke in vivo stabiliteit alsmede een goede opname en retentie in PC, toonden de analogen Pesin en AMBA zich de beste GRPR-agonisten. Receptor agonisten staan bekend om het stimuleren van groei van PC [30, 33, 34]. Vanwege binding en daaraan gerelateerde activering van GRPR positieve organen (pancreas en colon), kunnen receptor antagonisten voor bijwerkingen zorgen. Om dit te voorkomen is het gebruik van niet biologisch actieve receptor antagonisten sterk te prefereren [35-39]. Bovendien is de receptor antagonist Demobesin-1, in tegenstelling tot Pesin en AMBA [40, 41], hoog selectief voor de GRPR en heeft het dus geen affiniteit met andere BN receptor subtypen [42].

Groei van PC is afhankelijk van androgenen [43]. Fysiologische testosteron niveaus stimuleren de androgeenreceptor (AR) en zetten tumorcellen aan tot groei. De eerste therapie bij uitgezaaide PC is hormonale behandeling, welke als doel heeft om circulerende testosteronspiegels te reduceren of de androgeenreceptor (AR) te blokkeren. Na verloop van tijd gaat PC echter onafhankelijk van hormonen groeien. Op dit moment van progressie, wordt gesproken van een hormoon resistent of hormoon refractair prostaat carcinoom (ofwel castration resistant PC; CRPC) [44, 45]. Voor patiënten met CRPC resten enkel nog beperkte palliatieve behandelopties [44, 45]. Vanwege het feit, dat de meerderheid van patiënten met uitgezaaide PC hormonale therapie zal worden aangeboden, is het essentieel om het effect van hormoontherapie op

expressie van de GRPR te onderzoeken. Dit is met name nodig om de juiste plaats van GRPR-gerichte beeldvorming en therapie in het behandeltraject van PC te bepalen. Verder kan het van belang zijn voor de selectie van patiënten, die mogelijk baat zouden kunnen hebben bij BN-GRPR-gemedieerde modaliteiten. **Hoofdstuk 4 en 5** richten zich beiden op dit vraagstuk.

Eerdere studies van onze studiegroep toonden aan dat GRPR expressie in PC tumoren in een vroeg stadium, lager wordt na het onttrekken van androgenen aan PC-dragende muismodellen [46]. In de literatuur is tevens beschreven dat GRPR expressie laag is in CRPC [31]. In **hoofdstuk 4** onderzochten we, in meer detail, het effect van androgenen op GRPR expressie in drie humane PC dragende muismodellen in vivo (PC82, PC295 and PC310). De studie toonde aan, dat expressie van de GRPR in de geselecteerde androgeen gevoelige xenotransplantaties significant gereduceerd werd door het onttrekken androgenen. Tevens liet de studie zien dat reïntroductie van androgenen resulteert in hernieuwde toename van GRPR expressie tot aan het oorspronkelijke niveau. Resultaten suggereren dat patiënten onder hormoontherapie niet geschikt zijn voor BN-GRPR gemedieerde beeldvomring of therapie.

In de meeste PC studies naar BN-GRPR gerichte modaliteiten wordt gebruik gemaakt van de, androgeen-onafhankelijke en AR negatieve, PC3 cellijn. Een cellijn, die duidelijk niet geschikt is voor onderzoek naar de invloed van androgenen op de regulering van GRPR expressie. Om het effect van hormonale manipulatie en de AR status op de expressie van GRPR in PC verder te exploreren, maakte wij in **hoofdstuk 5** gebruik van de androgeen-afhankelijke humane cellijn VCaP. Een cellijn, verkregen uit een humane PC metastase, met hoge expressie van GRPR, welke bruikbaar is in zowel in vivo als in vitro studies. Resultaten lieten zien dat GRPR-specifieke opname van VCaP en PC-3 overeen kwamen. In tegenstelling tot onze eerdere observaties beschreven in hoofdstuk 4, bleek GRPR expressie in de VCaP cellijn niet te worden beïnvloed door hormonale manipulatie zowel in vivo als in vitro. Androgeen regulatie van de GRPR kon derhalve niet worden vastgesteld over de gehele linie van androgeen-afhankelijke PC cellijnen. Een verklaring voor de tegenstrijdige bevindingen uit hoofdstuk 4 en 5 kan zijn dat, in tegenstelling tot de xenotransplantaties, die representatief zijn voor vroege stadia van androgeen-gevoelige PC, de meer geavanceerde VCaP en PC3 cellijnen een constitutieve expressie van GRPR hebben. Ondanks een (deels) behouden androgeen-gereguleerde groei, lijkt GRPR expressie in VCaP ontsnapt te zijn aan de “normale” mechanismen van androgeen regulering.

In **hoofdstuk 5** hebben we ons tevens bezig gehouden met de relatieve mRNA expressie niveaus van de GRPR alsmede GRPR-specifieke binding in humane weefsels verkregen uit de weefselbank van het Erasmus MC. Deze weefselbank bevat PC materiaal van patiënten uit verschillende klinische stadia. Humane PC mRNA was veelal GRPR

positief, maar expressie was variabel. Alleen CRPC weefsels neigden naar lagere expressie van GRPR mRNA. Ondanks het feit, dat het aantal specimina in deze groep laag was, geeft deze observatie een indicatie, dat GRPR (in ieder geval initieel) androgeen gereguleerd is. PC toonde al met al een hogere mediane GRPR-specifieke opname dan benigne weefsels. Daarentegen was er wel een aanzienlijke overlap tussen de lagere opnames in PC en de opname in benigne weefsels. Deze bevindingen komen overeen met originele data uit een invloedrijk artikel van Markwalder et al. [31]. Een aangepaste puntgrafiek met data uit deze studie, vergelijkbaar met de grafiek vervaardigd met onze data, toonde een overeenkomstige overlap in GRPR expressie tussen PC en benigne tissue samples. Concluderend impliceren de data dat BN-GRPR gemedieerde beeldvorming van PC uitvoerbaar lijkt bij patiënten met een vroeg staduim PC vóór het ondergaan van hormonale therapie. Bovendien suggereren de resultaten aan, dat voorselectie van PC patiënten op GRPR expressie nodig lijkt bij de overweging om GRPR-BN gemedieerde technieken in te zetten voor post-operatieve monitoring of therapie.

In **hoofdstuk 6** wordt het effect van verscheidene experimentele condities op de farmacokinetiek van BN-specifieke binding in PC-dragende naakte muizen beschreven. Met deze kennis hoopten we de BN-GRPR-gemedieerde binding te kunnen verbeteren. Deze studie maakte duidelijk, dat receptorbinding niet alleen afhankelijk is van de affiniteit van BN-analoga voor de GRPR, maar dat variaties in experimentele protocollen, zoals *zuivering van analoga met behulp van high-performance liquid chromatography* (HPLC), het toedienen van injectievloeistof met verschillende specifieke activiteiten en saturatie van de GRPR (door het toedienen van niet radioactief gebonden BN vóór radioactief gebonden BN) van significante invloed kan zijn op peptide-receptor binding in vivo. Deze bevindingen onderstrepen de noodzaak om experimentele condities nauwkeurig te bepalen ten einde een volmaakt gestandaardiseerd protocol op te stellen voor BN-GRPR gemedieerde toepassingen.

Aangezien het metabolisme van kankercellen dikwijls hoog is, wordt er veel onderzoek gedaan naar beeldvorming van PC met behulp van metabole radiofarmaca. In **hoofdstuk 7** staat een PET-onderzoek met VCaP dragende muizen beschreven, waarin een BN-GRPR gemedieerd radiofarmacon, ^{68}Gallium-AMBA (^{68}Ga-AMBA), wordt vergeleken met een metabole radiotracer, ^{18}Flourine- methylcholine (^{18}F-CH). Opname in de VCaP tumor was flink hoger bij gebruik van ^{68}Ga-AMBA in vergelijking met ^{18}F-CH, terwijl opname in achtergrondorganen grotendeels lager was. De studie beschrijft derhalve superioriteit van peptide-receptor gemedieerde ten opzichte van metabolisme-gemedieerde beeldvorming met PET bij PC.

In dit proefschrift wordt preklinisch onderzoek beschreven naar de potentie van BN-GRPR gemedieerde specifieke beeldvorming en therapie van PC. Het belang van standaardisatie voor het

mogelijk maken van betrouwbare vergelijking werd duidelijk uit een kritische evaluatie van veelgebruikte experimentele condities in BN-gerelateerde literatuur. Voor zover ons bekend waren wij de eerste studiegroep, die BN analoga testte onder gestandaardiseerde experimentele condities. In overeenstemming met de literatuur [47-49], vonden we dat GRPR antagonisten superieur waren ten opzichte van gelijkwaardige agonisten. Onze studiegroep zal zich voor toekomstige klinische PC studies zodoende richten op het gebruik van GRPR antagonist. We hebben in dit proefschrift ook origineel onderzoek beschreven naar de rol van hormonen voor de expressie van de GRPR. Dit heeft meer inzicht opgeleverd in de expressie van de GRPR tijdens verschillende stadia van humane PC. Tenslotte, aangezien er geen gouden standaard is voor beeldvorming van PC, hebben we metabole radiofarmaca vergeleken met peptide-receptor radiofarmaca ten einde hun relatieve potentieel als beeldvormende tracer van PC te evalueren. Het veelbelovende karakter van BN-GRPR gemedieerde beeldvorming werd bevestigd door deze studie aangezien de tumorspecifieke binding ruim vier maal hoger was ten opzichte van de tumoropname van het metabole radiofarmacon. Bovendien was de opname in achtergrondorganen met BN radiofarmaca over het algemeen lager.

De gepresenteerde uitkomsten uit dit proefschrift vormen de basis voor toekomstige evaluatie van GRPR antagonisten inzake beeldvorming van PC in patiënten. Omdat ontwikkelingen van BN analoga snel verloopt, zal het door ons best bevonden analoog Demobesin-1 niet het radiofarmacon van eerste keus zijn in klinische studies. Het zal vervangen worden door een verbeterde nieuwe GRPR antagonist, ontwikkeld in een binnen onze studiegroep parallel lopend onderzoeksproject, die naast voor SPECT ook ingezet kan worden voor PET.

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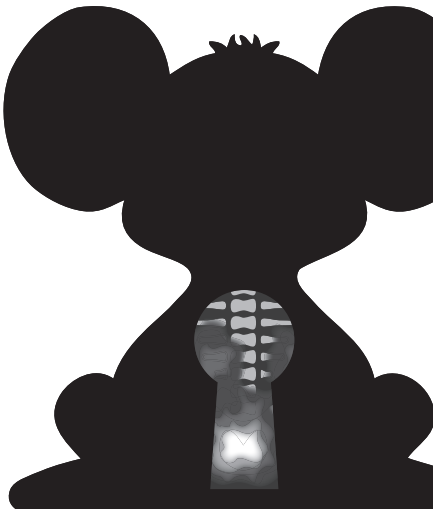
DANKWOORD



Na het behalen van mijn geneeskunde bul aan de Universiteit van Utrecht, besloot ik vol overtuiging te gaan voor het specialisme urologie. Het doorlopen van een promotietraject leek me de ideale eerste stap in mijn toekomstige carrière.

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Professor dr. ir. T.J. Visser en Professor dr. O.C. Boerman, leden van de kleine

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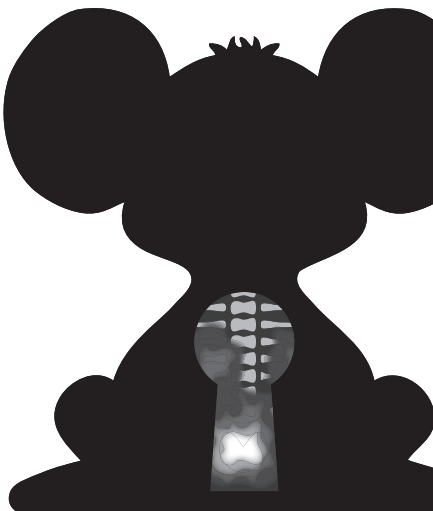
Lieve Kelly, als diëtiste in het OLVG had ik je al snel in de smiezen. Door mijn doortastend handelen zijn we inmiddels de belichaming van het medisch cliché, ”de doktersroman”, geworden. Ons officieuze samenwonen is sinds augustus officieel. In de periode waarin we nu samen zijn, heb ik je eigenlijk niet genoeg kunnen geven. De opleiding chirurgie, het gedoe rondom mijn huis én de promotie hebben hiervoor gezorgd. In plaats van “quality time” met jou zat ik veelal achter de laptop. Door jezelf enigzins weg te cijferen voor mijn werk, heb je me fantastisch geholpen. Op veel vlakken heb je me onvoorwaardelijk gesteund en bijgestaan. Vakantie zat er tijdens het eerste jaar van onze relatie nog niet in. Gelukkig hebben we in de alledaagse beslommeringen al veel plezier samen. Met de bestemming Zanzibar en Tanzania hebben we onze eerste en welverdiende droomreis nu wel voor de boeg. Dat er vele reizen mogen volgen, babe!

CURRICULUM VITAE



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1091RP Amsterdam
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Mobile phone: 06-54274964
Date of birth: 20-02-1980

Education

1998-2006 Medical school University of Utrecht
1991-1998 VWO/Atheneum (high school degree)

Experience

2010-now Surgical residency "Onze Lieve Vrouwe Gasthuis" (OLVG), Amsterdam
2006-2009 PhD Urology / Nuclear Medicine Erasmus MC Rotterdam
2003-2005 Vaccination of travellers at the Utrecht Public Health Service, or GGD (Gemeentelijke gezondheidsdienst)
2003-2004 Internship gynaecology, hospital Umberto I, University 'La Sapienza', Rome, Italy
2001-2002 President of the students union, "het Utrechts Studenten Corps", with over 1000 members
2000-2004 Student teacher Utrecht Medical Centre

Publications

- 'Improving radiopeptide pharmacokinetics by adjusting experimental conditions for bombesin receptor-targeted imaging of prostate cancer.'
  Rogier P.J. Schroeder[1, 2], Erik De Blois[1], Corine M.A. de Ridder[2], Wytske M. van Weerden[2], Wout A.P. Breeman[1], Marion de Jong[1]
  Departments of [1]Nuclear Medicine and [2]Experimental Urology, Erasmus MC, Rotterdam, the Netherlands
  Manuscript submitted in June 2011 to the Quarterly Journal of Nuclear Medicine and Molecular Imaging

- 'Gastrin-releasing peptide receptor-based targeting using bombesin analogues is superior to metabolism-based targeting using choline for in vivo imaging of human prostate cancer xenografts.'
  R.P.J. Schroeder[1, 2], W.M. van Weerden[2], E.P. Krenning[1], C.H. Bangma[2], S. Berndsen[1], C.H. Grievink-de Ligt[1], H.C. Groen[1], S. Reneman[2], E. de Blois[1], W.A. Breeman[1], M. de Jong[1]
  Departments of [1]Nuclear Medicine and [2]Experimental Urology, Erasmus MC, Rotterdam, the Netherlands
  Manuscript published in July 2011 in the European Journal of Nuclear Medicine and Molecular Imaging

- 'A standardised study to compare prostate cancer targeting efficacy of five radiolabelled bombesin analogues.'
  R.P.J. Schroeder[1, 2], C.M. Müller[1], S. Reneman[2], M.L. Melis[1], W. Breeman[1], C. Bangma[2], E. De Blois[1], E.P. Krenning[1], W.M. van Weerden[2] and M. de Jong[1]
  Departments of [1]Nuclear Medicine and [2]Experimental Urology,
  Erasmus MC, Rotterdam, the Netherlands
  Manuscript published in July 2010 in the European Journal of Nuclear Medicine and Molecular Imaging

- 'Androgen-Regulated Gastrin-Releasing Peptide Receptor Expression in Androgen-Dependent Human Prostate Tumour Xenografts'
  Rogier P.J. Schroeder[1,2], M. de Visser[1], Wytske M. van Weerden[2], Corrina M.A. de Ridder[2], Suzanne Reneman[2], Marleen Melis[1], Wout A.P. Breeman[1], Eric P. Krenning[1], Marion de Jong[1].
  Departments of [1]Nuclear Medicine and [2]Experimental Urology,
  Erasmus MC, Rotterdam, the Netherlands
  Manuscript published in June 2010 in the International Journal of Cancer

- 'Peptide receptor imaging of prostate cancer with radiolabelled bombesin analogues'
  R.P.J. Schroeder[1,2], W.M. van Weerden[2], C. Bangma[2], E.P. Krenning[1] and M. de Jong[1]
  Departments of [1]Nuclear Medicine and [2]Experimental Urology,
  Erasmus MC, Rotterdam, the Netherlands
  Manuscript published in June 2009 in Methods

Presentations

08-10-2010 'Visualisation and treatment of prostate cancer using radiolabelled bombesin' oral presentation, Dutch Association of Urology (NVU), Nieuwegein, The Netherlands.

13-10-2009 'Selection best bombesin-analogue for use in phase I trial imaging prostate cancer with SPECT' oral presentation, EANM congres 2009, Barcelona, Spain.

17-10-2008 'Radiolabelled Bombesin Analogues: a Promising Tool for Imaging and Radionuclide Therapy for Prostate cancer' poster presentation, European Society for Urological Research, Dublin, Ireland.

06-02-2008 'Characterisation of GRP-receptor expression in prostate cancer; a potential target for PET/SPECT imaging.' poster presentation, '12th Molecular Medicine Day' Erasmus Postgraduate School Molecular Medicine, Rotterdam, The Netherlands.

28-09-2007 'Androgen regulation of Gastrin-Releasing Peptide Receptor (GRPR), a target for imaging and therapy of human prostate cancer' poster presentation, European Society for Urological Research (8th meeting), Barcelona, Spain

15-11-2004 'Life birth rate after vasovasostomy' oral presentation, Association for Fertility study (Vereniging voor Fertiliteitstudie), Rotterdam, The Netherlands

Grants

11-02-2008 until 23-02-2008
'Testing new bombesin analogues which are suitable for peptide receptor radiotherapy'
Short-term scientific mission in the framework of the European Cooperation in Science and Technology (COST)
Institute of Radioisotopes – Radiodiagnostic Products (I/R-RP), National Center for Scientific Research 'Demokritos' (NCSRD), Athens, Greece

Memberships

04-2008-now Member European Association of Urology

Certificates

2011 Fundamental Critical Care Support
2010 Advanced Trauma Life Support
2006 'Animal handling' art 9
2006 'Safe Handling of Radioactive Materials and Sources' art 5B
2005 Teaching qualification, Centre of expertise for education of the Utrecht Medical Centre
2001 Media training, 'Headline media trainers'

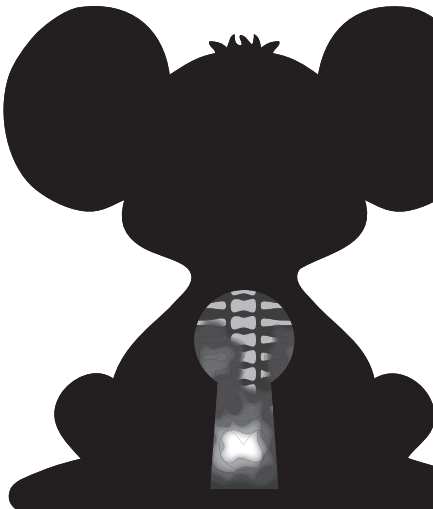
Languages

Dutch, English and Italian

Hobbies

Boxing, field hockey, tennis, golf, sailing and cooking

PhD PORTFOLIO



PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: R.P.J. Schroeder	PhD period: 10-2006 untill now
Erasmus MC Department:	Promotor: Prof. dr. ir. M. de Jong
Urology / Nuclear medicine	Co-promotor: Dr. ir. W.M. van Weerden

1. PHD TRAINING

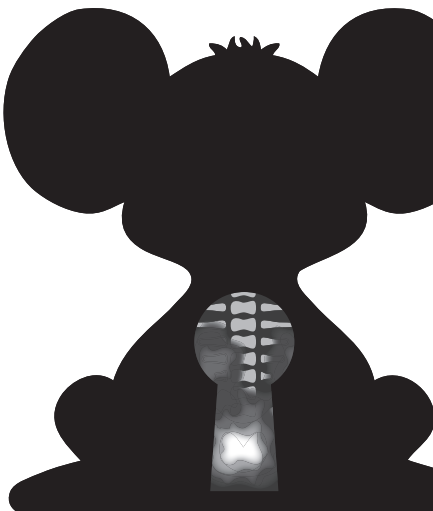
	Year	*Workload (Hours/ECTS)*
General courses		
- Biomedical English Writing and Communication	2009	4 ECTS
- Laboratory animal science	2006	3 ECTS
Specific courses (e.g. Research school, Medical Training)		
- 'Safe Handling of Radioactive Materials and Sources' art 5B		2 ECTS
Seminars and workshops		
- Department journal club	2006-2009	2 ECTS (28 hours)
- Department seminars Urology / nuclear medicine (in and outside Erasmus MC)	2006-2009	3 ETCS (50 hours)
- General seminars	2006-2009	3 ETCS (50 hours)
Presentations		
- JNI-meeting	2006-2009	5 ECTS (100 hours)
- lab meeting	2006-2009	2 ECTS (30 hours)
- seminars	2006-2009	2 ECTS (25 hours)
(Inter)national conferences		
- European Congress (poster presentations)	2006-2009	6 ECTS (100 hours)
- European Congress (oral presentation)	2006-2009	2 ECTS (50 hours)
- National Urological association (oral presentation)	2006-2009	2 ECTS (30 hours)

Other		
- Short-term scientific mission in framework of Cost (Athens)	2008	7 ECTS (300 hours)

2. TEACHING

Supervising practicals and excursions, Tutoring		
- Translational research practical	'07-'08-'09	2 ECTS (50 hours)
TOTAL		**45 ECTS (813 hours)**

LIST OF ABBREVIATIONS



LIST OF ABBREVIATIONS

PC	prostate cancer
GRP	Gastrin-Releasing Peptide
BN	bombesin
TURP	transurethral resection of prostate
TRUS	transrectal ultrasound
TUR	transurethal resection
LHRH	luteinizing hormone-releasing hormone
CT	computed tomography
MRI	magnetic resonance imaging
FDG	fluoro-2-deoxy-D-glucose
PRRT	peptide receptor radionuclide therapy
(i)PSA	(initial) prostate specific antigen
DRE	digital rectal examination
ERSPC	European Randomised Study of screening for Prostate Cancer
PSMA	prostate-specific membrane antigen
FDA	American Food and Drug Administration
PIN	prostatic intraepithelial neoplasias
BPH	benign prostate hyperplasia
NMB / BB_1	neuromedin B receptor
GRPR / BB_2	Gastrin-Releasing Peptide Receptor
BRS-3 / BB_3	bombesin receptor subtype 3
BB_4	bombesin receptor subtype 4
pmol	picomole
MBq	megabecquerel
SPECT	single positron emission tomography
PET	positron emission tomography
SST	somatostatin
DTPA	diethylenetriaminepenteacetic acid
DOTA	1,4,7,10-tetraazacyclododecane-N,N',N'', N'''-tetraacetic acid
N_4	1,4,8,11-tetraazaundecane
HE	haematoxylin-eosin
RCP	radiochemical purity
ITLC(-SG)	instant thin-layer chromatography (Silica gel)
HPLC	high-performance liquid chromatography
TFA	trifluoroacetic acid
FCS	fetal calf serum
DEC	Animal Experimental Committee / dierexperimentencommissie
EDTA	ethylenediaminetetraacetic acid
pi	post injection
%ID/g	percentage of the injected dose per gram of tissue
SD	standard deviation

RT-PCR	quantitative reverse transcriptase polymerase chain reaction
mRNA	messenger ribonucleic acid
HKG	housekeeping gene
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
PBGD	porphobilinogendeaminase
DLU/mm^2	Digital Light Units per per millimeter square
dpm/mg	disintegrations per minute per mg
cas	castrated
T	testosterone
R1881	synthetic androgen
AR	androgen receptor
PAP	prostatic acid phosphatise
DCC	steroid deprived serum
CRPC	castration resistant prostate cancer
RPMI	Roswell Park Memorial Institute
Rad PC	radical prostatectomy
LN(+)	lymph node (metastasis)
PIN	prostatic intraepithelial neoplasia
PMID	PubMed identifier
MW	molecular weight
n	number
t	timepoint
min	minutes
h	hours
RT	retention time
(^{18}F-)CH	(^{18}Flour-)methylcholine
NMR	Nuclear magnetic resonance
aq	aqueous
FBP	filtered backprojection
OSEM	ordered subsets expectation maximization
MAP	maximum a posteriori
VOI	volume of interest
IQR	interquartile range