Androgen-Regulated Gastrin-Releasing Peptide Receptor Expression in Androgen-Dependent Human Prostate Tumor 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.

Keywords: androgen-regulation, biodistribution, bombesin, Gastrin-Releasing Peptide Receptor, prostatic neoplasms, xenografts

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 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.

^{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 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).

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)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 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 androgen 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 is 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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Legends

Table 1

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 / mm^2 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 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 ($p < 0.05$).

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 mm^2 (net DLU/mm^2). 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.

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 ($p < 0.05$).

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

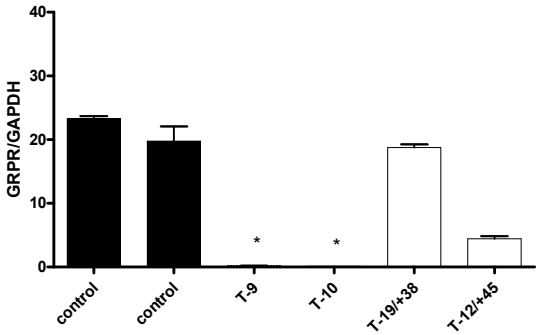
derived from castrated and testosterone resupplemented mice used in the biodistribution.

Table 1

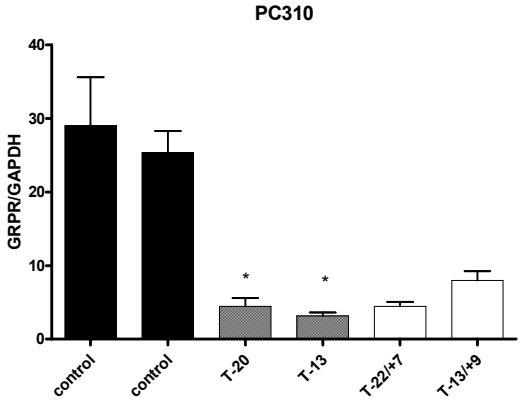
Group	study	PC295		PC82		PC310		PC3		Pancreas[#]	
		% control	(n)	% control	(n)	% control	(n)	% control	(n)	% control	(n)
control	A uptake ^{111}In-MP2653	100 ± 31	(11)	100 ± 31	(14)	100 ± 19	(4)	100 ± 33	(12)	100 ± 21	42
	B binding ^{125}I-GRP	100 ± 19		100 ± 37		100 ± 67		100 ± 53			
	C binding ^{111}In-MP2248	100 ± 36		100 ± 54		100 ± 25		100 ± 42			
cas 2	A uptake ^{111}In-MP2653	124 ± 29	(5)	109 ± 37	(3)	nd		nd		nd	
	B binding ^{125}I-GRP	72 ± 6.9*		67 ± 26							
	C binding ^{111}In-MP2248	55 ± 17*		67 ± 28							
cas 4	A uptake ^{111}In-MP2653	22 ± 7.3*	(5)	56 ± 30	(3)	nd		nd		nd	
	B binding ^{125}I-GRP	11 ± 8.1*		29 ± 27*							
	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)	nd		nd		nd	
	B binding ^{125}I-GRP	20 ± 6.0**		33 ± 4.6**							
	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)	nd		nd		nd	
	B binding ^{125}I-GRP	30 ± 9.7**		60 ± 23**							
	C binding ^{111}In-MP2248	17 ± 6.3**		56 ± 10**							
cas 7 T 14	A uptake ^{111}In-MP2653	nd		86 ± 46	(8)	99 ± 30**	(5)	95 ± 47	(5)	99 ± 17	20
	B binding ^{125}I-GRP			91 ± 30**		50 ± 19		112 ± 76			
	C binding ^{111}In-MP2248			87 ± 40**		83 ± 21		125 ± 49			

Figure 1

A


PC295
GRPR/GAPDH
40
30
20
10
0
*
*
control
control
T-9
T-10
T-19/+38
T-12/+45

C


PC310
GRPR/GAPDH
40
30
20
10
0
*
*
control
control
T-20
T-13
T-22/+7
T-13/+9

B

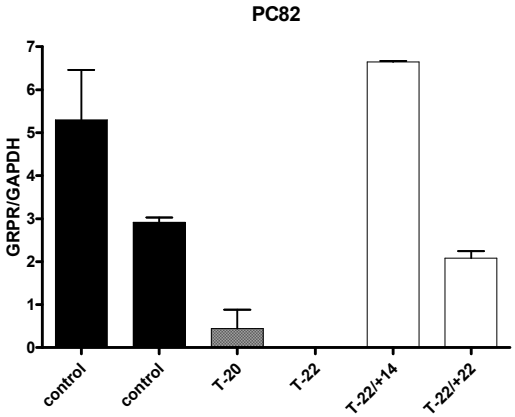

PC82
GRPR/GAPDH
7
6
5
4
3
2
1
0
control
control
T-20
T-22
T-22/+14
T-22/+22

Figure 2

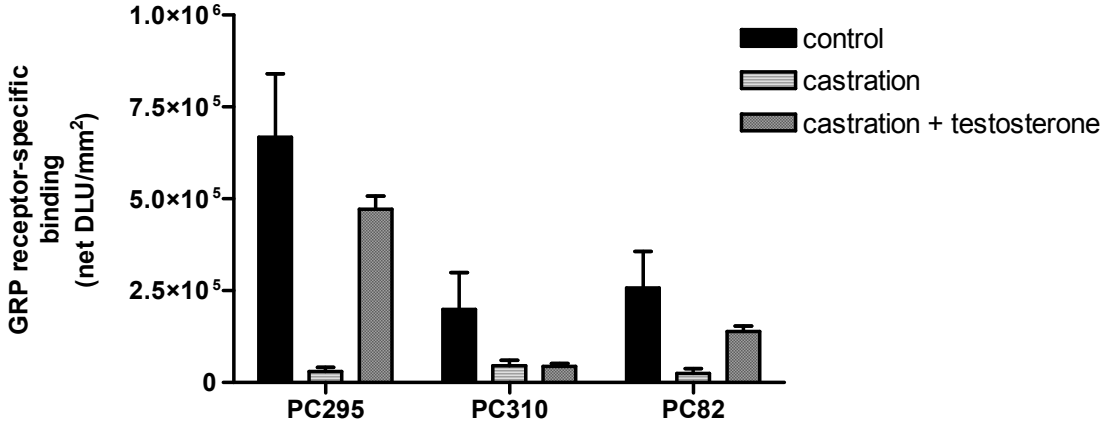

GRP receptor-specific binding (net DLU/mm²)
1.0×10⁶
7.5×10⁵
5.0×10⁵
2.5×10⁵
0
PC295
PC310
PC82
control
castration
castration + testosterone

Figure 3

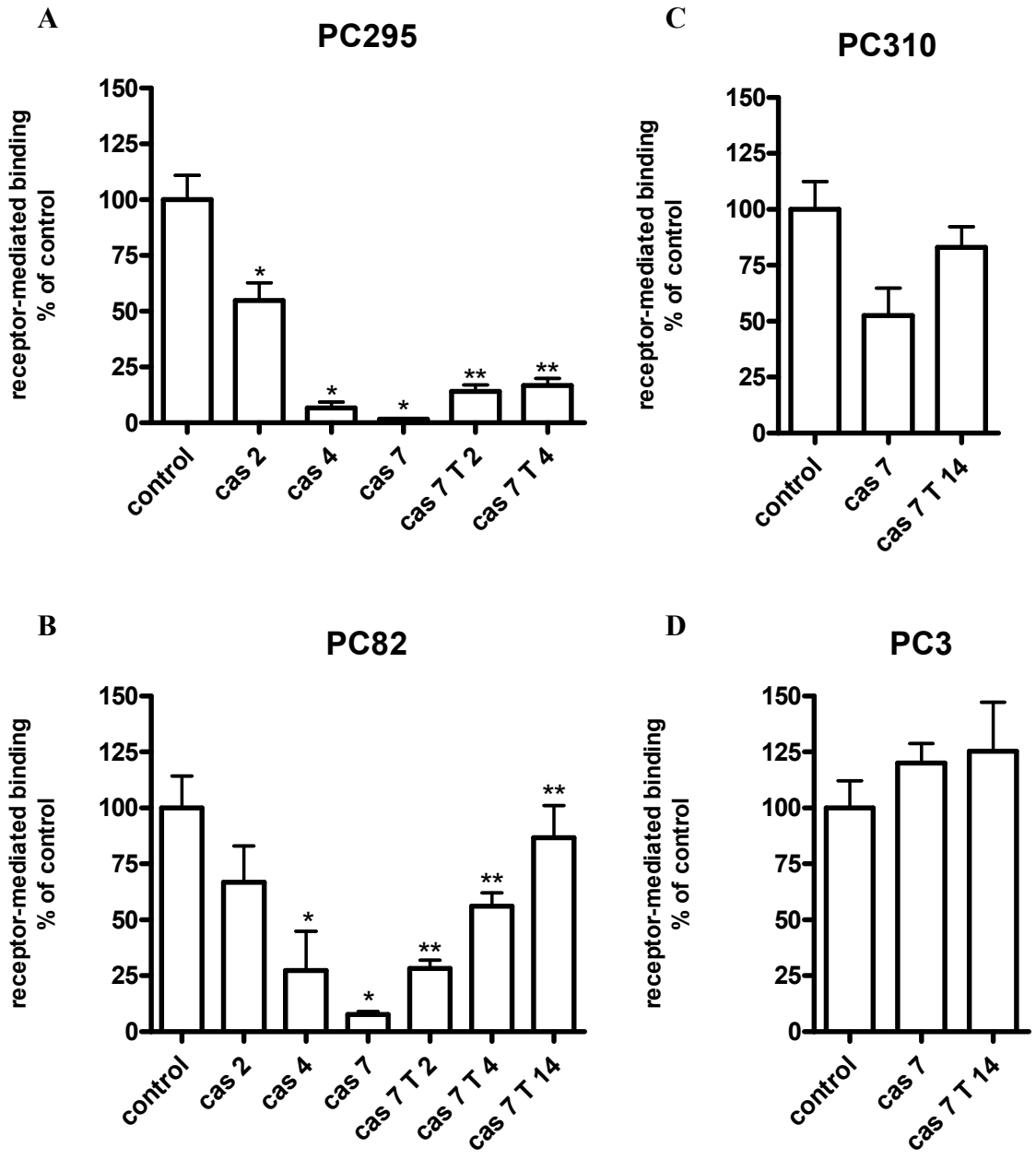
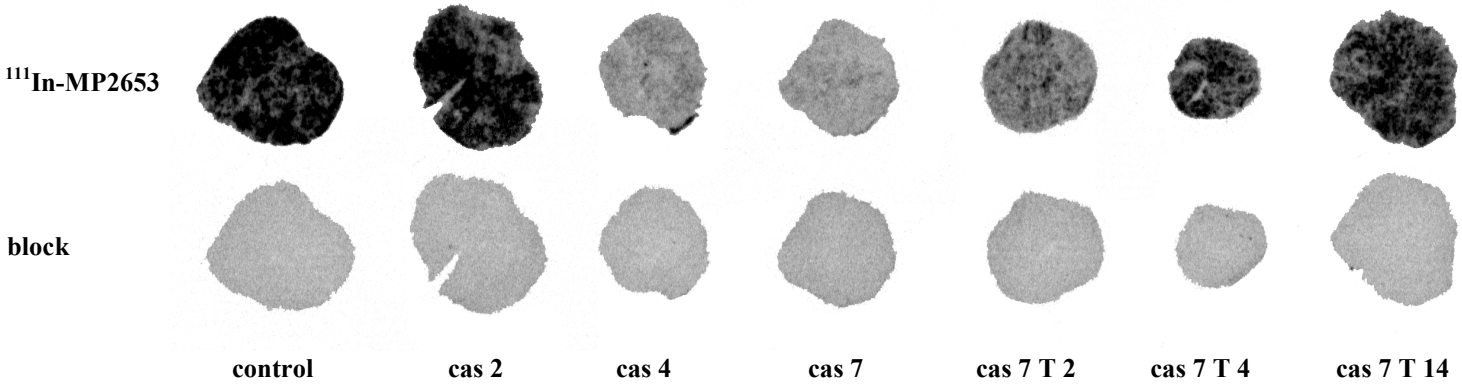

A
PC295
receptor-mediated binding
% of control
150
125
100
75
50
25
0
control
cas 2
cas 4
cas 7
cas 7 T 2
cas 7 T 4
C
PC310
receptor-mediated binding
% of control
control
cas 7
cas 7 T 14
B
PC82
receptor-mediated binding
% of control
control
cas 2
cas 4
cas 7
cas 7 T 2
cas 7 T 4
cas 7 T 14
D
PC3
receptor-mediated binding
% of control
control
cas 7
cas 7 T 14

Figure 4


^{111}In-MP2653
block
control
cas 2
cas 4
cas 7
cas 7 T 2
cas 7 T 4
cas 7 T 14