PSMA-617 inhibits proliferation of human prostate cancer cells and enhances their radiosensitivity to $^{177}$Lu-PSMA-617

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Research Article

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Abstract

The human prostate – specific membrane antigen (PSMA) is substantially up-regulated in metastatic prostate cancer (PCa) cells. PSMA can be targeted by $^{177}$Lu conjugated to PSMA-617, a high-affinity ligand for the PSMA. The binding of the radioligand, $^{177}$Lu-PSMA-617, results in its internalization and delivery of $\beta$-radiation into the cancer cells. However, PSMA-617, a component of the final product in the synthesis of the radioligand, may also play a role in the pathophysiology of PCa cells. The present study clarifies the effects of PSMA-617 (10, 50 and 100 nM) on the expression of PSMA in PSMA-positive LNCaP cells, their proliferation and damage, and on the $^{177}$Lu-PSMA-617-induced cell death by WST-1 and lactate dehydrogenase assays, immunohistochemistry, Western blotting, immunofluorescence staining and uptake of $^{177}$Lu-PSMA-617. PSMA-617 at 100 nM concentration induced cell-growth arrest, down-regulated cyclin D1 and cyclin E1 (by 43 and 36 %, respectively) and up-regulated the cyclin-dependent kinase inhibitor p21$^{\text{Waf1/Cip1}}$ (by 48 %). Immunofluorescence staining demonstrated reduced content of DNA, pointing to a lower rate of cell division. PSMA-617 (up to 100 nM) did not alter the uptake of $^{177}$Lu-PSMA-617 into the LNCaP cells. Interestingly, simultaneous treatment with $^{177}$Lu-PSMA-617 and PSMA-617 for 24 and 48 h substantially potentiated the cell-death promoting effects of the radioligand.

In conclusion, the impeding tumour cell proliferation by PSMA-617 and its potentiation of the radiation-induced cell death brought about by $^{177}$Lu-PSMA-617 in PCa cells may considerably improve the outcome of the radiation therapy with $^{177}$Lu-PSMA-617, especially in patients with decreased radiosensitivity of PCa cells to the radioligand.

Introduction

Radioligand therapy is a new, attractive therapeutic option for the treatment of metastatic castration-resistant prostate cancer (mPCA). The therapeutic approach is based on the interaction of small, high affinity ligands with the prostate-specific membrane antigen (PSMA). PSMA is a carboxypeptidase, also known as folate hydrolase I or glutamate carboxypeptidase II. PSMA is excessively up-regulated in advanced-stage PCa [1, 2]. Due to close relation to tumour progression, PSMA is an attractive target for a more efficient diagnosis and therapy. Peptide ligands, which bind to PSMA with high affinity and possess high stability in vitro and in vivo, have been developed [3, 4]. Ligand binding leads to internalisation and the endocytosis of PSMA results in an effective transportation of the ligand into the cells [5]. The most promising radioligand, which interacts with PSMA, is PSMA-617 (vipivotide tetraxetan), a high affinity PSMA inhibitor (Ki 0.37 nM), conjugated with $^{177}$Lu [6]. After binding of $^{177}$Lu-PSMA-617 to PSMA, the complex is internalised and transported into PCa cells, where $^{177}$Lu releases energetic beta radiation which preferentially destroys cancer cells [7]. Ongoing clinical trials demonstrate a substantial anti-tumour activity, favourable safety and few side effects [8, 9, 10].

Excessive amounts of the precursor, PSMA-617, are usually used in order to avoid the presence of the free, unbound $^{177}$Lu, which induces serious organ damage. The final product contains the radioligand and variable concentrations of the unlabelled precursor. The precursor may compete with the binding of
177Lu-PSMA-617 to PSMA and may thus reduce its internalisation and therapeutic efficacy. Moreover, the pathophysiological processes occurring in PCa cells exposed to PSMA-617 have not yet been intensively studied. We have therefore investigated the effects of PSMA-617 in PSMA-positive PCa cells, (LNCaP cells, Lymph Node Carcinoma of the Prostate), on tumour growth, cell-cycle arrest and cell death and the corresponding underlying mechanisms. We report that PSMA-617 inhibits proliferation and induces cell cycle arrest in the G1 stage in LNCaP cells and considerably potentiates the cytotoxic effect of 177Lu-PSMA-617.

Materials And Methods

Chemicals and reagents

**Antibodies (Ab)**

Rabbit monoclonal anti cyclin D1, mouse monoclonal anti Cyclin E1, mouse monoclonal anti p21$^{Waf1/Cip1}$ (p21) and rabbit monoclonal anti p27$^{Kip1}$ (p27) (Cell Signalling Technology); mouse monoclonal anti PSMA (Abcam); mouse monoclonal anti β-actin (Sigma-Aldrich); horseradish peroxidase-conjugated secondary anti-rabbit and anti-mouse IgGs (Amersham); Fluor® 488-conjugated donkey anti-mouse IgG (Molecular Probes).

**Chemicals and Kits**

PSMA-617 (ABX Advanced Biochemical Compounds); WST-1 assay Kit and Cytotoxicity Detection Kit (lactate dehydrogenase, LDH) (Roche); Propidium Iodide Reagent (Invitrogen); cresyl violet (Sigma-Aldrich); PRMI 1640 culture medium, HAM´-12 medium, foetal bovine serum (FBS) and penicillin/streptomycin solution (10,000 U/ml) (Gibco®); Cellytic™ MT Cell Lysis Reagent (Sigma-Aldrich); halt protease & phosphatase inhibitor and Pierce™ BCA Protein Assay Kit (Thermo Scientific); Western Blotting Luminol Reagent (Santa Cruz Biotechnology); Immobilon-P polyvinylidene difluoride membrane (PVDF) (Millipore); high performance chemiluminescence film (Amersham International plc); Western Bolt Stripping Buffer (Thermo Scientific). All other chemical substances which are not explicitly mentioned were purchased from Sigma-Aldrich or Merck.

Cell cultures

LNCaP cells (ACC 256), a human prostate carcinoma cell line, was purchased from DSMZ (German Collection of Microorganisms and Cell Culture GmbH). The cells were cultured in RPMI 1640 medium containing 10% heat inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. PC3 cells (ATCC CRL-1435), kindly provided by Dr. Tiwari from Molecular Imaging North Competence Centre, University of Kiel, Germany, were grown in the culture medium consisting of RPMI 1640 and HAM´-12, 1:1, supplemented with 10% heat inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All cells
grew as a monolayer in dishes at 37°C in a humidified atmosphere of air/CO₂ (19:1). For cell passages, trypsin/EDTA (0.025% trypsin and 0.02% EDTA) was used.

**WST-1 assay**

Cell proliferation was assessed by the WST-1 reagent according to the manufactures’ instructions. Cells were grown in 24-well culture plates and exposed to PSMA-617 (10, 50 or 100 nM) for 24 or 48 h, respectively. Aliquots of the culture medium from each well were taken for LDH assay (see below). The cells were then incubated with 10% WST-1 reagent for 4 h. 100 µl of supernatant were transferred into the 96-well plate and measured with Multi-Well Spectrophotometer (Elisa Reader, TECAN Infinite 200) at 460 nm wavelength. The absorbance directly correlates with the number of viable cells. Each sample was measured in triplicate.

**Lactate dehydrogenase (LDH) assay**

The assessment of the cell membrane integrity and cytotoxicity based on the measurement of LDH activity released from damaged cell into the culture medium were determined by the Cytotoxicity Detection Kit according to the manufacturers’ recommendations and as published previously [11]. 10 µl of the culture medium were used for the detection of LDH activity. Each sample was measured in triplicate.

**Protein isolation and Western blot analysis**

Cells were lysed in Cellytic™ MT Cell Lysis Reagent containing 1% of the halt protease & phosphatase inhibitor on ice for 30 min. After short incubation (5 min at 95°C), the lysates were sonicated and centrifuged (15,000 x g at 4°C for 15 min). The protein concentration in the supernatant was measured by the Pierce™ BCA Protein Assay Kit according to the manufacturer’s instruction. Extracts equivalent to 15 or 30 µg of total proteins per lane were loaded and separated on 10% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked and incubated overnight with primary Ab against PSMA (1:5000), cyclin D1 (1:1000), cyclin E1 (1:3000), p21 (1:2000), or p27 (1:1000). On the next day, the membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary Ab. Western blots were developed with Western Blotting Luminol Reagent on high performance chemiluminescence film. For the re-staining, the blots were stripped in the Restore Western Bolt Stripping Buffer, washed in Tween-Tris-Buffered saline, with 1% Tween and blotted again. To normalise the protein content of each lane, all membranes were blotted with anti-β-actin Ab (1:10,000). The films were scanned and quantified using the quantification software (Quantity One, Bio-Rad).

**Protocols**

The determination of PSMA in LNCaP- or PC3 cells was carried out in cells cultured in the appropriate culture medium till 80–90% confluence was reached. The effect of PSMA-617 on the expression of the
cell cycle regulators was quantified in LNCaP cells exposed to vehicle (controls) or PSMA-617 (10, 50 or 100 nM) at 37°C for 24 or 48 h, respectively.

**Immunofluorescence staining for the PSMA and DNA**

Cells grown on cover slips (Becton Dickinson) in 4-well plates were exposed to vehicle or PSMA-617 (100 nM) for 24 h. The cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature (RT), washed twice with 2xSSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), treated with 100 µg/ml DNase-free RNase diluted in 2xSSC buffer and incubated at 37°C for 20 min to destroy RNA. The cells were washed three times with phosphate buffered saline containing 0.02% of Triton (PBST), permeabilised with 0.2% saponin in 0.1% BSA for 30 min at RT, incubated in the block solution containing 1% BSA for 30 min at RT and exposed to the primary Ab, mouse anti-human PSMA (1:3000), at 4°C overnight. Following three washing steps with PBST, the cells were incubated with Alexa Fluor®488 - conjugated donkey anti-mouse 2nd Ab (1:500) in 0.5% BSA for 1 h at RT in dark. After the incubation and washing with PBST and twice with 2xSSC buffer, PI regent (500 nM in 2xSSC buffer) was added (300 µl/well) and the cells were incubated for 2 min at RT. The cells were washed three times with 2xSSC buffer, dried in the dark and mounted in Prolong® Gold Antifade Reagent. Immunofluorescence analyses was carried out by a microscope (Leica DMR Microscope system equipped with CC 12 digital camera).

**Cresyl violet staining**

LNCaP cells cultured in 24-well plates, were exposed to vehicle or to 10, 50 and 100 nM PSMA-617 for 24 or 48 h, respectively. The cells were washed three times with PBS and fixed with 4% PFA/PBS at RT for 1 h. After washing three times with PBS and twice with distilled water, the cells were incubated with 0.1% cresyl violet solution for 20 min at RT. Cresyl violet was washed out with distilled water, the stained cells were dried and photographed under an inverted light microscope (OLYMPUS IX73 Microscope system equipped with OLYMPUS DP74 digital camera).

**Synthesis and quality control of $^{177}$Lu-PSMA-617**

No-carrier added $^{177}$LuCl$_3$ solution (36–44 Gbq/ml) (EndolucinBeta®) was purchased from ITM (ITM Isotopen Technologien). PSMA-617 (60 µg) mixed with 740 µl of gentensic solution (3 mg/ml dissolved in 0.4 M sodium acetate, pH = 4.6) was added to 163 µl of the $^{177}$LuCl$_3$ solution. The buffered solution was heated for 20 min at 92°C and diluted with saline to reach a final activity of 1 GBq/ml. Quality control was performed using TLC scanner and HPLC according to the Guideline Monographs on Radiopharmaceutical Preparations spotting 0.3 µl aliquots on the Chromatography strips (Biodex) employing 0.1 M citric acid buffer (pH 5.5) as eluent or on the ITLC-SG plates (Varian) using 1 M NaAc/MeOH (1:1) as eluent. The strips and plates were scanned using a flat-bed scanner (Rita Star, Raytest-Isotopenmessgeräte GmbH). The radiochemical and chemical purity were determined by a gradient HPLC method. The analytical HPLC apparatus was equipped with a radiation detector and an UV (220 nm) detector. HPLC separations were performed on Chromolith columns (3 x 100 mm). The gradient eluent consisted of mobile phase A (water containing 0.1% TFA) and mobile phase B (acetonitrile...
containing 0.1% TFA) and a flow rate of 1.0 ml/min. Starting with 100%A/0%B, the gradient was increased to 100% B over 8 min. The retention time of free $^{177}$LuCl$_3$ was 0.8 min and that of $^{177}$Lu-PSMA-617 4.7 min, respectively.

**Effect of PSMA-617 on the uptake of $^{177}$Lu-PSMA-617 into LNCaP cells**

LNCaP cells (2 x $10^6$) were plated onto 10 cm-Petri dishes and incubated for 2–3 days. The cells were simultaneously treated with $^{177}$Lu-PSMA-617 (diluted with serum deprived culture medium, 2 MBq/4 ml/dish) and PSMA-617 (10, 50 or 100 nM) for 30 min, rinsed twice with 4 ml of ice-cold saline, scraped using 1 ml cold saline, transferred to an Eppendorf-tube and centrifuged (2000 rpm) for 5 min at 4°C. The radioactivity of the cell pellets was measured with a γ-counter (ISOMED 200). The total protein content of the cell pellets was measured as described above. The counts of individual samples were normalised for protein content. The data is expressed as cpm/mg of protein.

**Effects of PSMA-617 on the anti-cancer efficacy of $^{177}$Lu-PSMA-617 in LNCaP cells**

LNCaP cells cultured in a 24-well plate (approximately $10^5$ cells/well), were incubated with $^{177}$Lu-PSMA-617 (100 KBq/0.5 ml/well) and vehicle (controls) or with 50 or 100 nM PSMA-617 for 24 (n = 12) or 48 h (n = 8), respectively. 10 µl of the culture medium was used for LDH-assay. Each sample was measured in triplicate.

**Statistical Analyses**

The data is expressed as the mean ± SD of triplicate determinations from 3–5 separate experiments. The distribution of the sampled data was analysed by Kolmogorov-Smirnov’s test and the homogeneity of variance was tested by Bartlett’s test. The statistical evaluation of the data was carried out by one-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni test for pairwise comparisons or by the Kruskal-Wallis test followed by a post-hoc Dunn's test. Statistical significance was accepted at P < 0.05.

**Results**

**Expression of PSMA and affinity test in PC3- and LNCaP cells**

The expression of PSMA in the LNCaP- and PC3 cell lines was analysed in the protein fraction using Western blotting. As expected, PC3 cells did not express PSMA, however, high concentration of PSMA was detected in LNCaP cells (Fig. 1A). Accordingly, $^{177}$Lu-PSMA-617, displayed high potency uptake into LNCaP cells, but not into PC3 cells (Fig. 1B).

**Effect of PSMA-617 on PSMA expression**
Western blot analysis demonstrated that PSMA-617 tended to reduce, although not statistically significant, the PSMA concentration in LNCaP cells after 24 h of treatment, (n = 5) (one-way ANOVA) (Fig. 1C).

**PSMA-617 and cell death**

LDH release into the culture medium was quantified in LNCaP cells incubated with PSMA-617 (10, 50, 100 nM). Compared to vehicle-treated cells, PSMA-617 at either dose did not increase LDH release from the cells indicating that even high doses of PSMA-617 did not induce cell death (24 h: n = 12, 48 h: n = 10) (Fig. 2A).

**PSMA-617 and cell proliferation**

WST-1 analysis was carried out to detect the relative proliferation rates of LNCaP cells after their incubation with PSMA-617 for 24 (n = 12) or 48 h (n = 12). PSMA-617 (100 nM) significantly reduced proliferation of LNCaP cells after 24 h by 50% (P < 0.05) and 48 h by 26% (p < 0.01 (Fig. 2B). This effect was only detected in LNCaP cells but not in PC3 cells (data not shown), suggesting that the growth-inhibitory effect of PSMA-617 clearly depends on the PSMA expression. Additional experiments employing cresyl violet staining confirmed the anti-proliferative effects of PSMA-617 in LNCaP cells. Cells incubated in the absence of PSMA-617 (control group) showed an invasive growth, conglomerates of cell clusters, especially pronounced after incubation for 48 h. The cell numbers were lower in cells treated with PSMA-617 and the cells acquired an elongated morphology (Fig. 3).

**Mechanism of the anti-proliferative effects of PSMA-617 in LNCaP cells**

Tumour cells, which rapidly divide, contain high amounts of DNA. Labelling DNA with propidium iodide allows for fluorescence-based analysis of the cell cycle, as the amount of DNA doubles between G1 and G2 phases. Figure 4 shows double immunofluorescence staining for DNA (red) and PSMA (green) in LNCaP cells exposed to vehicle (controls) or PSMA-617 (100 nM) for 24 h. An intensive red fluorescent staining, irregular size and shape of the nuclei and multinucleate cells were observed in cells treated with vehicle (Fig. 4a). Treatment of LNCaP cells with PSMA-617 (100 nM) reduced qualitatively the DNA content, pointing to a lower rate of cell division (Fig. 4d). To further explore the effect of PSMA-617 on the cell cycle regulation, Western blotting was used to quantify the level of cyclin D1 and cyclin E1 in LNCaP cells incubated for 24 h with different concentrations of PSMA-617. PSMA-617 gradually decreased cyclin D1 (n = 5) and cyclin E1 (n = 5), a significant reduction (by 43 and 36%, respectively) was detected at concentration 100 nM (Fig. 5 upper panels). The cyclin-dependent kinase (CDK) inhibitors, p21 and p27, are important for the evaluation of the efficacy of anti-cancer drugs in the tumour therapy. The quantification of p21 (n = 5) and p27 (n = 9) levels in LNCaP cells treated with different concentration of PSMA-617 revealed, that p21 but not p27 was dose-dependently up-regulated (by 48%) (Fig. 5, lower panels).
Effect of PSMA-617 on uptake of $^{177}$Lu-PSMA-617 into LNCaP cells

Both $^{177}$Lu-PSMA-617 and its precursor, PSMA-617, bind to the PSMA on the cell membrane. Therefore, PSMA-617 can compete with the $^{177}$Lu-PSMA-617- binding to LNCaP cells and, consequently, reduce its cytotoxic effects. However, Fig. 6 shows that PSMA-617 concentrations up to 100 nM did not impair the $^{177}$Lu-PSMA-617 uptake into the LNCaP cells ($n=9$).

Effect of PSMA-617 on anti-cancer efficacy of $^{177}$Lu-PSMA-617 in LNCaP cells

To investigate whether the precursor, PSMA-617, interferes with cell death induced by $^{177}$Lu-PSMA-617, LNCaP cells were treated with $^{177}$Lu-PSMA-617 (100 KBq/well) combined with different concentrations of PSMA-617. PSMA-617 at 50 nM and 100 nM concentrations significantly potentiated the $^{177}$Lu-PSMA-617-induced LDH release from LNCaP cells after a 24 h incubation (each group $n=12$) (Fig. 7). Although $^{177}$Lu-PSMA-617 alone failed to induce cell death 48 h after incubation, strong cytotoxic effects were observed in cells incubated with both ligands (each group $n=8$) (Fig. 7).

Discussion

The PSMA-targeted radioligand, $^{177}$Lu-PSMA-617, is clinically tested in the therapy of PCa since 2015. Several clinical groups have reported on the successful treatment of advanced PSMA-positive PCa patients with $^{177}$Lu-PSMA-617, as evidenced by a significant reduction of PSA levels [7, 9, 10, 12]. In the present study, we demonstrate that the precursor, PSMA-617, used for the synthesis of $^{177}$Lu-PSMA-617, inhibits cell proliferation and cell growth of LNCaP cells via its binding to PSMA. The mechanisms of the anti-proliferative effects include the down regulation of cyclin D1 and cyclin E1 and the increase of the cyclin-dependent kinase inhibitor, p21, resulting in the cell cycle arrest in the G1 stage. Although PSMA-617 alone does not induce death of tumour cells, it significantly potentiates the cell death-promoting effect of $^{177}$Lu-PSMA-617 in LNCaP cells at concentrations which do not reduce the binding of $^{177}$Lu-PSMA-617.

Preparation of $^{177}$Lu-labelled PSMA-617

$^{177}$Lu-PSMA-617 has not yet been approved for the treatment of PCa patients in Europe. The tracer is currently evaluated in multi-centre trials and encouraging results have been achieved in treatment of mPCa patients so far [13]. $^{177}$Lu-PSMA-617 is usually prepared in the Radiopharmacy Laboratories of Departments of Nuclear Medicine. In principle, the chemical reaction of $^{177}$LuCl$_3$ with PSMA-617 is simple [14]. To obtain the $^{177}$Lu-PSMA-617 in very high radiochemical purity, several factors, such as pH, volume, the type of the buffer, the temperature, and the reaction time play an important role. Besides
these factors, the proportion of the precursor, PSMA-617, to $^{177}$LuCL$_3$ used for the synthesis is a key factor which ultimately determines the amount of free $^{177}$LuCL$_3$ in the final product. The unbound free $^{177}$LuCL$_3$ is radiotoxic and causes serious damage of numerous organs and tissues, such as the liver, heart and especially the kidney. Our preliminary experiments, in which 1, 3 or 10 µg of PSMA-617 per 1 GBq of $^{177}$LuCL$_3$ were used, have shown that 10 µg precursor per 1 GBq yields more than 99.99% of pure $^{177}$Lu-PSMA-617 (data not shown). The final product contains approximately 8 µg/ml of the unlabelled precursor. The precursor may interfere with the effect of $^{177}$Lu-PSMA-617, as both substances competitively bind to PSMA. Therefore, it was assumed so far, that the product should either be purified by chromatography methods, which entails a very complex and laborious process, or, alternatively, that one should use only a very slight excess of the precursor for the synthesis. With this study, however, we demonstrate that the unlabelled precursor up to 100 nM concentration does not interfere with the uptake of the $^{177}$Lu-PSMA-617, but even potentiates its death-promoting effects in PCa cells.

**Anti-proliferative effects of PSMA-617 in LNCaP cells**

The maintaining of cell turnover and the replacement of differentiated cells lost require the modulation of the balance between cell growth and cell death. Under pathologic condition, the balance between cell growth and cell death is impaired and such cells can transform into tumour cells. The key proteins controlling the cell cycle are cyclins, CDKs and CDK inhibitors. Although cyclin D1 is also involved in DNA repairing processes in some cell types, this cyclin supports cell proliferation of prostate cancer cells [15, 16]. Cyclin E1 promotes tumourigenesis in various cell types (for review see Hwang HC et al., 2005) [17]. Both cyclins play a critical role in the G1/S transition. After binding of cyclin D1 and cyclin E to the corresponding kinases, CDK4 and CDK2, respectively, and the formation of the maturation-promoting factors, they, together with several transcription factors, enable the expression of genes that regulate the entry of cells into the S phase [18]. The down-regulation of cyclin D1 and cyclin E by PSMA-617 prevents the formation and activation of the corresponding cyclin/CDK complexes and the maturation-promoting factors, which in turn arrests the G1/S transition. We have shown that PSMA-617 impaired the DNA duplication and, consequently, cell proliferation.

**p21 and p27 and the cell cycle in LNCaP cells**

The CDK inhibitors, p21 and p27, bind to cyclin-CDK complexes to inhibit their catalytic activity. p21 inhibits cyclin-CDK2, cyclin-CDK1, and cyclin-CDK4,6 complexes and thus, inhibits the cell cycle progression during G1 and S phases. The prognostic significance of the rate of p21 expression in PCa is controversial. A p21 overexpression was associated with poor clinical outcome [19]. On the other hand, an up-regulation of p21 was demonstrated to induce growth arrest in several PCa cell lines [20, 21]. p21 also inhibits the DNA replication via binding to the proliferating cell nuclear antigen (PCNA) [22]. Here, we report that, PSMA-617 up-regulates p21. Consequently, a number of tumour cells entered a G$_0$/quiescent state and did not continue to proliferate, an effect observed in the present study. The increase in p21 may also be intrinsically related to the reduced levels of cyclin E, as the E3 ubiquitin ligase complexes,
SCF^K2, CRL^CDT2 and APC/C^CDC20, promote the ubiquitylation and degradation of p21 only when it is bound by complexes of CDK2 with cyclin E or PCNA [22].

p27 binds and inhibits cyclin/CDK complexes and arrests cell cycle. Therefore, p27 is a putative tumour-suppressor and its reduced expression has been shown to correlate with poor prognosis in cancer patients [23, 24]. However, we did not observe any alterations in p27 levels after treatment of LNCaP cells with PSMA-617.

### Potentiation of the cell death – promoting effects of $^{177}$Lu-PSMA-617 by PSMA-617

PSMA-617 did not reduce the PSMA expression in LNCaP cells 24 h after treatment. This is an important finding, as PSMA is the target molecule for $^{177}$Lu-PSMA-617. The most prominent finding of this study is the considerable potentiation of the cell death-inducing activity of $^{177}$Lu-PSMA-617 by PSMA-617. Upon ligand binding, PSMA internalises via clathrin-coated pits and subsequent endocytosis results in an effective transportation of PSMA into the cell [25].

Highly efficient internalisation of PSMA has been shown in LNCaP cells after treatment with PSMA-617 [6]. The rate of exocytosis of $^{177}$Lu-PSMA-617 by PCa cells may be an important predictive factor for the outcome in terms of the therapy or prognosis in patients treated with the radioligand. The internalisation of the $^{177}$Lu-PSMA-617/PSMA- and PSMA-617/PSMA complexes is the prerequisite for their anti-cancer effects. Upon binding and intracellular internalisation of $^{177}$Lu-PSMA-617, tumour cells are destroyed by $^{177}$Lu through the delivery of the beta particle radiation. The underlying mechanisms may involve an effective alteration of the DNA structure and/or related molecular signalling pathways related to DNA repairing processes. In addition, PSMA-617 promotes cell-cycle arrest. The interference of PSMA-617 with the cell death results in anti-proliferative activity and substantial elimination of tumour cells. The approximate concentrations of PSMA-617 in patients, who receive 50 µg of the ligand, are 8–10 and 18–20 nM in blood and plasma, respectively. It is worth noticing that even a 5-times higher concentration of PSMA-617, used in our experiments, did not alter the uptake of $^{177}$Lu-PSMA-617 into LNCaP cells. Therefore, PSMA-617 administered to patients at the clinically relevant doses not only does not interfere with the uptake of $^{177}$Lu-PSMA-617 into PCa cells, it actually augments the cytotoxic effects of the radioligand.

Interestingly, $^{177}$Lu-PSMA-617 at a dose of 200 KBq/ml alone, which is the lowest dose inducing cell death in LNCaP cell, did not exert any cytotoxic effects after a 48 h exposure to the radioligand. We do not have any plausible explanation for this rather surprising finding. We assume that the majority of the radiosensitive cells were already killed during the 24 h exposure to the radioligand. The remaining cells most probably entered the G0/quiescent stage in which the tumour cells exhibit marked radioresistance. Nevertheless, the simultaneous treatment of LNCaP cells with the radioligand and its precursor, PSMA-617 did induce cell death. This finding substantiates the relevance of the PSMA-617 for the cytotoxic effects of $^{177}$Lu-PSMA-617 in PCa cells.
Conclusion

PSMSA-617 may considerably improve the radioligand therapy with $^{177}$Lu-PSMA-617 in patients suffering from mPCa harbouring genomic defects in DNA damage repair mechanisms. Such deleterious mutations affect the radiosensitivity of PCa cells and the patients are unlikely to respond to $^{177}$Lu-PSMA-617 alone [26].

Declarations

Compliance with Ethical Standards

Funding: no

Conflict of Interest: all authors declare no conflict of interest

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References


**Figures**
Figure 1

A: PSMA is highly expressed in LNCaP cells, but not in PC3 cells (n=3). B: LNCaP cells potently accumulate $^{177}$Lu-PSMA-617, however, no uptake of the radioligand was detected in PC3 cells. C: Western blot analysis of PSMA-617 in LNCaP cell treated with vehicle (V) (empty column) or 10, 50 and 100 nM PSMA-617 (solid columns) for 24 h. Representative blots and graphical analysis are shown. Data is
expressed as means ± SD. PSMA-617 does not alter PSMA expression in LNCaP cells (n=5) (one-way ANOVA).

Figure 2

Effect of PSMA-617 on cell death (upper panels) and proliferation of LNCaP cells (lower panels). Empty columns: cells treated with vehicle (V); solid columns: cells treated with PSMA-617. Exposure of LNCaP
cells to PSMA-617 for 24 (n=12) or 48 h (n=10) did not induce cell death, but significantly reduced their proliferation rates (WST-1 assay, n=12 each group). Results are expressed as means ± SD. Statistical comparison with vehicle-treated cells: ** P<0.01, calculated by Kruskall-Wallis test (24 h) or by one-way ANOVA (48 h) followed by a post-hoc Dunn’s test and Bonferroni test, respectively.

Figure 3
Effects of a 24 and 48 h incubation of LNCaP cell with PSMA-617 on their morphology. LNCaP cells treated with vehicle rapidly proliferate, display irregular size and shape and nuclear atypia, especially after incubation for 48 h. PSMA-617 decreased cell proliferation and, interestingly, the cells acquired an elongated morphology and formed processes (40 x magnification).

![Figure 4](image)

**Fig. 4**

**Figure 4**
Immunofluorescence staining for DNA (red, a,d) and PSMA (green, b,e) in LNCaP cells incubated with vehicle (left panels) or with 100 nM PSMA-617 (right panels) for 24 h. Vehicle-treated cells showed large amounts of DNA (intense red staining) and irregular form of nuclei (a). PSMA-617 did not alter the intensity of staining for PSMA (b,e). No overlapped immunoreactivity for DNA (localised in the nuclei) and PSMA (localised in the cytoplasm or cell membrane) was observed (c,f).
Western blot analysis of cyclin D1 (n=5) and cyclin E1 (n=5) (upper panels) and p21 (n=5) and p27 (n=9) (lower panels) in LNCaP cells treated with vehicle (controls) (empty columns) or exposed to various concentration of PSMA-617 (solid columns). Representative blots and graphical analysis are shown. Results are expressed as the means ± SD. Statistical comparison with controls: *P<0.05, **P<0.01, calculated by one-way ANOVA followed by a post-hoc Bonferroni test.

Figure 6

Effects of PSMA-617 (10, 50 and 100 nM) (solid columns) on the uptake of $^{177}$Lu-PSMA-617 into LNCaP cells. Empty column: cells treated with vehicle. No significant differences among the groups were found (one-way ANOVA).
Figure 7

Assessment of cell death in LNCaP cell incubated with vehicle (V) (empty columns) or with $^{177}$Lu-PSMA-617 in the presence (solid columns) or absence of PSMA-617 (hatched columns) for 24 (n=12) or 48 h (n=10). PSMA-617 potentiated the cell death-promoting effects of $^{177}$Lu-PSMA-617. Results are expressed as the means ± SD. Statistical comparisons with the vehicle-treated group: *P<0.05; **P<0.01
and ***P<0.001, and with the group exposed to $^{177}$Lu-PSMA-617: †P<0.05; ††P<0.01 and †††P<0.001, calculated by Kruskal-Wallis test followed by a post-hoc Dunn´s test.