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# Prostate Specific Membrane Antigen as Biomarker and Therapeutic Target for Prostate Cancer

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## 1. Introduction

The Prostate Specific Membrane Antigen (PSMA) is considered to be the most well established target antigen in prostate cancer, since it is highly and specifically expressed at all tumor stages on the surface of prostate tumor cells. This chapter outlines the structure, function, and expression of PSMA, its relevance as prognostic and diagnostic biomarker, and different therapeutic approaches targeting this antigen.

## 2. The Prostate Specific Membrane Antigen (PSMA)

### 2.1 Structure, function and expression

PSMA, also known as Glutamate Carboxypeptidase II (GCPII, EC 3.4.17.21), N-acetyl-α-linked acidic dipeptidase I (NAALADase) or folate hydrolase, is a type II transmembrane protein, which is anchored in the cell membrane of prostate epithelial cells (Carter et al., 1996; Pinto et al., 1996). In 1998, the gene encoding PSMA was mapped to chromosome 11p11-p12, where it encompasses 19 exons spanning about 60 kb of genomic DNA (O'Keefe et al., 1998). The cDNA of PSMA codes for a glycoprotein of 750 amino acids (aa) with a molecular mass of about 100 kDa. The protein is partitioned into a small intracellular domain of 19 aa, a transmembrane domain of 21 amino acids, and a large extracellular domain of 707 aa. Crystallization data revealed that the extracellular domain of PSMA folds into three distinct structural and functional domains: a protease domain (aa 56-116), an apical domain (aa 117-351), and a C-terminal domain (aa 592-750). Furthermore, it was shown that PSMA is expressed as a compact homodimer, which is highly glycosylated with oligosaccharides accounting up to 25% of the molecular weight (Davis et al., 2005; Mesters et al., 2006).

PSMA contains a binuclear zinc site and can act as glutamate carboxypeptidase or folate hydrolase, catalyzing the hydrolytic cleavage of glutamate from poly-γ-glutamated folates (Ghosh & Heston, 2003). Therefore, PSMA is thought to play a role in the folate metabolism of the prostate. This hypothesis is supported by recent studies, where PSMA expression correlated with proliferation and folate uptake of PSMA transfected cells (Yao & Bacich, 2006; Yao et al., 2009).

In contrast to other prostate-related antigens, like prostate specific antigen (PSA), prostate acidic phosphatase (PAP) or prostate secretory protein (PSP), PSMA is not secreted into

circulation. Instead of that, PSMA undergoes constitutive internalization, which is about threefold enhanced after antibody binding (Liu et al., 1998). It is therefore suggested that PSMA has transport function and that anti-PSMA antibodies might act as surrogates for a yet unknown ligand. The endocytic pathways of PSMA after antibody binding were specified in a recent study and comprise clathrin-mediated endocytosis, macropinocytosis, and clathrin-, calveolae-independent endocytosis (Liu et al., 2009b).

To examine the PSMA expression in the prostate, immunohistochemical analyses were performed. In a study with prostate tissue specimen from 184 patients with prostate cancer, the percentage of PSMA positive stained cells averaged about 69.5% (range 20%-90%) in the benign epithelium, 77.9% (range 30-100%) in high grade prostatic intraepithelial neoplasia (PIN) and was highest in adenocarcinomas with a mean of 80.2% (range 30-100%). In contrast, tumor stroma, urothelium, normal vasculature and, with rare exceptions, basal cells were PSMA negative (Bostwick et al., 1998). Other immunohistochemical studies demonstrated a heterogeneous, weak to moderate staining of normal prostate epithelial cells, and a homogeneous, extensive staining of prostate adenocarcinomas and metastases (Silver et al., 1997; Wolf et al., 2010a).

PSMA expression is highly organ specific. An extraprostatic expression was only detected in secretory cells of the salivary glands (Israeli et al., 1994; Troyer et al., 1995; Wolf et al., 2010a), in cryptic cells of the duodenal brush border (Chang et al., 1999; Wolf et al., 2010a), and in a subset of proximal renal tubules (Liu et al., 1997; Silver et al., 1997; Chang et al., 1999). In some studies, an additional expression was found in the brain and in the colon, but these results are controversially discussed (Troyer et al., 1995; Silver et al., 1997; Chang et al., 1999; Sacha et al., 2007). Nonetheless, potential side effects of anti-PSMA therapeutics against PSMA expressing normal organs were not described until today.

Interestingly, PSMA is also discussed as an unique anti-angiogenetic target, since it is expressed in the neovascularization of numerous solid tumors (bladder, kidney, breast, pancreas, lung, melanoma), but not in normal blood vessels (Liu et al., 1997; Chang et al., 1999; Chang et al., 2001; Baccala et al., 2007). In this respect, it was found that PSMA regulates cell invasion and tumor angiogenesis by modulating integrin signal transduction in endothelial cells (Conway et al., 2006).

### 2.2 PSMA as prognostic and diagnostic biomarker

Generally, prostate carcinoma tissues show a higher PSMA expression and an increased enzymatic activity of PSMA compared with normal prostate and benign prostate hyperplasia (BPH) tissues (Lapidus et al., 2000; Burger et al., 2002). Therefore, the question was raised, if PSMA might serve as a valuable biomarker for the management of prostate cancer.

Indeed, in different studies a direct correlation between PSMA expression and the Gleason score, which is used for the staging of prostate cancer, was determined for adenocarcinomas (Su et al., 1995; Kawakami & Nakayama, 1997; Burger et al., 2002). Moreover, an upregulation of PSMA was shown in tumor cells of patients with hormone-refractory prostate cancer (Wright et al., 1996; Kawakami & Nakayama, 1997). In a study with tissue specimen from 136 patients it was demonstrated that PSMA can serve as a prognostic biomarker, because it significantly correlates with adverse prognostic factors, like tumor grade, pathological stage, aneuploidy, and biochemical recurrence, and therefore independently predicts disease outcome (Ross et al., 2003).

Recently, a new splice variant (PSM-E) was described, which is specifically overexpressed in prostate carcinomas and which correlates with the Gleason score (Cao et al., 2007). PSM-E, which is expressed in the cytoplasm, could account for the lack of correlation between histological positive staining of anti-PSMA antibodies with clinical grade (stage) (Mannweiler et al., 2009).
Despite of such controversies, it is apparent that the enhanced expression and enzymatic activity of PSMA in aggressive prostate tumors is indicative of a selective advantage on the part of cells expressing it and that it contributes to prostate carcinogenesis.
PSMA was found to associate with the anaphase-promoting complex and to induce chromosomal instability (Rajasekaran et al., 2008). Moreover, PSMA favoured prostate cancer development in a permissive folate environment (Yao & Bacich, 2006; Yao et al., 2009). One mechanism by which PSMA contributes to prostate tumor growth is its ability to activate IL-6 and CCL5 synthesis. These cytokines acted synergistically to enhance the growth of LNCaP cells by activating the MAPK pathway (Colombatti et al., 2009).
Taken together, assessment of PSMA levels, either alone or in combination with PSA status, might prove useful in future for the diagnosis of metastatic prostate cancer, risk assessment, and the prognosis of disease outcome.

### 2.3 PSMA as therapeutic target

Specific characteristics of PSMA concerning its structure, function and expression make it an ideal candidate as a target antigen for the treatment of advanced prostate cancer. (1) Its high and specific expression on the prostate cancer cell surface and the fact that it is not shed into the circulation allows an effective systemic delivery of PSMA targeting therapeutics. (2) Its high organ specificity leads to a minimal binding of anti-PSMA drugs to normal organs and therefore to a maximal reduction of potential side effects. (3) Its expression at all tumor stages enables a therapeutic intervention at any time of the disease. (4) Its internalization after ligand binding can be used for the targeted delivery of intracellular acting drugs. (5) Its enzymatic activity allows the cleavage of prodrugs to active molecules on the surface of prostate cancer cells.
Many preclinical and clinical studies were performed in the last years, which used PSMA as target antigen. They include radioimmunotherapy, the use of immunotoxins, targeted virotherapy, retargeting of immune cells, PSMA vaccination, prodrug activation, photodynamic therapy, and PSMA targeting nanoparticles.

#### 2.3.1 Anti-PSMA radioimmunotherapy

Radioimmunoconjugates generally consist of an antibody moiety as target domain coupled to a therapeutic radionuclide (alpha- or beta-particle) with the biologic effect of high linear energy transfer (LET) radiation. Compared to conventional radiotherapy, radioimmunoconjugates allow the targeted delivery of reduced radiation doses to the tumor, which ideally leads to a reduction of side effects. Moreover, the radiation of a radioimmunoconjugate is not restricted to cells presenting the target antigen. It also affects neighboring cells with a heterogeneous antigen expression or insufficient vascularization, which is so called “bystander effect” (Rzeszowska-Wolny et al., 2009).
An initial immunoscintigraphic approach targeting PSMA was done with the $^{111}$Indium ($^{111}$In) labeled anti-PSMA monoclonal antibody 7E11 (Capromab Pendetide (Prosta Scint®), Cytogen, Philadelphia, PA) (Kahn et al., 1994; Sodee et al., 1996; Kahn et al., 1998). With this

radioimmunoconjugate a higher sensitivity was reached in the imaging of prostate cancer soft tissue metastases compared to Computed Tomography (CT) or Magnetic Resonance Tomography (MRT) (Murphy et al., 1998). Therefore, Prosta Scint® received approval from the U.S. Food and Drug Administration (FDA) for the detection and imaging of prostate cancer soft tissue metastases (Rosenthal et al., 2001). The reason, why Prosta Scint® is only suitable for the detection of soft tissue metastases, is based on the fact that the 7E11 antibody recognizes an intracellular epitope of PSMA. Therefore, it can not bind to viable tumor cells, but only to PSMA molecules in damaged, dead or dying cells. Lymph node or bone metastatic lesions tend to be relatively small and do not characteristically own a high percentage of apoptotic or necrotic cells. Indeed, in a radioimmunotherapeutic trail with the $^{90}$Yttrium-($^{90}$Y) labeled 7E11, no objective or biochemical remissions could be measured (Deb et al., 1996; Kahn et al., 1999).

Therefore, monoclonal antibodies, which bind to the extracellular domain of PSMA, were used for the construction of radioimmunoconjugates in subsequent studies. Three anti-PSMA antibodies, called 3/A12, 3/E7, and 3/F11, which recognize different extracellular epitopes, were labeled with $^{64}$Copper ($^{64}$Cu) and used for Positron Emission Tomography (PET) imaging of human prostatic tumors in the SCID mouse xenograft model. Whereas excellent tumor uptakes of all antibodies between 31.6 and 35.1%ID/g were measured in tumors of the PSMA expressing androgen-independent LNCaP subline C4-2, only activities at background levels were detected in PSMA negative DU 145 control xenografts (Elsasser-Beile et al., 2009; Alt et al., 2010). In a first preclinical experiment, the antibody 3/F11 was labeled with the beta particle emitter $^{177}$Lutetium ($^{177}$Lu) and was used for the radioimmunotherapy of mice bearing C4-2 tumors xenografts. Biodistribution studies revealed a tumor to muscle ratio of more than 70 and a tumor to blood ratio of more than 4.5 after 72 h. Treatment of mice with the conjugate resulted in tumor growth inhibition and in a more than 2-fold enhanced survival after application of a single dose of 1 MBq. However, in this study the therapeutic window was small, because mice treated with a dose of 2 MBq apparently died of myelotoxicity (Behe et al., 2011).

Another panel of antibodies binding to extracellular PSMA (J415, J533, and J591) was tested in preclinical and clinical trials for radioimmunotherapy. J591 was labeled with the alpha particle emitter $^{213}$Bismuth ($^{213}$Bi), which is well suited for the radiation of single cell neoplasms and micrometastases in a range between 0.07 and 0.1 mm. [$^{213}$Bi]J591 caused a high cytotoxicity against LNCaP cells *in vitro*. *In vivo*, a significant improvement of tumor free survival in nude bearing LNCaP tumors was reached, which was accompanied by a significant reduction of PSA serum levels (McDevitt et al., 2000). In another study, the biodistribution of the $^{131}$Iod ($^{131}$I) labeled antibodies J591, J415, and 7E11 was examined. High tumor uptakes were measured with all antibodies in LNCaP tumor xenografts, which were up to 20-fold higher than in PSMA negative DU 145 or PC-3 tumors. Autoradiographic studies showed that the extracellular binding antibodies J415 and J591 preferentially recognized areas of viable tumor cells, whereas the intracellular binding antibody 7E11 mainly detected necrotic tumor areas (Smith-Jones et al., 2003).

The antibody J591 was chosen for further radioimmunotherapeutic experiments. To reduce a possible immunogenicity of the mouse antibody in prostate cancer patients, J591 was humanized by site directed mutagenesis of putative B- and T-cell epitopes of the variable domains and by exchanging the mouse constant domains into human ones. The humanized antibody J591 (huJ591) was then labeled with $^{131}$I and $^{90}$Y. Doses of 3.7 to 11.1 MBq $^{131}$I-hu591

and of 3.7 to 4.7 MBq $^{90}$Y-hu591 led to a reduction of mean tumor volumes between 15 and 90% in nude mice bearing LNCaP tumors. Additionally, both radioimmunoconjugates effected an 2 to 3-fold increase of median survival relative to untreated controls (Vallabhajosula et al., 2004).

In a first phase I clinical trail, prostate cancer patients initially received the $^{111}$In-labeled huJ591 for immunoscintigraphy followed by application of $^{90}$Y-huJ591 for therapy. With $^{111}$In-huJ591 total body images demonstrated a significant metabolism of the radioimmunoconjugate in the liver and to a lesser extend in the kidneys and spleen. Additionally, bone and soft tissue metastases were efficiently targeted. One week later, $^{90}$Y-huJ591 was applicated and a maximal tolerated dose (MTD) of 17.5 mCi/m² could be determined. PSA stabilisation was noted in 6/29 patients and 2 patients showed a PSA decline of 70 and 85% lasting 8 and 8.6 months, respectively (Milowsky et al., 2004). Another cohort of 35 patients received $^{177}$Lu-labeled huJ591 at doses between 10 and 75 mCi/m². Blood and urinary pharmacokinetics were similar to those of $^{90}$Y-huJ591. But the MTD of 70 mCi/m² was about 4-fold higher. Patients treated with 75 mCi/m² $^{177}$Lu-huJ591 developed grade 3 and 4 thrombocytopenia and grade 4 neutropenia, but retreatment with 30 mCi/m² was well tolerated. In 4 patients a PSA decline of more than 50% and in 16 patients a PSA stabilisation was noted (Milowsky et al., 2004). In both clinical trials with radiolabeled huJ591 myelosuppression was dose-limiting. Whereas no clear correlation between myelotoxicity and therapeutic dose was determined for $^{90}$Y-huJ591, myelotoxicity and especially thrombocytopenia correlated well with the applicated doses and the bone marrow doses for $^{177}$Lu-huJ591 (Vallabhajosula et al., 2005).

To verify PSMA as a target for an anti-angiogenesis therapy, $^{111}$In-huJ591 was used for the imaging of known metastases in patients with different solid tumors. Indeed, this radioimmunoconjugate showed a high uptake in metastases of 7/10 kidney cancer patients, 4/4 colon carcinoma patients, 3/3 lung cancer patients, 3/3 pancreatic cancer patients, 1/3 bladder cancer patients, 2/3 breast cancer patients, and 1/1 melanoma patient (Milowsky et al., 2007).

### 2.3.2 Anti-PSMA immunotoxins

PSMA was also used as a target for the generation of immunotoxins against prostate cancer. Immunotoxins are constructs, where a PSMA binding domain (antibody, antibody fragment, RNA aptamer, peptide) is coupled to a toxin domain. The toxin domain is targeted by the PSMA binding domain to the prostate cancer cell and is cytotoxic after internalization.

Ricin from *Ricinus communis* acts as a very common toxin for the construction of immunotoxins. It consists of the ricin A chain and the ricin B chain held together by a disulfide bond. The ricin A chain is the enzymatically active subunit, which inactivates the protein biosynthesis machinery by irreversible hydrolysis of the N-glycosidic bond of an adenine (A4324) within the 28S rRNA. The ricin B chain binds ubiquitous to cell surface structures and facilitates membrane translocation and intracellular trafficking of the ricin A chain (Sandvig et al., 2002).

The first generation of anti-PSMA immunotoxins was made by chemically coupling of anti-PSMA antibodies to ricin A. An immunotoxin consisting of J591 and ricin A elicited a 50% reduction in cell viability ($IC_{50}$) of LNCaP cells at a concentration of about 265 pM and showed a more than 5000-fold potentation of cytotoxicity compared to the unconjugated

ricin A chain (Fracasso et al., 2002). For another ricin A-based immunotoxin with the rat monoclonal antibody E6 an $IC_{50}$ value of 60 pM was measured. Additionally, a significant inhibition of LNCaP tumor growth in the mouse xenograft model was reached with this molecule (Huang et al., 2004).

In a recent study, the humanized antibody huJ591 was linked to the plant toxin saporin. Saporin is produced is seeds and leaves of the plant *Saponaria officinalis* and belongs to class I ribosome inactivating proteins. With the saporin-based immunotoxin, a percentage of 60.3% apoptotic cells and an $IC_{50}$ value of 140 pM was determined on LNCaP cells after 72 h incubation. Furthermore, a significant inhibition of tumor growth was measured in the LNCaP tumor xenograft model. However, due to its high molecular weight of about 280 kDa, the immunotoxin is thought to have a high immunogenicity and a limited diffusion into tumor tissues. Therefore, further development is focused on saporin-based constructs of smaller size less inherent immunogenicity (Kuroda et al., 2010).

The anti-PSMA antibody J591 was also used for the construction of an immunotoxin containing the melittin-like peptide 101 from honey bee (*Apis mellifera*) venom. This construct successfully inhibited the growth of LNCaP-LN3 tumors and led to a slight improvement of the median survival of treated mice. However, the high affinity of peptide 101 to lipid bilayer membranes also led to a high non-specific cytotoxicity (Russell et al., 2004).

A further immunotoxin was generated by coupling huJ591 to the chemotherapeutic drug maytansinoid 1 (DM1) (Henry et al., 2004), which is a microtubule-depolymerizing analogue of maytansine (Chari et al., 1992). Maytansine is a naturally occurring ansa macrolide and was evaluated as a chemotherapeutic agent in the 1970s and 1980s. Unfortunately, maytansine caused severe, dose-limiting gastrointestinal and central neurological toxicities and was therefore not developed further (Blum et al., 1978). With the DM1-based anti-PSMA immunotoxin, called MLN2704, a growth delay of CWR22Rv1 tumor xenografts of more than 100 days was achieved at an optimized dosage schedule of 60 mg/kg every 14 days (Henry et al., 2004). MLN2704 was also tested in a clinical phase I trial in 9 patients with prostate cancer. Two of these patients, treated with 264 or 343 mg/m² immunotoxin respectively, had a more than 50% decrease in their PSA serum level. Additionally, the patient treated with 264 mg/m² showed a measurable tumor regression (Galsky et al., 2008).

In another approach, a fully human anti-PSMA antibody was generated in transgenic mice and was conjugated to monomethylauristatin E (MMAE), which is a potent inhibitor of tubulin polymerization. With this construct $IC_{50}$ values of 83 pM on LNCaP cells and of 65 pM on C4-2 cells could be determined. Furthermore, a significant improvement of the median survival of tumor bearing mice 9-fold relative to the controls was reached without any signs of toxicity. Interestingly, 2/5 animals treated with a maximal dose of 6 mg/kg immunotoxin had no detectable tumor or measurable PSA at day 500 and could therefore considered as cure (Ma et al., 2006).

Obstacles of chemically conjugated immunotoxins to be optimal therapeutic agents comprise a high immunogenicity, a possible influence of chemical modifications on antigen binding, and inhomogeneous preparations. This can, at least in part, be overcome by second generation, recombinant immunotoxins.

For the construction of the first recombinant immunotoxin against PSMA, an anti-PSMA single chain antibody fragment (scFv), consisting of one variable domain of the heavy chain ($V_H$) and one variable domain of the light chain ($V_L$) connected by a flexible linker, was generated from the monoclonal antibody 3/A12 by phage display. This scFv was called A5.

As toxin domain, the truncated form of *Pseudomonas* exotoxin A (PE40), consisting of the transmembrane domain and the enzymatically active domain of the toxin, was used. The virulence factor *Pseudomonas* Exotoxin A from the human pathogenic bacterium *Pseudomonas aeruginosa* is able to ADP-ribosylate the eukaryotic elongation factor 2 (eEF-2) of a target cell, which leads to the inhibition of protein biosynthesis and finally to apoptosis (Wolf & Elsasser-Beile, 2009). The bacterially expressed anti-PSMA immunotoxin, called A5-PE40, specifically bound to prostate cancer cells with $IC_{50}$ values in the low pM range. Moreover, it induced a significant growth inhibition of C4-2 tumors in the SCID mouse xenograft model (Wolf et al., 2006; Wolf et al., 2008).

A similar immunotoxin was recently generated by using the scFv D7 from the anti-PSMA antibody 3/F11. This immunotoxin, termed D7-PE40, also showed a high binding to C4-2 cells and led to a significant growth inhibition of subcutaneously implanted tumors. In toxicity studies, D7-PE40 was well tolerated in mice at single, but at higher doses the immunotoxin was lethal. Blood analyses indicated that the death of the animals was based on a severe hepatotoxicity, which was marked by increased aspartate transaminase (AST) and alanine transaminase (ALT) serum levels. Histopathological examinations revealed a marked damage of the hepatic parenchyma with disappearance of sinusoidal structures based on apoptosis and vacuolar degeneration of hepatocytes (Wolf et al., 2010b). Hepatotoxicity is a very common side effect of *Pseudomonas* Exotoxin A based immunotoxins and is presumably attributed to a TNF-alpha release of Kupffer cells (Onda et al., 1999). Different strategies are therefore under investigation to reduce the hepatotoxicity, e.g. by PEGylation or lowering of the isoelectric points of the immunotoxins (Onda et al., 2001). Another main research comprises the reduction of the immunogenicity by elimination of immunodominant B-cell epitopes (Onda et al., 2006).

Alternatively to antibodies or antibody fragments, RNA aptamers can be used for the construction of immunotoxins, which are considered to be advantageous with respect to a higher stability, ease of synthesis and lower production costs. One immunotoxin was constructed by coupling of an anti-PSMA RNA aptamer to the plant toxin gelonin from *Gelonium multiflorum*, which has high N-glycosidase activity on the 28S RNA unit of eukaryotic ribosomes. This molecule was found to be toxic against prostate cancer cells with an $IC_{50}$ value of 27 nM (Onda et al., 2006).

Recently, a chemical ligand, termed 2-[3-(1,3-dicarboxypropyl)ureido] pentanedioic acid (DUPA), was synthesized that selectively binds to PSMA (Kularatne et al., 2010). After coupling to different chemotherapeutic drugs, this molecule was capable to mediate the targeted killing of LNCaP cells (Kularatne et al., 2010).

### 2.3.3 Targeted virotherapy

In a recent study, measles viruses of a live attenuated strain were used for a targeted anti-PSMA virotherapy (Liu et al., 2009a). Measles viruses are very effective against a variety of tumor types, including prostate cancer (Blechacz & Russell, 2008; Msaouel et al., 2009). Generally, measles viruses infect host cells via one of two measles receptors, CD64 or SLAM. CD64 is ubiquitously present on the surface of human cells, whereas SLAM is expressed on immune cells. The viruses take their oncolytic effect by induction of an extensive intracellular fusion between infected cells and neighboring cells to form non-viable multinucleated structures (syncytia). For the construction of the virotherapeutic conjugate, called MVG-αPSMA, the anti-PSMA antibody huJ591 was coupled to a coat protein of

measles virus, in which alanine substitutions of specific residues ablated the viral interaction with CD64 and SLAM. After propagation and infection, a MVG-αPSMA mediated cytopathic killing of PSMA expressing LNCaP and PC3/PIP cells was detected. Moreover, a regression or growth inhibition of tumor xenografts could be achieved (Msaouel et al., 2009). A crucial obstacle for a future clinical use of MVG-αPSMA could be pre-existing anti-viral antibodies in patients, who have been vaccinated or infected by wild type measles viruses. These antibodies might quickly neutralize the virus domain after application. Therefore, efforts are undertaken to circumvent the problem, e.g. by intratumoral application of the construct, by hiding the virus in cell carriers, or by the use of immunosuppressive drugs to dampen the patient's immune response (Liu et al., 2009a).

### 2.3.4 Retargeting of immune cells

The therapeutic concept of immune cell retargeting comprises the activation of T lymphocytes for the targeted cytolysis of tumor cells. For the retargeting of prostate cancer cells via PSMA, two strategies were pursued: the construction of diabodies and the generation of fusion receptors.

Generally, diabodies for T cell retargeting consist of two antibody domains. One domain binds to the tumor antigen and the other one to a T cell activating antigen. The diabody builds a bridge between the tumor cell and the immune effector cell, which then triggers the cytotoxic responses that include perforin and granzyme release.

Anti-PSMA diabodies were constructed by fusing the anti-PSMA scFvs A5 or D7 to a scFv against the CD3 T cell receptor. With these constructs, a retargeting of CD4+ and CD8+ blood lymphocytes with subsequent lysis of C4-2 cells was obtained. Moreover, a significant inhibition of C4-2 tumor growth could be achieved (Buhler et al., 2008; Buhler et al., 2009; Fortmuller et al., 2011).

A fusion receptor targeting PSMA, also referred to as chimeric antigen receptor (CAR), was generated by coupling an anti-PSMA scFv to the zeta-chain of the CD3 T-cell receptor. The CAR can be expressed at the surface of CAR-transfected T cells. Then the CAR can recruit and activate the T cell by binding to PSMA. This mechanism is independent of a human leukocyte antigen expression. Using CAR transfected peripheral blood lymphocytes, a specific killing of PSMA-expressing prostate cancer cells and an elimination of orthotopically or subcutaneously implanted PSMA-positive tumors was reached (Gade et al., 2005). In a subsequent study, the effects of CAR could be optimized by adding combined CD28 and 4-1BBL costimulatory signaling domains. With this strategy, an enhanced cytokine release, a higher *in vivo* T cell survival, and an enhanced anti-tumor activity could be measured in tumor bearing SCID mice (Zhong et al., 2010).

A similar fusion receptor against PSMA, designated as chimeric immunoglobulin T-cell receptor (IgTCR), was also used for the retargeting of immune cells. IgTCR consists of an anti-PSMA scFv from the monoclonal antibody 3D8 and a signaling portion of the CD3 zeta chain. IgTCR transfected T-cells were activated after PSMA binding, which was followed by cytokine release and specific lysis of the prostate cancer cells. Additionally, this molecule showed a high anti-tumor activity in a mouse xenograft model (Ma et al., 2004).

### 2.3.5 PSMA vaccination

Vaccination with PSMA peptides, which aims for boosting the patient's immune response against PSMA expressing tumor cells, represent another weapon in the battle against prostate cancer. One approach utilizes the patient's dentritic cells (DCs), to present PSMA

peptides in association with the MHC class I peptide complex to naïve cytotoxic T lymphocytes (CTL) (Melief, 2008). For DC preparation, patients were leucophoresed, and peripheral blood mononuclear cells (PBMCs) were isolated. Adherent cells were differentiated with GM-CSF and IL-4. Then the obtained DCs were pulsed with recombinant PSMA peptides, which were identified in function of their ability to recruit CTLs and to be recognized as CTL targets. In a first phase I/II clinical trial involving 33 prostate cancer patients, 9 partial responders were identified with an average response duration of 225 days (Tjoa et al., 1998). In a phase II study, 2/33 patients with hormone refractory metastatic disease showed a complete response and another 6 patients a partial response (Murphy et al., 1999). In another clinical trial with 37 patients, one complete and 10 partial responses were identified (Salgaller et al., 1998; Tjoa et al., 1999).

Two subsequent protocols used DCs to present a PSMA peptide in combination with peptides from other tumor antigens to treat hormone-refractory patients. In one study, CTL responses and transient decline of serum PSA was observed in 4/8 patients, who received 4 intradermal vaccinations every other week (Fuessel et al., 2006). In the other study, 3 patients were administered with 6 vaccines intradermally at biweekly intervals and showed partial remissions. However, no CTL response against the PSMA peptide could be observed (Waeckerle-Men et al., 2006). The same PSMA peptide was loaded as a single peptide to PBMCs, which were used to treat 12 patients with hormone-resistant tumors. However, no clinical advantages were observed and no immune responses were detected in this trial (Knight et al., 2009).

A further development was the transfection of DCs with plasmids containing the extracellular domain of PSMA to activate autologous lymphocytes in an *in vitro* model. Indeed, PSMA-expressing DCs were able to generate antigen-specific cytotoxic T cell responses (Mincheff et al., 2003).

In a recent study, replication deficient adenoviruses were used to introduce a truncated form of PSMA and the T cell stimulatory molecule 4-1BBL into murine DCs. After infection of the DCs, PSMA-specific proliferative responses and an upregulation of CD80 and CD86 costimulatory molecules were detected. Moreover, vaccination of mice with the transfected DCs induced a potent protective and therapeutic anti-tumor immunity (Kuang et al., 2010).

Other strategies for PSMA vaccination are the application of DNA plasmids or viral immunizations. In a phase I study, prostate cancer patients were intradermally immunized with an expression plasmid or a replication-defective adenoviral vector bearing the PSMA gene according to six different drug regimens. All vaccinations were well tolerated and no immediate- or long-time side effects were reported (Mincheff et al., 2000). Anti-PSMA antibodies were found in 21% of patients at baseline and in 12-50% of patients at longitudinal time points ranging from 3 to 36 months after immunization (Todorova et al., 2005).

In a preclinical study it was shown that immunization of mice with xenogenic PSMA protein followed by a boosting with a vector that encoded autologous PSMA gave the best protection (Mincheff et al., 2006). These data provided the basis for a clinical study with DNA plasmid vaccines, in which 36 patients with recurrent prostate cancer received three vaccinations with mouse or human PSMA. Vaccination was well tolerated and PSA serum levels were maximally reduced at the highest dose level (Gregor et al., 2007).

### 2.3.6 Prodrug activation

The glutamate carboxy peptidase activity of PSMA can be used for the activation of prodrugs to a fully active compound on the surface of prostate cancer cells. Different

methotrexate-based peptide analoges were screened to identify PSMA selective substrates that are stable to unspecific hydrolysis in human and mouse plasma. Analogs containing α-linked or γ-linked glutamic or aspartic acids were most efficiently hydrolyzed by PSMA to release the cytolytic anti-metabolite methotrexate. As a consequence thereof, these analoges showed the highest cytotoxicity against PSMA-expressing prostate cancer cells (Mhaka et al., 2004). In a subsequent study, these peptides were coupled to a cytotoxic analogue of the plant toxin thapsigargin that induces apoptosis by inhibition of the endoplasmatic reticulum Ca-ATPase pump. PSMA hydrolysis of these peptide prodrugs led to a cytotoxicity against PSMA-positive prostate cancer cells that was 10- to 60-fold higher than against PSMA-negative ones. One of these prodrugs was also tested in mice with CWR22H xenografts and elicited tumor growth delay or tumor regressions following a single 3-day or 10-day course of administration (Mhaka et al., 2006).

### 2.3.7 Photodynamic therapy

The lack of specific delivery of photosensitizers, chemical compounds that can be excited by light of a specific wavelength for the destruction of tumor tissues, represents a significant limitation for photodynamic therapies (PDT). Therefore, a conjugate was generated containing the photosensitizer pyropheophorbide-a and a PSMA inhibitor for the treatment of prostate cancer. This construct demonstrated a high and specific cytotoxicity against LNCaP cells after irradiation, whereas PSMA-negative PC-3 cells remained unaffected. PDT-mediated effects of the photodynamic conjugate were extensively studied and involved cell membrane permeabilization, rapid disruption of microtubules (α-/β-tubulin), microfilaments (actin), and intermediate filaments (cytokeratin 8/18) in the cytoplasm, activation of caspase-3, -8, and -9, Poly [ADP-ribose] Polymerase (PARP)-cleavage, and DNA fragmentation (Liu et al., 2009c; Liu et al., 2010a; Liu et al., 2010b).

### 2.3.8 Nanoparticles targeting PSMA

Nanotechnology represents a new alternative for the treatment of prostate cancer. The production of nanoparticles enables the targeted delivery and controlled release of thousands of drug molecules per vehicle into the tumor cells and is a promising strategy to overcome the lack of specificity and limited efficacy of conventional chemotherapeutic agents.

One of the first therapeutic nanoparticle against prostate cancer was a docetaxel-encapsulated nanoparticle formulated with biocompatible and biodegradable poly(D,L-lactic acid-co-glycolic acid)-block-poly(ethyleneglycol) copolymer (PLGA-b-PEG), which surface was derivatized with the anti-PSMA RNA-aptamer A10. With this construct, an enhanced cytotoxicity, compared to non-targeted nanoparticles that lack the aptamer, was shown. After a single intratumoral injection, the nanoparticle elicited a complete tumor reduction in 5/7 mice with LNCaP tumor xenografts of about 300 $mm^3$ in size. The survival rate of these mice in a 109 days study was 100%, compared to 57% of mice treated with the non-targeted nanoparticle and to 14% of mice treated with docetaxel alone (Farokhzad et al., 2006). In a subsequent study, the cisplatin prodrug Pt(IV) was encapsulated in the anti-PSMA nanoparticle. Endocytosis was detected using fluorescence microscopy by colocalization of the encapsulated green fluorescent labeled cholesterol with early endosome marker EEA-1. In a series of *in vitro* cytotoxic assays, the $IC_{50}$ value was determined as 0.03 μM for the nanoparticele compared to 0.13 μM for the non-targeted nanoparticle and to 2.4 μM

for free cisplatin on LNCaP cells. However, a high background toxicity of the nanoparticle with an $IC_{50}$ value of 0.11 μM was also detected on PSMA-negative PC-3 cells (Dhar et al., 2008). In a recent study, the anti-PSMA aptamer based nanoparticle was optimized to a self-assembly polymeric nanoparticle carrying cisplatin and docetaxel to prostate cancer cells with synergistic cytotoxicity. The controlled released of both chemotherapeutics was observed over a time period of 48 to 72 h and formation of cisplatin 1,2d(GpG) intrastrand crosslinks could be detected. *In vitro* cytotoxicity of the targeted nanoparticle with an $IC_{50}$ value of 0.09 μM on LNCaP cells was shown to be superior over single drug or non targeted nanoparticles (Kolishetti et al., 2010).

Epigallocatechin 3-gallate (EGCG) is a green tea catechin, which has protective effects against some common types of cancer (Yang et al., 2009). Since it was shown to be chemopreventive against prostate cancer (Bettuzzi et al., 2006; Brausi et al., 2008), an EGCG loaded nanoparticle consisting of PLGA-PEG copolymers was functionalized with an urea-based PSMA inhibitor (Sanna et al., 2011), which is capable of targeting PSMA with a similar affinity and specificity like antibodies and aptamers (Sanna et al., 2011). In *in vitro* experiments LNCaP cells were incubated for 1 or 3 h with the anti-PSMA nanoparticles. In this assay, a significant antiproliferative effect of the nanoparticles to the tumor cells, marked by a growth inhibition up to 60% after 72 h, could be measured (Sanna et al., 2011).

## 3. Conclusion

In the last years, PSMA arouse increasing interest as a prognostic and diagnostic biomarker as well as an attractive target antigen for new therapeutic approaches against prostate cancer.

Anti-PSMA radioimmunoconjugates demonstrated efficient targeting of soft tissue and bone metastases and led to objective anti-tumor responses in a subset of patients. Moreover, high efficacy and tolerability of anti-PSMA immunotoxins was shown in many preclinical and clinical trials. The future strategy in this field is the recombinant production of immunotoxins, by which different limitations of chemically linked immunotoxins can be overcome.

Retargeting of cytotoxic lymphocytes *in vitro* and *in vivo* led to anti-tumor activities in different preclinical studies. These effects could be enhanced by the evocation of costimulatory signaling. Additionally, early vaccination trials showed that an anti-PSMA immune response can be generated without general toxicity in prostate cancer patients.

The development of PSMA-targeted prodrugs, photosensitizers and nanoparticles is still in an early stage, but such constructs also represent alternative therapeutics in the near future.

It has to be considered that most of the discussed clinical treatments were tested in patients with advanced prostate cancer. However, as shown in preclinical trials, these new therapeutic drugs seem likely to be more effective in patients with minimal residual disease or metastases after primary therapies.

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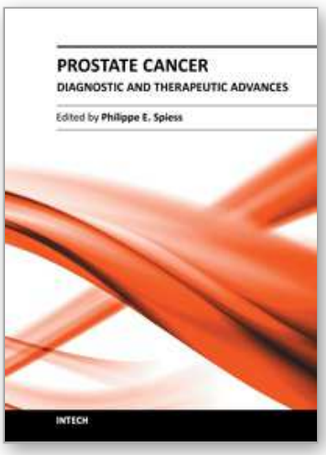


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In this book entitled "Prostate Cancer - Diagnostic and Therapeutic Advances", we highlight many of the significant advances made in our treatment armamentarium of prostate cancer. The book is subdivided into four sections termed: 1) novel diagnostic approaches, 2) surgical treatments options, 3) radiation therapy and its potential sequelae, and 4) medical management and its treatment complications. After reading the present book , readers will be very familiar with the major clinical advances made in our multifaceted treatment approach to prostate cancer over the past decade.This book is a tribute to our pioneering urologists and allied healthcare professionals who have continually pushed forward our traditional therapeutic envelope.



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