(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 953 375 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

(15) Correction information:
Corrected version no 1 (W1 B1)
Corrections, see
Sequence listing
Remarks
Sequence listing replaced or added

(48) Corrigendum issued on:
04.10.2023 Bulletin 2023/40

(45) Date of publication and mention of the grant of the patent:
07.06.2023 Bulletin 2023/23

(21) Application number: **20716487.2**

(22) Date of filing: **08.04.2020**

(51) International Patent Classification (IPC):
C07K 14/47 *(2006.01)* ***A61K 38/17*** *(2006.01)*

(52) Cooperative Patent Classification (CPC):
C07K 14/47; A61K 38/17

(86) International application number:
PCT/EP2020/060043

(87) International publication number:
WO 2020/208083 (15.10.2020 Gazette 2020/42)

(54) NOVEL PSMA SPECIFIC BINDING PROTEINS FOR CANCER DIAGNOSIS AND TREATMENT

NEUARTIGE PSMA SPEZIFISCHE BINDEPROTEINE FÜR DIE KREBSDIAGNOSE UND BEHANDLUNG

NOUVELLES PROTÉINES DE LIAISON SPÉCIFIQUES À PSMA POUR LE DIAGNOSTIC ET LE TRAITEMENT DU CANCER

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **10.04.2019 EP 19168525**

(43) Date of publication of application:
16.02.2022 Bulletin 2022/07

(60) Divisional application:
22180667.2 / 4 108 680

(73) Proprietor: **NAVIGO PROTEINS GMBH**
06120 Halle/Saale (DE)

(72) Inventors:
- **BOSSE-DOENECKE, Eva**
  06120 Halle/Saale (DE)
- **GLOSER-BRÄUNIG, Manja**
  06120 Halle/Saale (DE)
- **SETTELE, Florian**
  06120 Halle/Saale (DE)
- **FIEDLER, Erik**
  06120 Halle/Saale (DE)
- **HAUPTS, Ulrich**
  06120 Halle/Saale (DE)

(74) Representative: **Grünecker Patent- und Rechtsanwälte**
PartG mbB
Leopoldstraße 4
80802 München (DE)

(56) References cited:
WO-A1-2016/124702 **WO-A1-2017/013136**
WO-A1-2017/149002 **WO-A2-2006/028429**

- **DUANWEN SHEN ET AL: "Evaluation of Phage Display Discovered Peptides as Ligands for Prostate-Specific Membrane Antigen (PSMA)", PLOS ONE, vol. 8, no. 7, 25 July 2013 (2013-07-25), page e68339, XP055622882, DOI: 10.1371/journal.pone.0068339**

Processed by Luminess, 75001 PARIS (FR)

(Cont. next page)

- **AGGARWAL SAURABH ET AL: "A dimeric peptide that binds selectively to prostate-specific membrane antigen and inhibits its enzymatic activity", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 66, no. 18, 15 September 2006 (2006-09-15), pages 9171-9177, XP002793201, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-06-1520**
- **MEHDI EVAZALIPOUR ET AL: "Generation and characterization of nanobodies targeting PSMA for molecular imaging of prostate cancer", CONTRAST MEDIA & MOLECULAR IMAGING, vol. 9, no. 3, 6 May 2014 (2014-05-06), pages 211-220, XP055353576, GB ISSN: 1555-4309, DOI: 10.1002/cmmi.1558**
- **LEUNG ISABEL ET AL: "A Highly Diverse and Functional Naïve Ubiquitin Variant Library for Generation of Intracellular Affinity Reagents", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 429, no. 1, 22 November 2016 (2016-11-22), pages 115-127, XP029861440, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2016.11.016**
- **LOREY S ET AL: "Novel ubiquitin-derived high affinity binding proteins with tumor targeting properties", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 289, no. 12, 21 March 2014 (2014-03-21), pages 8493-8507, XP002742090, ISSN: 0021-9258, DOI: 10.1074/JBC.M113.519884 [retrieved on 2014-01-28]**

Remarks:

The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

FIELD OF THE INVENTION

[0001] The present invention relates to new binding proteins that are specific for prostate specific membrane antigen (PSMA). The invention further refers to PSMA binding proteins that further comprise a diagnostically or therapeutically active component. Further aspects of the invention refer to the use of these PSMA binding proteins in medicine, for example, in diagnosis and therapy of cancer associated with PSMA expression.

BACKGROUND OF THE INVENTION

[0002] The prostate specific membrane antigen (PSMA) is expressed in tumors, particularly in prostate cancer. Over-expression of PSMA has also been shown in the neovasculature of other solid tumors for example in breast, kidney, lung, ovarian, colorectal, bladder, gastric or brain cancers (e.g. glioblastoma), and multiple myeloma. It increases with progression of the cancer with particular high levels in metastatic disease. The three-domain glycoprotein (extracellular, transmembrane, and short intracellular domain) is mediating tumor nutrition and cell proliferation. Targeted PSMA specific monoclonal antibodies were developed for diagnosis and treatment of cancer, in particular prostate cancer. So far, the only approved agent for diagnostic imaging and staining of newly diagnosed and recurrent prostate cancer patients is a radiolabeled murine monoclonal antibody Capromab Pendetide. However, the antibody is not of therapeutic benefit due to the binding to an intracellular epitope of PSMA. Second generation monoclonal antibodies binding to extracellular epitopes of PSMA were developed such as murine monoclonal antibody J591. J591 was tested for *in vivo* imaging of progressive solid tumors or for capturing of metastatic circulating tumor cells. Its therapeutic use is confined by toxic side effects and short serum halflives.

[0003] PSMA-specific monoclonal antibodies as agents for diagnostic and therapeutic approaches, for example for PSMA-radioimmunotherapy and PSMA-radioimaging, have further major disadvantages. One is the complex molecular structure and the corresponding complicated production process. The other is their large size, resulting in poor tissue penetration *in vivo.* In combination with long circulation times antibody based compounds for imaging applications may result in poor contrast due to a high background signal.

[0004] Further, the frequent development of resistance to initially effective treatments constitutes a need for additional and improved therapeutics for prostate cancer and other cancers overexpressing PSMA.

[0005] Diagnosis and treatment of PSMA related cancer is not adequately addressed by existing options, and as a consequence, many patients do not adequately benefit from current strategies. Needless to say that there is a strong need for novel strategies for diagnosis and treatment of PSMA related tumors.

[0006] Shen et al. (PLoS ONE 2013, Vol. 8(7): e68339) relates to the identification of potential ligands of PSMA based on a 15-mer phage display random peptide library. Aggarwal et al. (Cancer Res 2006; 66(18): 9171-7) relates to 12 amino acid peptides that bind to PSMA and inhibit its enzymatic activity. WO 2006/028429 A2 relates to PSMA binding peptides consisting of 9 amino acids as well as pharmaceutical compositions thereof.

[0007] One objective of the present invention is the provision of molecules for specific targeting of PSMA for allowing targeted diagnostic and treatment options, including detection of PSMA positive tumors. Targeting this tumor-associated protein may offer benefit to patients with unmet need for novel diagnostic and therapeutic routes. Targeting PSMA suggests potentially non-toxic diagnostic and treatment approach, due to low and restricted distribution of PSMA in normal tissues. Thus, binding proteins with specificity for PSMA may enable effective medical options for cancer, and finally improve quality of life for patients. The present disclosure relates to novel provides novel PSMA bindina molecules for new and improved strategies in the diagnosis and treatment of PSMA related cancer.

[0008] The above-described objectives and advantages are achieved by the subject-matters disclosed herein.

[0009] The present invention meets the needs presented above bv providing examples for PSMA binding proteins. The above overview does not necessarily describe all problems solved by the present invention.

SUMMARY OF THE INVENTION

[0010] The present invention is defined by the appended claims. In particular, the present invention provides the following [1] to [12]:

[1] A prostate specific membrane antigen (PSMA) binding protein, comprising one or more ubiquitin mutein(s) having at least 85 % sequence identity to SEQ ID NO: 1 and comprising an amino acid binding motif GFAHR, or a motif with 80 % identity thereto, at amino acid residues that correspond to positions 62, 63, 64, 65, 66 of SEQ ID NO: 1.
[2] The PSMA binding protein according to [1], wherein amino acids corresponding to positions 6 and 8 of SEQ ID NO: 1 are substituted.

[3] A PSMA binding protein, wherein the PSMA binding protein is a multimer comprising of a plurality of the PSMA binding protein according to claim 1 or 2.

[4] A PSMA binding protein, wherein the PSMA binding protein is a dimer of the PSMA binding protein according to any one of claims 1-2.

[5] The PSMA binding protein according to any one of [1]-[4], comprising or consisting of an amino acid sequence selected from the group of SEQ ID NOs: 3-15, 20-22, 24, 52, 54, and 55.

[6] The PSMA binding protein according to any one of [1]-[5], wherein the PSMA binding protein has a specific binding affinity to the extracellular domain of PSMA of 500 nM or less.

[7] The PSMA binding protein according to any one of [1]-[6], further comprising one or more coupling sites for the coupling of chemical moieties, preferably wherein the chemical moieties are selected from any of chelators, drugs, toxins, dyes, and small molecules.

[8] The PSMA binding protein according to any one of [1]-[7], further comprising at least one diagnostically active moiety, optionally selected from a radionuclide, fluorescent protein, photosensitizer, dye, or enzyme, or any combination of the above, or further comprising at least one therapeutically active moiety, optionally selected from a monoclonal antibody or a fragment thereof, a binding protein, a radionuclide, a cytotoxic compound, a cytokine, a chemokine, an enzyme, or derivatives thereof, or any combination of the above.

[9] The PSMA binding protein according to any one of [1]-[8], further comprising at least one moiety modulating pharmacokinetics optionally selected from a polyethylene glycol, a human serum albumin, an albumin-binding protein, an immunoglobulin binding protein, or an immunoglobulin or immunoglobulin fragment, a polysaccharide, or an unstructured amino acid sequence comprising amino acids alanine, glycine, serine, proline.

[10] The PSMA binding protein according to any one of [1]-[9], for use in diagnosis or treatment of PSMA related tumors, preferably for imaging tumors and radiotherapy treatment of PSMA related tumors.

[11] A composition comprising the PSMA binding protein according to any one of [1]-[9] for use in medicine, preferably for use in the diagnosis or treatment of PSMA related tumors, preferably for imaging tumors and radiotherapy treatment of PSMA related tumors.

[12] A method of producing the PSMA binding protein according to any one of [1]-[9], comprising the steps of a) culturing a host cell under conditions suitable to obtain said PSMA binding protein and b) isolating said PSMA binding protein produced.

[0011] This summary does not necessarily describe all features of the present invention. Other embodiments come apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0012] The Figures show: **FIG. 1-3** show examples for PSMA binding proteins with characteristic motifs in amino acids corresponding to positions 62-66 of SEQ ID NO: 1 (ubiquitin). Structural characteristics of such ubiquitin muteins are shown by the corresponding amino acids that are substituted in ubiquitin (numbers in the top row) or inserted between position 9 and 10 of ubiquitin (numbers in the top row: 9a, 9b, 9c, 9d, 9e, 9f in FIG. 2). Functional characteristics are shown as affinity to PSMA as determined by SPR (Biacore), thermal stability as determined by DSF, and cellular binding as described in Examples.

FIG. 1 shows PSMA binding proteins with 5-amino acid motif WWNPN in positions 62-66. Amino acids corresponding to positions 6, 8, 9, 10, 12, 42, 44, 46, 62-66, 68, 70, 72 of ubiquitin are substituted. In some binding proteins, further substitutions are found, as indicated in the column further substitution.

FIG. 2 shows PSMA binding proteins with 5-amino acid motif KHNTW corresponding to positions 62-66. Amino acids corresponding to positions 42, 44, 62-66, 68, 70, 72 of ubiquitin are substituted, and 6 amino acids are inserted between position 9 and 10 of ubiquitin. In some binding proteins, further substitutions are found, as indicated in the column further substitution".

FIG. 3 shows PSMA binding proteins with 5-amino acid motif GFAHR or GWAHR (or similar) and/or 5-amino acid WTTTF, WTPSI, WTPTI, or GDGDV. Amino acids corresponding to positions 6, 8, 62-66 of ubiquitin are substituted; two ubiquitin muteins are linked. In some PSMA binding proteins, further substitutions are found for example in positions 11, 33, 48, 51, 74 of ubiquitin, as indicated in the column "further substitution".

FIG. 4. Functional characterization of PSMA binding proteins as determined by flow cytometry. The histograms confirm binding of SEQ ID NO: 4 (referred to as 191871) on PSMAoverexpressing HEK293-cells or PSMA-expressing LNCaP-cells (grey peak); no binding on control cell lines HEK293-pEntry or PC3. Unmodified ubiquitin (bis-ubi; white peak) shows no binding on cells.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The present inventors have developed a solution to meet the strong ongoing need in the art for expanding medical options for the diagnosis and treatment of cancer by providing novel PSMA binding proteins. The PSMA specific proteins as defined herein are functionally characterized by high specific affinity for PSMA. In particular, the invention relates to PSMA binding proteins based on ubiquitin muteins (also known as Affilin® molecules). The PSMA binding proteins as described herein provide molecular formats with favorable physicochemical properties, high-level expression in bacteria, and allow easy production methods. The novel PSMA binding proteins may broaden so far unmet medical strategies for the diagnosis and therapy of PSMA related cancer. In particular, the PSMA binding proteins may be used for diagnostic or imaging purposes, for example, for the presence of tumor cells expressing PSMA, and for radiotherapy treatment of tumors expressing PSMA.

[0014] Before the present invention is described in more detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects and embodiments only. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. This includes a skilled person working in the field of protein engineering and purification, but also including a skilled person working in the field of developing new target-specific binding molecules for use in technical applications and in therapy and diagnostics.

[0015] Preferably, the terms used herein are defined as described in A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

[0016] Throughout this specification and the claims, which follow, unless the context requires otherwise, the word "comprise", and variants such as "comprises" and "comprising", was understood to imply the inclusion of a stated integer or step, or group of integers or steps, but not the exclusion of any other integer or step or group of integers or steps. The term "comprise(s)" or "comprising" may encompass a limitation to "consists" or "consisting of", should such a limitation be necessary for any reason and to any extent.

[0017] All sequences referred to herein are disclosed in the attached sequence listing that, with its whole content and disclosure, forms part of the disclosure content of the present specification.

GENERAL DEFINITIONS

[0018] The term "PSMA" refers to a prostate specific membrane antigen. Human PSMA is a glycoprotein of about 100 kD with a short intracellular domain (residues 1-19), a transmembrane domain (residues 20-43), and an extracellular domain (residues 44-750). PSMA is represented by the UniProt ID Q04609 (version of March 15, 2017); human PSMA mRNA is represented by the NCBI reference sequence NM_004476.1. The term PSMA comprises polypeptides which show a sequence identity of at least 70 %, 80 %, 85 %, 90 %, 95 %, or 100 % to Q04609.

[0019] The term "PSMA binding protein" refers to a protein with high affinity binding to PSMA.

[0020] The terms "protein" and "polypeptide" refer to any chain of two or more amino acids linked by peptide bonds, and does not refer to a specific length of the product. Thus, peptides, protein, amino acid chain, or any other term used to refer to a chain of two or more amino acids, are included within the definition of polypeptide", and the term polypeptide may be used instead of, or interchangeably with, any of these terms. The term polypeptide is also intended to refer to the products of post-translational modifications of the polypeptide, which are well known in the art. The term "modification" or "amino acid modification" refers to a substitution, a deletion, or an insertion of an reference amino acid at a particular position in a parent polypeptide sequence by another amino acid. Given the known genetic code, and recombinant and synthetic DNA techniques, the skilled scientist can readily construct DNAs encoding the amino acid variants. The term "mutein" as used herein refers to derivatives of, for example, ubiquitin according to SEQ ID NO: 1 or bis-ubiquitin according to SEQ ID NO: 2, or similar proteins, which differ from said amino acid sequence by amino acid exchanges, insertions, deletions or any combination thereof, provided that the mutein has a specific binding affinity to PSMA.

[0021] The term "Affilin®" (registered trademark of Navigo Proteins GmbH) refers to non-immunoglobulin derived binding proteins.

[0022] The term "substitution" is understood as exchange of an amino acid by another amino acid. The term "insertion" comprises the addition of amino acids to the original amino acid sequence.

[0023] The term ubiquitin refers to ubiquitin in accordance with SEQ ID NO: 1 or to bis-ubiquitin of SEQ ID NO: 2 and to proteins with at least 95 % identity, such as for example with point mutations in positions 45, 75, 76 which do not influence binding to a target (PSMA).

[0024] The terms binding "affinity" and "binding activity" may be used herein interchangeably, and they refer to the ability of a polypeptide to bind to another protein, peptide, or fragment or domain thereof. Binding affinity is typically

measured and reported by the equilibrium dissociation constant (K_D), which is used to evaluate and rank order strengths of bimolecular interactions. The term fusion protein" relates to a protein comprising at least a first protein joined genetically to at least a second protein. A fusion protein is created through joining of two or more genes that originally coded for separate proteins. Fusion proteins may further comprise additional domains that are not involved in binding of the target, such as but not limited to, for example, multimerization moieties, polypeptide tags, polypeptide linkers or moieties binding to a target different from PSMA.

[0025] The term "amino acid sequence identity" refers to a quantitative comparison of the identity (or differences) of the amino acid sequences of two or more proteins. Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. To determine the sequence identity, the sequence of a query protein is aligned to the sequence of a reference protein or polypeptide. Methods for sequence alignment are well known in the art. For example, for determining the extent of an amino acid sequence identity of an arbitrary polypeptide relative to the amino acid sequence of the SIM Local similarity program as known in the art is preferably employed. For multiple alignment analysis, Clustal Omega is preferably used, as known to someone skilled in the art.

[0026] The term "multimeric binding molecules" refers to binding proteins comprising at least two, three, four, five or more binding proteins. Said binding proteins may bind specifically to the same or overlapping epitopes on a target antigen, for example overlapping epitopes of PSMA, or they may bind to different epitopes on a target antigen, for example different epitopes of PSMA.

[0027] The term "conjugate" as used herein relates to a protein comprising of at least a first protein, for example the PSMA binding protein of the invention, attached chemically to other substances such as to a non-proteinaceous (chemical) moiety or to a second protein.

[0028] The term "epitope" includes any molecular determinant capable of being bound by a binding protein as defined herein and is a region of a target antigen (i.e. PSMA) that is bound by a binding protein, and may include specific amino acids that directly contact the binding protein.

DETAILED DESCRIPTION

[0029] **Structural characterization of PSMA binding proteins.** The prostate specific membrane antigen (PSMA) binding protein as defined herein comprises at least one amino acid binding motif selected from the group consisting of GFAHR, GWAHR, SFAHR, SYAHR, GFAHL, WTTTF, WTPSI, WTETI, GDGDV, KHNTW, VAYRP, and WWNPN (SEQ ID Nos: 56-61 and 72-77), or amino acid motifs with may at least 80 % identity thereto, respectively. The PSMA binding protein may comprise a mutein of ubiquitin (SEQ ID NO: 1). Described herein is a PSMA binding protein that comprise an amino acid binding motif selected from the group of GFAHR, GWAHR, SFAHR, SYAHR, GFAHL, WTTTF, WTPSI, WTETI, GDGDV, KHNTW, VAYRP, and WWNPN, or similar motifs, wherein the amino acid residues of the binding motif correspond to positions 62, 63, 64, 65, 66 of ubiquitin according to SEQ ID NO: 1. As further described herein the PSMA binding protein may comprise a mutein of ubiquitin according to SEQ ID NO: 1 wherein the ubiquitin mutein comprises at least one amino acid binding motif selected from GFAHR, GWAHR, SFAHR, SYAHR, GFAHL, WTTTF, WTPSI, WTETI, GDGDV, KHNTW, VAYRP, or WWNPN, or similar motifs, in positions that correspond to positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1).

PSMA binding proteins with motif WWNPN

[0030] Further described herein are PSMA binding proteins having a characteristic amino acid motif WWNPN in positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1). The PSMA binding protein may comprise a mutein of ubiquitin according to SEQ ID NO: 1 wherein the ubiquitin mutein comprises an amino acid binding motif WWNPN in positions that correspond to positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1), and further substitutions in positions corresponding to position 6, 8, 9, 10, 12, 42, 44, 46, 68, 70, 72 of SEQ ID NO: 1. PSMA binding proteins may comprise or consist of at least one ubiquitin mutein modified by, in addition to Q62W, K63W, E64N, S65P, and T66N, further substitutions selected from the group of K6Y or K6W; L8R or L8A or L8K; T9A, T9V, T9E, or T9Q; G10L or G10F; T12Q; R42M or R42K or R42T; As further I44F or I44K; A46K or A46R; H68S; V70N or V70D; R72D or R72N or R72G. As further described herein PSMA binding proteins may comprise ubiquitin muteins with additional 1, 2, or 3 substitutions, for example but not limited to selected from the group of E16A, E18G, I23V, K29R, Q31R, K33R, I36T, K48R, L71R, and L73R (for example, see SEQ ID NOs: 38, 39, 40, 41, 47, 48, 49, 50, 51).

[0031] The PSMA binding protein may comprise or consist of an ubiquitin mutein with substitutions in amino acid positions 6, 8, 9, 10, 12, 42, 44, 46, 62, 63, 64, 65, 66, 68, 70, and 72 of SEQ ID NO: 1 wherein the ubiquitin mutein has a characteristic five amino acid motif WWNPN in positions 62, 63, 64, 65, 66. **FIG. 1** shows examples for PSMA binding proteins with amino acid substitutions in positions 6, 8, 9, 10, 12, 42, 44, 46, 62, 63, 64, 65, 66, 68, 70, and 72 of SEQ

ID NO: 1.

[0032] The PSMA binding protein may comprise one or more amino acids selected from the group consisting of SEQ ID NOs: 35-51, for example but not limited to SEQ ID NO: 40 (Affilin-187191)(differences to SEQ ID NO: 1 that result in PSMA binding are underlined). MQIFVYTRALKQITLEVEPSDTIENVKAKIQDKEGIPPDQQMLFWK-GRQLEDGRTLSDYNIWWN PNLSLNLDLRAA.

PSMA binding proteins with motif KHNTW or VAYRP

[0033] Further disclosed herein are PSMA binding proteins having a characteristic amino acid motif KHNTW in positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1). The PSMA binding proteins may comprise or consist of an ubiquitin mutein modified by substitutions selected from R42H, I44Y, Q62K, K63H, E64N, S65T, T66W, H68E, V70M, and R72F of ubiquitin (SEQ ID NO: 1).

[0034] As further described herein. the PSMA binding protein may have a characteristic amino acid motif VAYRP in positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1). Further, the PSMA binding proteins may comprise or consist of an ubiquitin mutein modified by substitutions selected from R42I, I44W, Q62V, K63A, E64Y, S65R, T66P, H68Y, V70T, and R72A of ubiquitin (SEQ ID NO: 1).

[0035] The PSMA binding protein may comprise an ubiquitin mutein that is additionally modified by an insertion of 4-8 amino acids between amino acids T9 and G10 of ubiquitin (SEQ ID NO: 1), in particular by an insertion of 6 amino acids. The PSMA binding protein may have insertion of an 6 amino acid motif between position 9 and 10, wherein the amino acid in the 6^{th} position may be an aromatic amino acid, or M. Amino acid motifs of such insertion may include but are not limited to: FEHXSF, wherein X is selected from any amino acid, preferably P, H, K, or N; PQPPEX, wherein X is selected from W, F, or Y; PPFAFW, PIPPDW, or DMYRFM.

[0036] The PSMA binding protein may comprise or consist of a mutein of ubiquitin according to SEQ ID NO: 1 wherein the ubiquitin mutein comprises at least one amino acid binding motif selected from KHNTW or VAYRP in positions that correspond to positions 62, 63, 64, 65, 66 of ubiquitin (SEQ ID NO: 1), and further substitutions in positions corresponding to position 42, 44, 68, 70, 72 of ubiquitin (SEQ ID NO: 1), and an insertion of 4-8 amino acids between amino acids T9 and G10 of ubiquitin (SEQ ID NO: 1).

[0037] As further described herein the PSMA binding protein may comprise or consist of an ubiquitin mutein with substitutions in amino acids corresponding to positions 42, 44, 62, 63, 64, 65, 66, 68, 70, and 72 of SEQ ID NO: 1 and an insertion of 6 amino acids at position 9 of SEQ ID NO: 1 and wherein the ubiquitin muteins have a characteristic five amino acid motif KHNTW or VAYRP corresponding to positions 62, 63, 64, 65, 66. Additional 1, 2, or 3 positions might be substituted, for example, but not limited to, A46V of ubiquitin (see SEQ ID NO: 29).

[0038] **FIG. 2** shows examples for specific amino acids in positions 42, 44, 62, 63, 64, 65, 66 of SEQ ID NO: 1 and an insertion of 6 amino acids between position 9 and 10 (shown as 9a, 9b, 9c, 9d, 9e, 9f in **FIG. 2**) of SEQ ID NO: 1.

[0039] As disclosed herein the PSMA binding may comprise amino acids selected from the group consisting of SEQ ID NOs: 25-34, for example SEQ ID NO: 25 (Affilin-164667) MQIFVKTLTFEHPSFGKTITLEVEPSDTIENVKAKIQDKE-GIPPDQQHLYWAGKQLEDGRTLSDY NIKHNTWLELMLFLRAA (differences to SEQ ID NO: 1 that result in PSMA binding are underlined).

PSMA binding proteins with motif GWAHR/GFAHR or similar and or WTTTF or similar

[0040] As further described herein the PSMA binding protein may comprise a ubiquitin mutein with characteristic five amino acid motif GWAHR or GFAHR or SFAHR or SYAHR or GFAHL or variants thereof in positions 62, 63, 64, 65, 66 of ubiquitin. Disclosed herein is a PSMA binding protein comprises one or more ubiquitin mutein(s) based on ubiquitin according to SEQ ID NO: 1 and comprising an amino acid binding motif GFAHR, or a motif with 80 % identity thereto, at amino acid residues that correspond to positions 62, 63, 64, 65, 66 of ubiquitin according to SEQ ID NO:

[0041] Further disclosed herein is a PSMA binding protein that comprises one or more ubiquitin mutein(s) based on ubiquitin according to SEQ ID NO: 1 and comprising an amino acid binding motif $X_1X_2AHX_3$, wherein X_1 is selected from G or S, X_2 is selected from an aromatic amino acid, preferably W or F, X_3 is selected from R or L, at amino acid residues that correspond to positions 62, 63, 64, 65, 66 of ubiquitin according to SEQ ID NO: 1. Further disclosed herein is a PSMA binding protein that comprises one or more ubiquitin mutein(s) based on ubiquitin according to SEQ ID NO: 1 and comprising an amino acid binding motif G(X)AHR, wherein X is an aromatic amino acid residue, preferably wherein X is selected from F or W. Further disclosed herein is a PSMA binding protein that comprises one or more ubiquitin mutein(s) based on ubiquitin according to SEQ ID NO: 1 and comprising an amino acid binding motif GFAHR, GWAHR, SFAHR, or SYAHR or GFAHL, at amino acid residues that correspond to positions 62, 63, 64, 65, 66 of ubiquitin according to SEQ ID NO: 1.

[0042] Also disclosed herein is a PSMA binding protein that comprises a ubiquitin mutein with characteristic five amino acid motif WTTTF (SEQ ID NO: 58) or WTPSI (SEQ ID NO: 75) or WTETI (SEQ ID NO: 76) or GDGDV (SEQ ID NO:

77) or variants thereof in positions 62, 63, 64, 65, 66 of ubiquitin.

[0043] The PSMA binding protein may comprise or consist of two ubiquitin moieties where the first ubiquitin moiety has the motif GFAHR (SEQ ID NO: 56) or GWAHR (SEQ ID NO: 57) or SFAHR (SEQ ID NO: 72) or SYAHR (SEQ ID NO: 73) or GFAHL (SEQ ID NO: 74) or similar motif or the second ubiquitin moiety has the motif WTTTF or WTPSI or WTETI or GDGDV or similar motifs. Further, the PSMA binding protein may comprise or consist of two ubiquitin moieties where the first ubiquitin moiety has the motif GFAHR or GWAHR or SFAHR or SYAHR or GFAHL, or similar motif, and the second ubiquitin moiety has the motif WTTTF or WTPSI or WTETI or GDGDV, or similar motif. **FIG. 3** shows examples for specific amino acids in positions 6, 8, 62, 63, 64, 65, 66 in PSMA binding proteins consisting of two ubiquitin moieties.

[0044] Also, the PSMA binding protein may comprise or consist of two ubiquitin muteins with substitutions in amino acids corresponding to positions 6, 8, 62, 63, 64, 65, 66 of SEQ ID NO: 1 fused to each other, wherein the N-terminal located ubiquitin mutein has a characteristic five amino acid motif GFAHR or GWAHR or SFAHR or SYAHR or GFAHL corresponding to positions 62, 63, 64, 65, 66, and the C-terminal located ubiquitin mutein has a characteristic five amino acid motif WTTTF or WTPSI or WTETI or GDGDV corresponding to positions 62, 63, 64, 65, 66, as shown in **FIG. 3.**

[0045] As further described herein, an ubiquitin mutein may have substitutions selected from K6R or K6W; L8M, L8P, L8Q, L8W, L8D, L8G, L8H, or L8I; Q62G, Q62S, or Q62W; K63F, K63W, K63G, K63P, or K63Y; E64A or E64G; S65H or S65D; and T66R, T66Q, or T66L. In some embodiments, the ubiquitin mutein has substitutions selected from K6M, K6L, K6A, or K6H; L8Q, L8R, L8F, L8N, or L8H; Q62W or Q62G; K63T, K63H, or K63D; E64T, E64P, E64E, or E64G; S65T, S65Y, or S65D, and T66F, T66I, T66L, or T66V.

[0046] The PSMA binding protein may comprise an ubiquitin mutein additionally modified by further substitutions, for example but not limited to positions 10, 11, 33, 48, 48, 51, 74 of ubiquitin, for example selected from the group of G10Q, K11Q, K33T, K48T, E51A, R74C of ubiquitin (for example, see SEQ ID NOs: 11, 12, 17, 19).

[0047] As further disclosed herein, PSMA binding proteins comprise of two ubiquitin muteins linked to each other, i.e. PSMA binding proteins comprise of muteins of bis-ubiquitin (SEQ ID NO: 2). As disclosed herein, the PSMA binding protein may comprise one or more amino acids selected from the group consisting of SEQ ID NOs: 3-24, for example (differences to SEQ ID NO: 1 or SEQ ID NO: 2 that result in PSMA binding are underlined).

SEQ ID NO: 3 (Affilin-162462)

MQIFVRTPTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDYNIGFAH RLHLVLRLRAAMQIFVMTQTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGR TLSDYNIWTTTFLHLVLRLRAA;

SEQ ID NO: 4 (Affilin-191871)

MQIFVRTQTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDYNIGWAH RLHLVLRLRAAMQIFVHTNTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGR TLSDYNIWTETILHLVLRLRAA.

[0048] Disclosed herein is a PSMA binding protein that comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 3-55. The PSMA binding protein may comprise an amino acid sequence that exhibits at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95%, at least 96%, at least 97 %, at least 98 %, at least 99 %, or 100 % sequence identity to one or more of the amino acid sequences of SEQ ID NOs: 3-55. For example, but not limited to, amino acid sequences of selected PSMA binding proteins are shown in **Figure 1, Figure 2,** and **Figure 3.** may have

[0049] The PSMA binding protein as disclosed herein may have at least 74 %, 75 %, 76 %, 77 %, 78 %, or 79 % sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. The PSMA binding protein as disclosed herein has at least 80% sequence identity to the amino sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or has at least 81 %, 82 %, 83 %, 84 %, or 85 % sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. As further disclosed herein, the PSMA binding protein as disclosed herein may have any amino acid identity between 74 % identity and 90 % identity to the amino acid sequence of ubiquitin (SEQ ID NO: 1) or bis-ubiquitin (SEQ ID NO: 2).

[0050] As further disclosed herein the PSMA binding protein as disclosed herein may have an amino acid identity of at least 90 % to the amino acid sequence of SEQ ID NO: 3, or may have an amino acid identity of at least 90 % to the amino acid sequence of SEQ ID NO: 4, or may have any amino acid identity of at least 90 % to the amino acid sequence of SEQ ID NO: 25, or may have any amino acid identity of at least 90 % to the amino acid sequence of SEQ ID NO: 26,

or or may have any amino acid identity of at least 90 % to the amino acid sequence of SEQ ID NO: 40.

[0051] Multimers. The PSMA binding protein may be a multimer comprising of a plurality of the PSMA binding protein as defined herein. A multimer may comprise two, three, four, or more PSMA binding proteins. For example the PSMA binding protein may comprise 2, 3, 4, or more PSMA binding proteins linked to each other, i.e. the PSMA-binding protein can be a dimer, trimer, or tetramer, etc. The multimer may a dimer of the PSMA binding protein as defined above. The PSMA binding protein may be homodimer. A homodimeric PSMA binding protein as understood herein is a protein wherein two PSMA binding proteins with identical amino acid sequences are linked to each other. Homo-dimers can be generated by fusing two identical proteins of any one of the group of SEQ ID NO: 3-51 or of any of the amino acid sequences with at least 90 % identity thereto. For example, but not limited to, homodimers are exemplied in SEQ ID NO: 52 (dimer of SEQ ID NO: 3) and SEQ ID NO: 53 (dimer of SEQ ID NO: 25).

[0052] As further described herein, the multimer may be a heterodimer, e.g. the two amino acid sequences of the PSMA binding proteins are different. For example, heterodimers were generated, as shown in SEQ ID NO: 54 (dimer of SEQ ID NO: 25 and SEQ ID NO: 3, from N- to C-terminus) and SEQ ID NO: 55 (dimer of SEQ ID NO: 3 and SEQ ID NO: 25, from N- to C-terminus). Two or more PSMA binding proteins may be directly linked. Two or more PSMA binding proteins may be linked by a peptide linker. Two or more PSMA binding proteins are linked via a peptide linker of up to 30 amino acids. In other embodiments, two or more PSMA binding proteins are linked via a peptide linker of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids. Specifically, two PSMA binding proteins may be linked by 16 amino acids.

[0053] Disclosed herein is a linker that is comprised of amino acids selected from at least two or more of the group of glycine, serine, alanine, or proline. Two or more PSMA may be binding protein may be linked via a peptide linker of the amino acid sequence according to any one of SAPAASPSPAAPAPSPASPAPASPASAPSAPASAPPAASA (SEQ ID NO: 62) or PAAPAPSPASPAPASPASAPS (SEQ ID NO: 63) or peptide linkers with at least 90 % identity thereto. Other linkers for the fusion of proteins are known in the art and can be used. **Functional characterization.** The PSMA binding protein as described herein binds to PSMA expressed on cells as determined by FACS and/or has a binding affinity to PSMA of 500 nM or less as determined by surface plasmon resonance assays.

[0054] The PSMA binding protein as described herein may have a binding affinity (K_D) of less than 500 nM for PSMA. The PSMA binding proteins bind PSMA with measurable binding affinity of less than 500 nM, less than 200 nM, less than 100 nM, less than 50 nM, less than 20 nM, less than 10 nM, less than 5 nM, and less than 1 nM. The appropriate methods are known to those skilled in the art or described in the literature. The methods for determining the binding affinities are known *perse* and can be selected for instance from the following methods known in the art: enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), kinetic exclusion analysis (KinExA assay), Bio-layer interferometry (BLI), flow cytometry, fluorescence spectroscopy techniques, isothermal titration calorimetry (ITC), analytical ultracentrifugation, radioimmunoassay (RIA or IRMA), and enhanced chemiluminescence (ECL). Some of the methods are described in the Examples below. Typically, the dissociation constant K_D is determined at the range of temperatures between 20°C and 30°C, for example at 20°C, 25°C, or 30°C. If not specifically indicated otherwise, the K_D values recited herein are determined at 25°C by SPR. The lower the K_D value, the greater the binding affinity of the biomolecule for its binding partner. The higher the K_D value, the weaker the binding partners bind to each other. Examples are provided in **FIG. 1, FIG. 2, FIG. 3** and in the **Examples.** The binding of the PSMA binding protein as described herein is highly specific for PSMA. Ubiquitin (SEQ ID NO: 1) does not bind to PSMA. The PSMA binding protein as described herein binds to PSMA but does not detectably bind to human Fc-domain of immunoglobulin IgG_1, as determined by surface plasmon resonance assays. A binding to PSMA with K_D less than 500 nM may be important for targeted medical applications for PSMA related cancer. Further, a protein with PSMA binding with K_D less than 500 nM may have reduced potential toxic side effects. As disclosed herein the PSMA binding protein may comprise a ubiquitin mutein that has (a) a five amino acid residue motif at positions corresponding to positions 62, 63, 64, 65, and 66 of ubiquitin (SEQ ID NO: 1) selected from the group of GFAHR, GWAHR, SFAHR, SYAHR, GFAHL, WTTTF, WTPSI, WTETI, GHEYL, GDGDV, KHNTW, VAYRP, and WWNPN, or motifs with 80 % identity thereto; (b) 80 % to 93 % sequence identity to SEQ ID NO: 1; and (b) a binding affinity (K_D) of less than 500 nM for PSMA. The half maximal effective concentration EC_{50} refers to the concentration of a PSMA binding protein which induces a response halfway between the baseline and maximum after a specified exposure time and thus represents the concentration of a PSMA binding protein where 50 % of its maximal effect is observed, in this case half-maximal fluorescence intensity signal in a cell binding, flow cytometry experiment. The PSMA binding protein as described herein may have an EC_{50} of less than 100 nM for PSMA-expressing cells, less than 50 nM, less than 20 nM, less than 10 nM, less than 5 nM, and less than 1 nM. The PSMA binding protein as described herein has may have an EC_{50} to PSMA-expressing cells of less than 1 nM after incubation in the presence of mouse serum for at least 24 h at 37°C. The appropriate methods are known to those skilled in the art. The lower the EC_{50} value, the greater the binding of the PSMA binding protein for PSMA. Examples for PSMA binding proteins that are stable even in the presence of serum are provided in Table 4.

[0055] As further disclosed herein, the PSMA binding protein as described herein is stable at high temperatures, up to 85°C. For stability analysis, for example spectroscopic or fluorescence-based methods in connection with chemical or physical unfolding are known to those skilled in the art. For example, the stability of a molecule can be determined

by measuring the thermal melting (T_m) temperature, the temperature in °Celsius (°C) at which half of the molecules become unfolded, using standard methods. Typically, the higher the T_m, the more stable the molecule. Temperature stability was determined by differential scanning fluorimetry (DSF), as described in further detail in **Examples** and in **FIG. 1, FIG. 2, FIG. 3.**

[0056] **Competitive binding experiments** comparing PSMA binding proteins show that PSMA binding proteins with different motifs may bind non identical or non-overlapping epitopes (see Examples). For example, SEQ ID NO: 25 with motif KHNTW binds to a different epitope than SEQ ID NO: 15 with motifs GFAHR and WTTTF. Thus, certain PSMA binding proteins do not compete for PSMA binding, and are particularly suitable for certain diagnostic or therapeutic applications. Additional functional characterization was performed by **cellular PSMA binding analysis** with PSMA overexpressing cells. Immunofluorescence microscopy and flow cytometric analysis confirmed the specific binding of PSMA binding proteins as described herein to PSMA-positive tumor cell lines from human origin and to PSMA on live cells (see **Examples).**

[0057] **Coupling sites.** The PSMA binding protein as described herein may further comprise one or more coupling site(s) for the coupling of chemical moieties. A coupling site is capable of reacting with other chemical groups to couple the PSMA binding protein to chemical moieties. The defined number and defined position of coupling sites enables site-specific coupling of chemical moieties to the PSMA binding proteins as described herein. Thus, a large number of chemical moieties can be bound to a PSMA binding protein if required. The number of coupling sites can be adjusted to the optimal number for a certain application by a person skilled in the art to adjust the amount of the chemical moieties accordingly. The coupling site may be selected from the group of one or more amino acids which can be labeled with specific chemistry such as one or more cysteine residues, one or more lysine residues, one or more tyrosine residues, one or more tryptophan residues, or one or more histidine residues. The PSMA binding protein may comprise 1 to 20 coupling site(s), such as 1 to 6 coupling site(s), such as 2 coupling sites, or one coupling site.

[0058] **Coupling domains.** Disclosed herein is a PSMA binding protein that comprises at least one coupling domain of 1 to 80 amino acids comprising one or more coupling sites. The coupling domain of 1 to 80 amino acids may comprise alanine, proline, or serine, and as coupling site cysteine. An example for a PSMA binding protein with a coupling domain of amino acids SAC is provided in SEQ ID NO: 5 (Affilin-191871 with c-terminal amino acids SAC). The coupling domain of 1 to 80 amino acid residues may consist of alanine, proline, serine, and as coupling site cysteine. The coupling domain is consisting of 20 - 60 % alanine, 20 - 40 % proline, 10 - 60 % serine, and one or more cysteine residues as coupling site(s) at the C- or N-terminal end of the PSMA binding protein as described herein. The amino acids alanine, proline, and serine may be randomly distributed throughout a coupling domain amino acid sequence so that not more than a maximum of 2, 3, 4, or 5 identical amino acid residues are adjacent, preferably a maximum of 3 amino acids. The composition of the 1 to 20 coupling domains can be different or identical.

[0059] Amino acid compositions of selected examples for coupling domains with coupling site (Cysteine) are shown in Table 1.

Table 1. Amino acid compositions of examples for coupling domains

amino acid sequence	SEQ ID NO:
SAPAPSAPAASAPPAPAAPCAPAAPASAPAPASAPAASP CPAAPAPSPASPAPASPASAPS	64
SAPAPSAPAASAPPAPAAPCAPAAPASAPAPASAPAASP C	65
SAPAPSAPAASAPPAPAAPC	66
APAAPASAPAPASAPAASPC	67
SAPAPSAPAASAPPAPAAPAAPAAPASAPAPAC	68
APAASPSPAAPAPSPASPAPASPASAPSAPASC	69

[0060] The chemical moieties may be selected from any of chelators, drugs, toxins, dyes, and small molecules. At least one of the chemical moieties may be chelator designed as a complexing agent for coupling one or more further moieties to the targeted compound to the PSMA binding protein as disclosed herein. Further disclosed herein is a PSMA binding protein wherein the chelator is a complexing agent for coupling one or more radioisotopes or other detectable labels, as described in the **Examples.**

[0061] **Diagnostic moiety.** The PSMA binding protein may further comprise a diagnostic moiety. The PSMA binding protein may further comprise more than one diagnostic moiety. Such diagnostic moiety may be selected from radionuclides, fluorescent proteins, photosensitizers, dyes, or enzymes, or any combination of the above. A PSMA binding protein that comprises at least one diagnostic moiety can be employed, for example, as imaging agent, for example to evaluate presence of tumor cells or metastases, tumor distribution, and/or recurrence of tumor. Methods for detection or monitoring of cancer cells involve imaging methods. Such methods involve imaging PSMA related cancer cells by, for example, radioimaging or photoluminescens or fluorescence.

[0062] **Therapeutic moiety**. The PSMA binding protein may further comprise a therapeutically active moiety. The PSMA binding protein further may comprise more than one therapeutically active moiety. Such therapeutically active moiety may be selected from a monoclonal antibody or a fragment thereof, a binding protein such as an ubiquitin mutein (Affilin), an extracellular domain of a receptor or fragments thereof, a radionuclide, a cytotoxic compound, a cytokine, a chemokine, an enzyme, or derivatives thereof, or any combination of the above. The PSMA binding protein that comprises a therapeutically active component may be used in targeted delivery of any of the above listed components to the PSMA expressing tumor cell and accumulate therein, thereby resulting in low levels of toxicity to normal cells.

[0063] Radionuclides. Suitable radionuclides for applications in imaging *in vivo or in vitro* or for radiotherapy include for example but are not limited to the group of gamma-emitting isotopes, the group of positron emitters, the group of beta-emitters, and the group of alpha-emitters. In some embodiments, suitable conjugation partners include chelators such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or diethylene triamine pentaacetic acid (DTPA) or their activated derivatives, nanoparticles and liposomes. In various embodiments, DOTA may be suitable as complexing agent for radioisotopes and other agents for imaging, as described in the **Examples** in further detail.

[0064] **Moiety modulating pharmacokinetics.** The PSMA binding protein further comprise/ at least one moiety modulating pharmacokinetics optionaly selected from a polyethylene glycol, a human serum albumin, an albumin-binding protein, an immunoglobulin binding protein or an immunoglobulin or immunoglobulin fragments, a polysaccharide (for example, hydroxylethyl starch), or an unstructured amino acid sequence which increases the hydrodynamic radius such as a multimer comprising amino acids alanine, glycine, serine, proline.

[0065] Said moiety may increase the half-life of the PSMA binding protein at least 1.5 fold. Several techniques for producing PSMA binding protein with extended half-life are known in the art, for example, direct fusions of the moiety modulating pharmacokinetics with the PSMA binding protein as described above or chemical coupling methods. The moiety modulating pharmacokinetics can be attached for example at one or several sites of the PSMA binding protein through a peptide linker sequence or through a coupling site as described above.

[0066] Conjugation of proteinaceous or non-proteinaceous moieties to the PSMA binding protein may be performed applying chemical methods well-known in the art. Coupling chemistry specific for derivatization of cysteine or lysine residues may be applicable. Chemical coupling can be performed by chemistry well known to someone skilled in the art, including but not limited to, substitution, addition or cycloaddition or oxidation chemistry (e.g. disulfide formation).

[0067] **Molecules for purification/detection.** Additional amino acids can extend either at the N-terminal end of the PSMA binding protein or the C-terminal end or both. Additional sequences may include for example sequences introduced e.g. for purification or detection. Additional amino acid sequences include one or more peptide sequences that confer an affinity to certain chromatography column materials. Typical examples for such sequences include, without being limiting, Strep-tags (see e.g. SEQ ID NO: 71), oligohistidinetags, glutathione S-transferase, maltose-binding protein, inteins, intein fragments, or the albumin-binding domain of protein G.

[0068] **Use in medicine.** The present disclosure relates to the PSMA binding protein as disclosed herein for use in medicine. The PSMA binding protein may be used in medicine to diagnose or treat cancer associated with PSMA expression. The PSMA binding proteins as disclosed herein allow selective **diagnosis and treatment** of PSMA related cancer cells or cancer tissues. PSMA is known to be upregulated in tumor cells.. PSMA is highly expressed in prostate cancer and metastases thereof, for example selected from but not limited to, hepatic, thyroid, B-cell follicular lymphoma, lymph node, and bone metastases, and other solid tumors, preferably selected from breast cancer, renal/kidney cancer, multiple myeloma, brain tumor, lung cancer, ovarian cancer, colorectal cancer, bladder cancer and gastric cancer.

[0069] The present disclosure relates to diagnosing (including monitoring) a subject having PSMA related cancer, the method of diagnosis (monitoring) comprising administering to the subject the PSMA binding protein as described, optionally conjugated to radioactive molecules. The PSMA binding protein as disclosed herein may be used for diagnosis of PSMA related cancer, optionally wherein the PSMA binding protein is conjugated to a radioactive molecule. Imaging methods using the PSMA binding protein with labels such as radioactive or fluorescent can be employed to visualize PSMA on specific tissues or cells, for example, to evaluate presence of PSMA related tumor cells, PSMA related tumor distribution, recurrence of PSMA related tumor, and/or to evaluate the response of a patient to a therapeutic treatment.

[0070] The present disclosure also relates to treating a subject having PSMA related cancer, the method of treatment comprising administering to the subject the PSMA specific binding protein as described, optionally conjugated to a radioactive molecule and/or a cytotoxic agent. The PSMA binding protein as disclosed herein may be used for treatment of PSMA related cancer, optionally wherein the PSMA binding protein is conjugated to a cytotoxic agent and/or to a

radioactive molecule. The disclosure relates to the use of the PSMA binding protein labelled with a suitable radioisotope or cytotoxic compound for treatment of PSMA related tumor cells, in particular to control or kill PSMA related tumor cells, for example malignant cells. Curative doses of radiation may be selectively delivered to PSMA related tumor cells but not to normal cells.

[0071] Compositions. The disclosure relates to a composition comprising the PSMA binding protein as disclosed herein. A composition comprising the PSMA binding protein as defined above in particular for use in medicine, in particular for use in the diagnosis or treatment of various PSMA related cancer tumors, such as in prostate cancer and metastases thereof, hepatic, thyroid, B-cell follicular lymphoma, lymph node, and bone metastases, renal/kidney cancer, multiple myeloma, brain tumor, lung cancer, ovarian cancer, colorectal cancer, bladder cancer, gastric cancer, and others. Compositions comprising the PSMA binding protein as described above may be used for clinical applications for both diagnostic and therapeutic purposes. In particular, compositions comprising the PSMA binding protein as described above may be used for clinical applications for imaging, monitoring, and eliminating or inactivating pathological cells that express PSMA.

[0072] The disclosure further relates to a diagnostic composition for the diagnosis of PSMA related cancer comprising the PSMA binding protein as defined herein and a diagnostically acceptable carrier and/or diluent. These include for example but are not limited to stabilizing agents, surface-active agents, salts, buffers, coloring agents etc. The compositions can be in the form of a liquid preparation, a lyophilisate, granules, in the form of an emulsion or a liposomal preparation.

[0073] The diagnostic composition comprising the PSMA binding protein as described herein can be used for diagnosis of PSMA related cancer, as described above.

[0074] The disclosure relates The disclosure relates to a pharmaceutical (e.g. therapeutical) composition for the treatment of diseases comprising the PSMA binding protein as disclosed herein, and a pharmaceutically (e.g. therapeutically) acceptable carrier and/or diluent. The pharmaceutical (e.g. therapeutical) composition optionally may contain further auxiliary agents and excipients known *per se*. These include for example but are not limited to stabilizing agents, surface-active agents, salts, buffers, coloring agents etc.

[0075] The pharmaceutical composition comprising the PSMA binding protein as defined herein can be used for treatment of diseases, as described above.

[0076] The compositions contain an effective dose of the PSMA binding protein as defined herein. The amount of protein to be administered depends on the organism, the type of disease, the age and weight of the patient and further factors known *per se*. Depending on the galenic preparation these compositions can be administered parentally by injection or infusion, systemically, intraperitoneally, intramuscularly, subcutaneously, transdermally, or by other conventionally employed methods of application.

[0077] The composition can be in the form of a liquid preparation, a lyophilisate, a cream, a lotion for topical application, an aerosol, in the form of powders, granules, in the form of an emulsion or a liposomal preparation. The type of preparation depends on the type of disease, the route of administration, the severity of the disease, the patient and other factors known to those skilled in the art of medicine.

[0078] The various components of the composition may be packaged as a kit with instructions for use.

[0079] Preparation of PSMA binding proteins. PSMA binding proteins as described herein may be prepared by any of the many conventional and well known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques, fragment ligation techniques or by commercially available automated synthesizers. On the other hand, they may also be prepared by conventional recombinant techniques alone or in combination with conventional synthetic techniques. Furthermore, they may also be prepared by cell-free *in vitro* transcription/translation. The disclosure relates to a **polynucleotide** encoding a PSMA binding protein as disclosed herein. Disclosed herein is an expression **vector** comprising said polynucleotide, and a host cell comprising said isolated polynucleotide or the expression vector.

[0080] The disclosure further relates The disclosure further relates to a **method for the production** of a PSMA binding protein as disclosed herein comprising culturing of a host cell under suitable conditions which allow expression of said PSMA binding protein and optionally isolating said PSMA binding protein. For example, one or more polynucleotides which encode for the PSMA binding protein may be expressed in a suitable host and the produced PSMA binding protein can be isolated. A host cell comprises said nucleic acid molecule or vector. Suitable host cells include prokaryotes or eukaryotes. A vector means any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage or virus) that can be used to transfer protein coding information into a host cell. Various cell culture systems, for example but not limited to mammalian, yeast, plant, or insect, can also be employed to express recombinant proteins. Suitable conditions for culturing prokaryotic or eukaryotic host cells are well known to the person skilled in the art. Cultivation of cells and protein expression for the purpose of protein production can be performed at any scale, starting from small volume shaker flasks to large fermenters, applying technologies well-known to any skilled in the art.

[0081] Disclosed herein is a method for the preparation of a binding protein as detailed above, said method comprising the following steps: (a) preparing a nucleic acid encoding a PSMA binding protein as defined herein; (b) introducing said nucleic acid into an expression vector; (c) introducing said expression vector into a host cell; (d) cultivating the host cell;

(e) subjecting the host cell to culturing conditions under which a PSMA binding protein is expressed, thereby producing a PSMA binding protein as defined herein; (f) optionally isolating the PSMA binding protein produced in step (e); and (g) optionally conjugating the PSMA binding protein with further functional moieties as defined herein.

[0082] In general, isolation of purified PSMA binding protein from the cultivation mixture can be performed applying conventional methods and technologies well known in the art, such as centrifugation, precipitation, flocculation, different embodiments of chromatography, filtration, dialysis, concentration and combinations thereof, and others. Chromatographic methods are well-known in the art and comprise without limitation ion exchange chromatography, gel filtration chromatography (size exclusion chromatography), or affinity chromatography.

[0083] For simplified purification, the PSMA binding protein can be fused to other peptide sequences having an increased affinity to separation materials. Preferably, such fusions are selected that do not have a detrimental effect on the functionality of the PSMA binding protein or can be separated after the purification due to the introduction of specific protease cleavage sites. Such methods are also known to those skilled in the art.

EXAMPLES

[0084] The following Examples are provided for further illustration of the invention. The invention is particularly exemplified by particular modifications of ubiquitin (SEQ ID NO: 1 or SEQ ID NO: 2) resulting in binding to PSMA. The invention, however, is not limited thereto, and the following Examples merely show the practicability of the invention on the basis of the above description.

Example 1. Identification of PSMA binding proteins

[0085] *Library construction and cloning.* Ubiquitin libraries comprising randomized amino acid positions were either synthesized by triplet technology (MorphoSys SIonomics, Germany) or in house by randomized oligonucleotides generated by synthetic trinucleotide phosphoramidites (ELLA Biotech) to achieve a well-balanced amino acid distribution with simultaneously exclusion of cysteine and other amino acid residues at randomized positions.

[0086] Several libraries were used to identify PSMA binding proteins

library SPV2: Ubiquitin (SEQ ID NO: 1) randomized in amino acid positions 6, 8, 9, 10, 12, 42, 44, 46, 62, 63, 64, 65, 66, 68, 70, and 72.

library SPL27: Ubiquitin (SEQ ID NO: 1) randomized in amino acid corresponding to positions 62, 63, 64, 65, 66, 68, 70, and 72 and an insertion of six randomized amino acids introduced between T9 and G10 of ubiquitin. The occurrence of amino acid residues Cys, Ile, Leu, Val and Phe have been omitted in the insertion.

library SPVF19: Bis-ubiquitin (SEQ ID NO: 2) randomized in amino acid positions 6, 8, 62, 63, 64, 65, 66, 68, 70, 72, 82, 84, 138, 139, 140, 141, 142 (this corresponds to randomization of positions 6, 8, 62, 63, 64, 65, 66 in both ubiquitin moieties; in library SPVF19, two ubiquitin moieties are directly linked).

[0087] The corresponding cDNA library was amplified by PCR and ligated with a modified pCD87SA phagemid (herein referred to as pCD12) using standard methods known to a skilled person. The pCD12 phagemid comprises a modified *torA* leader sequence to achieve protein processing without additional amino acids at the N-terminus. Aliquots of the ligation mixture were used for electroporation of *Escherichia coli* ER2738 (Lucigen). Unless otherwise indicated, established recombinant genetic methods were used, for example as described in Sambrook, J & Russel, D.W. [2001] (Cold Spring Harbor Laboratory, NY.

[0088] *Target expression, purification and analysis.* A DNA sequence encoding the extracellular domain of human PSMA (uniprot Accession Number Q04609; residues 45 - 750) was genetically fused with the Fc region of human IgG_1 followed by a His-tag at the N-terminus. Full length cDNA with human codon usage was provided by GeneArt (Thermo Scientific), cloned into the mammalian expression vector pCEP4 and expressed in mammalian Expi293F cells at a scale of 250 ml in shaking flasks. Expression was analyzed by SDS-PAGE and by immunoblot analysis with antibodies directed against PSMA and the Fc-part of human IgG1. 130 ml cell culture supernatant of the large scale expression were centrifuged and filtrated for application to affinity chromatography on a Protein A HP 1mL column (GE Healthcare). The target protein was eluted by a gentle pH shift (pH 4) and applied to a Superdex XK16/600 gel filtration column. 9 mg of His-Fc-PSMA could be recovered; SDS-PAGE analysis and SE-HPLC analysis confirmed the purity of the target protein. The enzymatic activity of the target protein towards the substrate N-acteyl-L-aspartyl-L-glutamate (NAAG) was confirmed.

[0089] *Primary selection by TAT Phage Display.* The naive library was enriched against the target PSMA using TAT phage display as selection system. After transformation of competent bacterial ER2738 cells (Lucigene) with phagemid pCD12 carrying the library, phage amplification and purification was carried out using standard methods known to a skilled person. For selection the target protein was immobilized as Fc-fusion of the extracellular domain of human PSMA on Dynabeads® Protein A or Dynabeads® Protein G. The target concentration during phage incubation was lowered

from 200 nM (first round) to 100 nM (second round) and 50 nM (third round) and 25 nM (fourth round). In some of the selection rounds mouse serum was added to select molecules with increased serum stability. Target phage complexes were magnetically separated from supernatant and washed several times. Target bound phages were eluted by trypsin. To deplete the phage library of Fc-binding variants a preselection of phages with immobilized Fc-fragment of IgG_1 (Athens Research & Technology) was performed prior to round two and three. To identify target specific phage pools, eluted and reamplified phages of each selection round were analysed by phage pool ELISA. Wells of a medium binding microtiter plate (Greiner Bio-One) were coated with PSMA-Fc (2.5 μg/ml) and Fc-fragment of IgG_1 (2.5 μg/ml), respectively. Bound phages were detected using α-M13 HRP-conjugated antibody (GE Healthcare).

[0090] *Cloning of target binding phage pools into an expression vector.* Selection pools showing specific binding to the target in phage pool ELISA were amplified by PCR according to methods known in the art, cut with appropriate restriction nucleases and ligated into a derivative of the expression vector pET-28a (Merck, Germany) comprising a Strep-Tag II (IBA GmbH).

[0091] *Single colony hit analysis.* After transformation of BL21 (DE3) cells (Merck, Germany) kanamycinresistant single colonies were grown. Expression of the target-binding modified ubiquitin variants was achieved by cultivation in 384 well plates (Greiner Bio-One) using auto induction medium. Cells were harvested and subsequently lysed chemically or enzymatically by BugBuster reagent (Novagen) and mechanically by freeze/thaw cycles, respectively. After centrifugation the resulting supernatants were screened by ELISA with immobilized target on High Bind 384 ELISA micrrotiter plates (Greiner Bio-One). Detection of bound protein was achieved by *Strep*-Tactin® HRP Conjugate (IBA GmbH) in combination with TMB-Plus Substrate (Biotrend, Germany). The reaction was stopped by addition of 0.2 M H_2SO_4 solution and measured in a plate reader at 450 nm versus 620 nm.

[0092] *Construction of maturation library.* For maturation of each selected variant a module shuffling approach was used wherein the binding molecule is split into two modules. For ubiquitin muteins based on SEQ ID NO: 1, the first module comprises amino acids 1 - 40 and the second module amino acids 32 - 76. For bis-ubiquitin muteins based on SEQ ID NO: 2, the first module comprises amino acids 1 - 77 and the second module amino acids 71 - 152. For cloning of the module shuffling maturation libraries either module one of the variants was kept constant and fused with a native second module of the original library or vice versa. The fusion of the two modules was achieved by overlap extension PCR. The obtained cDNA of maturation libraries was ligated with pCD12 as described above. Alternatively, an error prone PCR approach was used where additional mutations in predefinded molecules or pools were induced.

[0093] *Maturation selection and analysis.* For affinity maturation two rounds of panning were performed. For both rounds a preselection with Fc-fragment of IgG_1 was performed. In some of the selection rounds mouse serum was added to select molecules with increased serum stability. To analyse the matured and selected pools for specific target binding a phage pool ELISA was performed followed by cloning of positive pools into expression vector pET-28a and hit ELISA as described above.

Example 2. Expression and purification of PSMA-binding proteins

[0094] PSMA binding molecules were cloned into an expression vector using standard methods known to a skilled person, purified and analyzed as described below.

[0095] All constructs were expressed in *Escherichia coli* BL21(DE3) using a low copy plasmid system under regulation of a T7 promoter. Proteins were produced cytoplasmatically in mostly soluble form after induction by lactose included in the medium (autoinduction medium). All overnight cultures were inoculated from a single colony after a fresh transformation with a defined plasmid. PSMA binding proteins were produced in ZYM5052 autoinduction medium according to Studier et al. (2005). Overnight cultures were grown up to saturation in shake flasks in a volume of 20-100 mL in 2xYT medium. Main cultures were inoculated to an OD600 of 0.05 to 0.1 and incubated in ZYM5052 with 50 μg/mL kanamycin for up to 24 h at 30 °C on a rotary shaker at 200 rpm in shake flasks with or without baffles. Depending on the expression levels either in 1 L shake flasks with 350 mL medium each or 5 L flasks with 1 L medium each.

[0096] Affilin proteins with affinity tag were purified by affinity chromatography and gel filtration. After affinity chromatography purification a size exclusion chromatography (SE HPLC or SEC) has been performed using an Äkta system and a Superdex™ 200 HiLoad 16/600 column (GE Healthcare). The column has a volume of 120 ml and was equilibrated with 2 CV. The samples were applied with a flow rate of 1 ml/min. Fraction collection starts as the signal intensity reaches 10 mAU. Following SDS-PAGE analysis positive fractions were pooled and their protein concentrations were measured.

[0097] Dimeric PSMA binding proteins without affinity tag were purified using cation exchange chromatography (SP Sepharose HP, GE Healthcare) followed by anion exchange chromatography (Q Sepharose HP, GE Healthcare) to reduce the amount of endotoxin.

[0098] Finally. a size exclusion chromatography (Sephacryl S200HR, GE Healthcare) was performed. Further analysis included SDS-PAGE, SE-HPLC and RP-HPLC. Protein concentrations were determined by absorbance measurement at 280 nm using the molar absorbent coefficient. For example, the purity of SEQ ID NO: 5 is 98% according to SE-HPLC. RP chromatography (RP-HPLC) has been performed using a Dionex HPLC system and a PLRP-S (5μm, 300 Å) column

(Agilents).

Example 3. PSMA binding proteins are stable at high temperatures

[0099] Thermal stability of the binding proteins of the invention was determined by Differential Scanning Fluorimetry (DSF). Each sample was transferred at concentrations of 0.1 μg/μL to a LightCycler® 480 Multiwell Plate 96 (Roche), and SYPRO Orange dye was added at suitable dilution. A temperature ramp from 20 to 90 °C was programmed with a heating rate of 1 °C per minute (LightCycler® 480, Roche). Fluorescence was constantly measured at an excitation wavelength of 465 nm and the emission wavelength at 580 nm (LightCycler® 480, Roche). The midpoints of transition for the thermal unfolding (Tm, melting points) are shown for selected muteins in FIG. 1, **FIG. 2, and FIG. 3.** PSMA binding proteins of the invention have melting temperatures up to 85°C. The T_m of SEQ ID NO: 5 is 69.3 °C and the T_m of SEQ ID NO: 6 is 84.2 °C. See **Table 2** for temperature stability of selected dimeric PSMA binding proteins.

Example 4. Analysis of PSMA binding proteins (Surface Plasmon Resonance, SPR)

[0100] A CM5 sensor chip (GE Healthcare) was equilibrated with SPR running buffer. Surface-exposed carboxylic groups were activated by passing a mixture of EDC and NHS to yield reactive ester groups. 700-1500 RU PSMA-Fc (on-ligand) were immobilized on a flow cell, IgG-Fc (off- ligand) was immobilized on another flow cell at a ratio of 1:3 (hIgG-Fc:Target) to the target. Injection of ethanolamine after ligand immobilization was used to block unreacted NHS groups. Upon ligand binding, protein analyte was accumulated on the surface increasing the refractive index. This change in the refractive index was measured in real time and plotted as response or resonance units (RU) versus time. The analytes were applied to the chip in serial dilutions with a flow rate of 30 μl/min. The association was performed for 120 seconds and the dissociation for 360 seconds. After each run, the chip surface was regenerated with 30 μl regeneration buffer and equilibrated with running buffer. A dilution series served as positive control, whereas a dilution series of unmodified ubiquitin represents the negative control. The control samples were applied to the matrix with a flow rate of 30 μl/min, while they associate for 60 seconds and dissociate for 120 seconds. Regeneration and re-equilibration were performed as previously mentioned. Binding studies were carried out by the use of the Biacore 3000 (GE Healthcare); data evaluation was operated via the BIAevaluation 3.0 software, provided by the manufacturer, by the use of the Langmuir 1:1 model (RI=0). After fitting the data with a 1:1 langmuir model, for example, K_D values were calculated and shown in **FIG. 1, FIG. 2, FIG. 3,** and **Table 2.** Evaluated dissociation constants (K_D) were standardized against off-target and indicated. For example, the K_D of SEQ ID NO: 5 is 1.8 nM vs. hPSMA-Fc (1100 RU immobilized via Protein A), the K_D of SEQ ID NO: 6 is 113 nM, the K_D of SEQ ID NO: 22 is 484 nM, the K_D of SEQ ID NO: 45 is 33 nM, and K_D of SEQ ID NO: 26 is 9,3 nM. **Table 2** shows binding affinity as determined by SPR and temperature stability (see Example 3) of dimeric PSMA binding proteins.

Table 2. Binding affinity and temperature stability of dimeric PSMA binding proteins

SEQ ID NO:	Dimer of	Affinity to PSMA (M)	DSF (oC)
52	SEQ ID NO: 3	2.94 e-13	68.04
53	SEQ ID NO: 25	5.17 e-13	64.02
54	SEQ ID NO: 25 and SEQ ID NO: 3	6.32 e-12	77.60
55	SEQ ID NO: 3 and SEQ ID NO: 25	8.27 e-14	76.93

Example 5. Functional characterization: Binding to cell surface expressed PSMA (Flow Cytometry)

[0101] Flow cytometry was used to analyze the binding of PSMA binding proteins to cell surface-exposed PSMA. PSMA overexpressing human prostate carcinoma cell line LNCaP, PSMA overexpressing transfected HEK293-PSMA-cells, PSMA non-expressing PC3-cells and empty vector control HEK293-pEntry-cells were used. Cells were trypsinized and resuspended in medium containing FCS, washed and stained in pre-cooled FACS blocking buffer. A cell concentration of 1×10^6 cells/ml was prepared for cell staining and 100 μl were respectively filled into the wells of a 96 well plate (Greiner) in triplicate for each cell line. Different concentrations, for example 50 nM of PSMA binding proteins or 0,5 μg/ml monoclonal anti-human-PSMA antibody (clone LNI-17; Biolegend; 342502) as positive control were added to PSMA overexpressing and control cells in several experiments. PSMA-binding proteins included C-terminal Strep-tags (see e.g. SEQ ID NO: 71) for purification and detection purposes. After 45 min the supernatants were removed and 100 μl/well rabbit anti-Strep-Tag antibody (obtained from GenScript; A00626), 1:300 diluted in FACS blocking buffer were added. Anti-PSMA antibody was detected with anti-mouse-IgG-Alexa 488 (Invitrogen; A-10680) with a dilution of 1:1000

in the positive control wells. After removal of the anti-Strep-Tag antibody from the other wells goat anti-rabbit IgG Alexa Fluor 488 antibody (obtained from Invitrogen; A11008) was applied in a 1:1000 dilution. Flow cytometry measurement was conducted on the Guava easyCyte 5HT device from Merck-Millipore at excitation wavelength 488 nm and emission wavelength 525/30 nm. All PSMA binding proteins of the invention (including dimers) showed binding to surface expressed PSMA on LNCaP-cells and HEK293-PSMA-cells (see **FIG. 1, FIG. 2, FIG. 3;** binding is indicated in the Figures by "yes") and no binding on PSMA-negative cell lines HEK293-pEntry or PC3 cells. Ubiquitin showed no binding on LNCaP-cells and HEK293-PSMA-cells. Positive binding on PSMA-expressing cells was also observed for anti-PSMA-antibody. For example, SEQ ID NO: 4 showed strong cell binding to HEK-PSMA and LNCaP cells.

Example 6. Binding to cell surface expressed PSMA (immunocytochemistry and fluorescence microscopy)

[0102] A concentration of 50 nM was tested on PSMA-expressing LNCaP-cells and control cell line PC3 (no PSMA expression). Bis-Ubiquitin was used as control for a non-PSMA-binding protein and 1 μg/ml anti-PSMA-ab served as positive control for PSMA binding. Cells were seeded with a concentration of 1×10^5 cells/ml in Poly-D-Lysin coated Lab-Tek® Chamber-Slides (Sigma-Aldrich). After cultivation over 72 h the cells were fixed with methanol (5 min, -20 °C), followed by blocking (5 % Fetal Horse Serum in PBS, 1 h) and incubation with 50 nM PSMA binding protein for 45 min at rt. PSMA binding was detected by incubation with rabbit-anti-Strep-Tag-antibody (1:500) for 1 h and subsequently with anti-rabbit-IgG-Alexa488-antibody (1:1000) for 1 h. The nuclei were stained with 4 μg/ml DAPI. All incubation steps were done at room temperature. PSMA binding of dimer SEQ ID NO: 54, dimer SEQ ID NO: 55 and monomer SEQ ID NO: 25 on LNCaP-cells was confirmed, whereas no binding to PC3-cells could be observed.

Example 7. Functional characterization: PSMA binding proteins bind to PSMA that is expressed on tumor tissue (immunohistochemistry)

[0103] Tissue sections of frozen LNCaP-xenograft-tumor and F9-syngraft-tumor slices were used to analyze binding proteins of the invention. Tissue slices were fixed with ice-cold acetone for 10 min. Dimers of PSMA-binding proteins included C-terminal Strep-tags (see e.g. SEQ ID NO: 71) for purification and detection purposes. After blocking and incubation with 50 nM and 10 nM of dimer of SEQ ID NO: 54, 100 nM and 10 nM of dimers of SEQ ID NOs: 52 and SEQ ID NO: 53 and 100 nM of control protein unmodified bis-ubiquitin, slices were incubated with rabbit anti-StrepTag-antibody (1:500) for 1 h. Sections were then processed with Novolink™ Polymer (Leica, RE7290-CE). The slices were incubated with AEC-solution (DAKO) for 1 min to visualize binding of proteins. Nuclei were stained with Mayer's hemalum solution (Merck Millipore, cat-no. 109249). All incubation steps were done at room temperature. 2 μg/ml of anti-PSMA-ab GCP-04 (Novus Biologicals) and 14 μg/ml GCP-05 (Thermo Scientific) served as positive control. Strong PSMA binding of 50 nM heterodimer (SEQ ID NO: 54) and 100 nM homodimers (SEQ ID NO: 52 and SEQ ID NO: 53) on LNCaP-tumor tissue was confirmed. The anti-PSMA-antibodies GCP-04and GCP-05showed the same staining pattern whereas un-modified ubiquitin showed no staining. No unspecific staining on F9-tumor tissue was observed.

Example 8. Competition Analysis of PSMA binding proteins

[0104] To investigate whether the isolated PSMA-Affilin-proteins bind to identical or different PSMA epitopes, the following assay was performed: PSMA-Fc fusion protein (60 nM) was immobilized on a CM5 Biacore chip that was coupled with recombinant Protein A using NHS/EDC chemistry resulting in 1000 response units (RU). In a first experiment, SEQ ID NO: 25 (motif KHNTW) and SEQ ID NO: 15 (motif GFAHR and WTTTF) were injected at one defined concentration (0.5 μM) at a flow of 30 μl/min PBST 0.005 % Tween 20. In the second experiment, the same flow channel was first pre-loaded with 500 nM SEQ ID NO: 25 until the chip surface was saturated. In the next step, 500 nM SEQ ID NO: 15 was identically applied as in the first experiment. Alternatively, in the second experiment, the same flow channel was first pre-loaded with 500 nM SEQ ID NO: 15 until the chip surface was saturated, followed by loading of 500 nM SEQ ID NO: 25.

[0105] The experiment showed that the binding of a PSMA binding protein with motif KHNTW was not influenced by the presence of a PSMA binding protein with motif GFAHR and WTTTF, and vice versa. Thus, no competition was observed that leads to the conclusion that these PSMA binding proteins bind to different or non-overlapping PSMA-epitopes, i.e. to different surface exposed amino acids.

Example 9: Labeling of fusion protein with DOTA

[0106] Dimeric proteins were incubated with 20-fold excess of Maleimide- DOTA (2,2',2"-(10-(2-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid, CheMatech) in 50 mM HEPES, 150 mM sodium chloride, 5 mM EDTA pH 7.0 for 3 h at room temperature. In order to reduce metal

ions that might interact with DOTA-molecules all columns and AKTA devices (GE Healthcare) were incubated with 0.1 M EDTA solution for 30 minutes. For preparing solutions only metal-free or metal-reduced components were used. After incubation the samples were separated from unbound DOTA molecules via gelfiltration (Superdex S200, GE Healthcare) in 100 mM sodium acetate pH 5.0-5.8. Samples of labeled proteins were also incubated with 5 mM iron(II)chloride for 1h at room temperature to prove that DOTA-molecules are available for coupling with radio isotopes. After the incubation unbound iron was removed using a HiTrap Desalting column (GE Healthcare). MALDI-TOF analysis was used to determine the degree of labeling.

Example 10: Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) Mass Spectrometry

[0107] Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was carried out as followed: Fusion proteins were purified and concentrated using C18-P10-ZipTips (Millipore; catalog number ZTC18S096). The tips were washed with 0,1% (v/v) trifluoroacidic acid (TFA) in water and eluted with 50% (v/v) acetonitrile/0,1% TFA . Samples were treated with 2 % (v/v) TFA in water and embedded in 2,5-dihydroxyacetophenone (DHAP) matrix (Bruker, catalog number 8231829). The mass of fusion proteins was measured on an autoflexTM speed mass spectrometer (Bruker). Protein calibration standards (Bruker, part no. 8206355 and part no. 8207234) were used for tuning of the autoflex speed mass spectrometer.

[0108] Fusion proteins with and without DOTA label were analyzed by MALDI-TOF mass spectra and peaks were compared. MALDI-TOF analysis shows that the DOTA molecules labeled to the dimeric PSMA-binding proteins are available for coupling with iron(II)chloride molecules. Although the K_D is slightly altered after labeling of the fusion proteins, labeling does not significantly affect the affinity of the fusion proteins to the target. Results are summarized in Table 3.

Table 3. Affinity analysis of labelled PSMA binding proteins using SPR

SEQ ID NO:	label	Mr_{calc}	Mr_{exp}	target affinity K_D
5	none			1.8 nM
5	DOTA			4.2 nM
52	none	43027 Da	43019 Da	2.94 e-13 M
52	DOTA	44080 Da	44072 Da	2.99 e-14 M
52	DOTA+iron	44192 Da	44178 Da	n.d.
54	none	35209 Da	35216 Da	6.32 e-12 M
54	DOTA	36263 Da	36266 Da	6.39 e-10 M
54	DOTA+iron	36374 Da	36363 Da	n.d.
55	none	27391 Da	27384 Da	8.27 e-14 M
55	DOTA	28444 Da	28433 Da	2.04 e-13 M
55	DOTA+iron	28556 Da	28535 Da	n.d.

Example 11. Serum stability of PSMA binding proteins (Flow cytometry)

[0109] The stability of PSMA binding proteins even in the presence of serum was analyzed. PSMA-binding proteins included C-terminal FLAG-tags (DYKDDDDK; see e.g. SEQ ID NO: 78) for purification and detection purposes. PSMA binding proteins based on ubiquitin muteins with GFAHR, or a motif with 80 % identity thereto, e.g. SEQ ID NO: 4 or SEQ ID NO: 11, were incubated with a dilution series from 1 μM to 5.6 pM in 100 % mouse serum for 0 h or for 24 h at 37°C. 100 μl Affilin-serum solution was used to analyze the serum stability on HEK293-PSMA-cells. After supernatants were removed, binding was proven with 1 μg/ml anti-FlagTag-ab (Sigma-Aldrich; F1804) and anti-mouse-IgG-Alexa488 (Invitrogen; A10680). FACS analysis confirmed PSMA binding even after 24 h incubation in mouse serum (see Table 4). Further PSMA binding proteins were tested and binding to PSMA was confirmed also in the presence of serum.

Table 4. Binding of PSMA binding proteins in the presence of serum (Flow cytometry)

SEQ ID NO:	EC50 (0h) nM	EC50 (24h) nM	Decrease (fold)	Serum stability
4	1.6	4.5	2.8	yes

(continued)

SEQ ID NO:	EC50 (0h) nM	EC50 (24h) nM	Decrease (fold)	Serum stability
11	2.6	6.6	2.5	yes

Example 12. Serum stability of PSMA binding proteins (ELISA)

[0110] High binding 96 well plates (Greiner, 781061) were immobilized with 2.5 μg/ml PSMA-Fc over night at 4°C. Dilution series of Affilin-191871 (with c-terminal SAC; SEQ ID NO: 5) and SEQ ID NO: 5 Dota labeled with Lutetium Lu3+ were incubated in 100 % mouse serum overnight at 37°C. ELISA-plates were washed with 1x PBS and blocked with 3 % BSA/0.5 % Tween/ PBS 2 h at RT. Dilution series after 0 h or 24 h incubation in the presence of serum were incubated on ELISA-plates 1 h at rt. After washing with PBST, wells were incubated with biotinylated anti-ubiquitinantibody (1:1000) 1 h at rt. The binding was visualized with Streptavidin-HRP (1:10.000). The PSMA binding proteins show no significant shift of K_D after 24h serum incubation. For example, ELISA analysis confirmed the binding of SEQ ID NO: 5 and SEQ ID NO: 5 Dota labeled with Lutetium Lu3+ to PSMA even after 24 h incubation in mouse serum with K_D of 0.75+/- 0.02 (compared to a K_D of 0.53+/- 0.02 at 0 h incubation in mouse serum).

Claims

1. A prostate specific membrane antigen (PSMA) binding protein, comprising one or more ubiquitin mutein(s) having at least 85 % sequence identity to SEQ ID NO: 1 and comprising an amino acid binding motif GFAHR, or a motif with 80 % identity thereto, at amino acid residues that correspond to positions 62, 63, 64, 65, 66 of SEQ ID NO: 1.

2. The PSMA binding protein according to claim 1, wherein amino acids corresponding to positions 6 and 8 of SEQ ID NO: 1 are substituted.

3. A PSMA binding protein, wherein the PSMA binding protein is a multimer comprising of a plurality of the PSMA binding protein according to claim 1 or 2.

4. A PSMA binding protein, wherein the PSMA binding protein is a dimer of the PSMA binding protein according to any one of claims 1-2.

5. The PSMA binding protein according to any one of claims 1-4, comprising or consisting of an amino acid sequence selected from the group of SEQ ID NOs: 3-15, 20-22, 24, 52, 54, and 55.

6. The PSMA binding protein according to any one of claims 1-5, wherein the PSMA binding protein has a specific binding affinity to the extracellular domain of PSMA of 500 nM or less.

7. The PSMA binding protein according to any one of claims 1-6, further comprising one or more coupling sites for the coupling of chemical moieties, preferably wherein the chemical moieties are selected from any of chelators, drugs, toxins, dyes, and small molecules.

8. The PSMA binding protein according to any one of claims 1-7, further comprising at least one diagnostically active moiety, optionally selected from a radionuclide, fluorescent protein, photosensitizer, dye, or enzyme, or any combination of the above, or further comprising at least one therapeutically active moiety, optionally selected from a monoclonal antibody or a fragment thereof, a binding protein, a radionuclide, a cytotoxic compound, a cytokine, a chemokine, an enzyme, or derivatives thereof, or any combination of the above.

9. The PSMA binding protein according to any one of claims 1-8, further comprising at least one moiety modulating pharmacokinetics optionally selected from a polyethylene glycol, a human serum albumin, an albumin-binding protein, an immunoglobulin binding protein, or an immunoglobulin or immunoglobulin fragment, a polysaccharide, or an unstructured amino acid sequence comprising amino acids alanine, glycine, serine, proline.

10. The PSMA binding protein according to any one of claims 1-9, for use in diagnosis or treatment of PSMA related tumors, preferably for imaging tumors and radiotherapy treatment of PSMA related tumors.

11. A composition comprising the PSMA binding protein according to any one of claims 1-9 for use in medicine, preferably for use in the diagnosis or treatment of PSMA related tumors, preferably for imaging tumors and radiotherapy treatment of PSMA related tumors.

12. A method of producing the PSMA binding protein according to any one of claims 1-9 comprising the steps of a) culturing a host cell under conditions suitable to obtain said PSMA binding protein and b) isolating said PSMA binding protein produced.

Patentansprüche

1. Ein Prostata-spezifisches Membran-Antigen (PSMA)-Bindeprotein, umfassend ein oder mehrere Ubiquitin-Mutein(e) mit mindestens 85 % Sequenzidentität zu SEQ ID NR: 1 und umfassend ein Aminosäure-Bindungsmotiv GFAHR oder ein Motiv mit 80 % Identität dazu, an Aminosäureresten, die den Positionen 62, 63, 64, 65, 66 von SEQ ID NR: 1 entsprechen.

2. Das PSMA-Bindeprotein nach Anspruch 1, wobei die Aminosäuren, die den Positionen 6 und 8 von SEQ ID NR: 1 entsprechen, substituiert sind.

3. Ein PSMA-Bindeprotein, wobei das PSMA-Bindeprotein ein Multimer ist, umfassend eine Vielzahl des PSMA-Bindeproteins nach Anspruch 1 oder 2.

4. Ein PSMA-Bindeprotein, wobei das PSMA-Bindeprotein ein Dimer des PSMA-Bindeproteins nach einem der Ansprüche 1-2 ist.

5. Das PSMA-Bindeprotein nach einem der Ansprüche 1-4, umfassend oder bestehend aus einer Aminosäuresequenz ausgewählt aus der Gruppe der SEQ ID NR: 3-15, 20-22, 24, 52, 54 und 55.

6. Das PSMA-Bindeprotein nach einem der Ansprüche 1-5, wobei das PSMA-Bindeprotein eine spezifische Bindungsaffinität zur extrazellulären Domäne von PSMA von 500 nM oder weniger aufweist.

7. Das PSMA-Bindeprotein nach einem der Ansprüche 1-6, ferner umfassend eine oder mehrere Kopplungsstellen für die Kopplung von chemischen Einheiten, wobei die chemischen Einheiten vorzugsweise aus Chelatoren, Wirkstoffen, Toxinen, Farbstoffen und niedermolekularen Verbindungen (small molecules) ausgewählt sind.

8. Das PSMA-Bindeprotein nach einem der Ansprüche 1-7, ferner umfassend mindestens eine diagnostisch aktive Einheit, optional ausgewählt aus einem Radionuklid, einem fluoreszierenden Protein, einem Photosensibilisator, einem Farbstoff oder einem Enzym, oder eine Kombination der genannten, oder ferner umfassend mindestens eine therapeutisch aktive Einheit, optional ausgewählt aus einem monoklonalen Antikörper oder einem Fragment davon, einem Bindungsprotein, einem Radionuklid, einer zytotoxischen Verbindung, einem Zytokin, einem Chemokin, einem Enzym oder Derivaten davon, oder einer Kombination der genannten.

9. Das PSMA-Bindeprotein nach einem der Ansprüche 1-8, ferner umfassend mindestens eine pharmakokinetisch modulierende Einheit, optional ausgewählt aus einem Polyethylenglykol, einem humanen Serumalbumin, einem Albumin-Bindeprotein, einem Immunglobulin-Bindeprotein oder einem Immunglobulin oder Immunglobulinfragment, einem Polysaccharid oder einer unstrukturierten Aminosäuresequenz, umfassend die Aminosäuren Alanin, Glycin, Serin, Prolin.

10. Das PSMA-Bindeprotein nach einem der Ansprüche 1-9 zur Verwendung in der Diagnose oder Behandlung von PSMA-bedingten Tumoren, vorzugsweise für die Bildgebung von Tumoren und die radiotherapeutische Behandlung von PSMA-bedingten Tumoren.

11. Eine Zusammensetzung, umfassend das PSMA-Bindeprotein nach einem der Ansprüche 1-9 zur Verwendung in der Medizin, vorzugsweise zur Verwendung bei der Diagnose oder Behandlung von PSMA-bedingten Tumoren, vorzugsweise zur Bildgebung von Tumoren und zur radiotherapeutischen Behandlung von PSMA-bedingten Tumoren.

12. Ein Verfahren zur Herstellung des PSMA-Bindeproteins nach einem der Ansprüche 1-9, umfassend die Schritte: a)

Kultivieren einer Wirtszelle unter Bedingungen, die geeignet sind, das PSMA-Bindeprotein zu erhalten, und b) Isolieren des hergestellten PSMA-Bindeproteins.

Revendications

1. Protéine de liaison à l'antigène membranaire spécifique de la prostate (PSMA), comprenant une ou plusieurs mutéine(s) d'ubiquitine présentant une identité de séquence d'au moins 85 % à la SEQ ID NO : 1 et comprenant un motif GFAHR de liaison à un acide aminé, ou un motif présentant une identité de 80 % à celui-ci, au niveau de résidus d'acides aminés qui correspondent aux positions 62, 63, 64, 65, 66 de la SEQ ID NO : 1.

2. Protéine de liaison au PSMA selon la revendication 1, dans laquelle les acides aminés correspondant aux positions 6 et 8 de la SEQ ID NO : 1 sont substitués.

3. Protéine de liaison au PSMA, la protéine de liaison au PSMA étant un multimère comprenant plusieurs exemplaires de la protéine de liaison au PSMA selon la revendication 1 ou 2.

4. Protéine de liaison au PSMA, la protéine de liaison au PSMA étant un dimère de la protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 2.

5. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 4, comprenant ou constitué par une séquence d'acides aminés choisie dans le groupe constitué par les SEQ ID NO : 3 à 15, 20 à 22, 24, 52, 54 et 55.

6. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 5, la protéine de liaison au PSMA ayant une affinité de liaison spécifique au domaine extracellulaire du PSMA inférieure ou égale à 500 nM.

7. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 6, comprenant en outre un ou plusieurs sites de couplage destinés au couplage de fragments chimiques, les fragments chimiques étant de préférence choisis parmi des agents chélatants, des médicaments, des toxines, des colorants et des petites molécules.

8. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 7, comprenant en outre au moins un fragment ayant une activité diagnostique, choisi éventuellement parmi un radionucléide, une protéine fluorescente, un agent photosensibilisant, un colorant ou une enzyme, ou une quelconque combinaison des précédents, ou comprenant en outre au moins un fragment ayant une activité thérapeutique choisi éventuellement parmi un anticorps monoclonal ou un fragment de celui-ci, une protéine de liaison, un radionucléide, un composé cytotoxique, une cytokine, une chimiokine, une enzyme ou des dérivés de ceux-ci, ou une combinaison quelconque de ceux-ci.

9. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 8, comprenant en outre au moins un fragment modulant la pharmacocinétique, éventuellement choisi parmi un polyéthylène glycol, de l'albumine de sérum humain, une protéine de liaison à l'albumine, une protéine de liaison à l'immunoglobuline, ou une immunoglobuline ou un fragment d'immunoglobuline, un polysaccharide, ou une séquence d'acides aminés non structurée comprenant les acides aminés alanine, glycine, sérine, proline.

10. Protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 9, destinée à être utilisée lors du diagnostic ou du traitement de tumeurs liées au PSMA, de préférence à des fins d'imagerie de tumeurs et de traitement par radiothérapie de tumeurs liées au PSMA.

11. Composition comprenant la protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 9 destinée à être utilisée à des fins médicales, de préférence destinée à être utilisée lors du diagnostic ou du traitement de tumeurs liées au PSMA, de préférence à des fins d'imagerie de tumeurs et de traitement par radiothérapie de tumeurs liées au PSMA.

12. Procédé de production de la protéine de liaison au PSMA selon l'une quelconque des revendications 1 à 9, comprenant les étapes consistant a) à cultiver une cellule hôte dans des conditions appropriées pour obtenir ladite protéine de liaison au PSMA et b) à isoler ladite protéine de liaison au PSMA produite.

FIGURE 1. Structural and functional characteristics of PSMA binding proteins based on ubiquitin; motif WWNPN

																			KD_PSMA	Thermal transition	cell binding
SEQ ID NO:	Affilin	6	8	9	10	12	42	44	46	62	63	64	65	66	68	70	72	further subst.	Biacore in M	DSF in °C	
40	187191	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	N	D	(48.K>R)	3.37E-11	72.62	yes
41	187210	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	N	D	(33.K>R)	2.77E-11	70.31	yes
42	187131	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	N	G		5.20E-09	67.45	yes
43	187170	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	N	N		7.87E-10	70.26	yes
44	187096	Y	R	E	L	Q	M	F	K	W	W	N	P	N	S	N	D		3.81E-08	72.7	yes
50	187295	Y	R	V	L	Q	M	F	K	W	W	N	P	N	S	N	D	(73.L>R)	8.64E-14	63.42	yes
47	187134	Y	R	V	L	Q	M	F	K	W	W	N	P	N	S	N	D	(16.E>A)	2.43E-11	49.51	yes
48	187186	Y	R	V	L	Q	M	F	K	W	W	N	P	N	S	N	D	(29.K>R)	6.73E-09	58.68	yes
49	187283	Y	R	V	L	Q	M	F	K	W	W	N	P	N	S	N	D	(71.L>R)	3.40E-09	58.17	yes
36	187092	Y	A	E	L	Q	M	F	K	W	W	N	P	N	S	N	D		1.47E-08	70	yes
37	187064	Y	K	Q	L	Q	M	F	K	W	W	N	P	N	S	N	D		1.73E-09	70	yes
39	187165	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	D	D	(23.I>V)	4.09E-09	69.81	yes
38	187154	Y	R	A	L	Q	M	F	K	W	W	N	P	N	S	D	D	(18.E>G).(31.Q>R)	1.26E-09	68.88	yes
35	187093	W	R	E	F	Q	M	F	K	W	W	N	P	N	S	N	D		1.46E-08	74	yes
51	187178	Y	R	V	L	Q	T	F	R	W	W	N	P	N	S	N	D	(36.I>T)	4.25E-13	53.42	yes

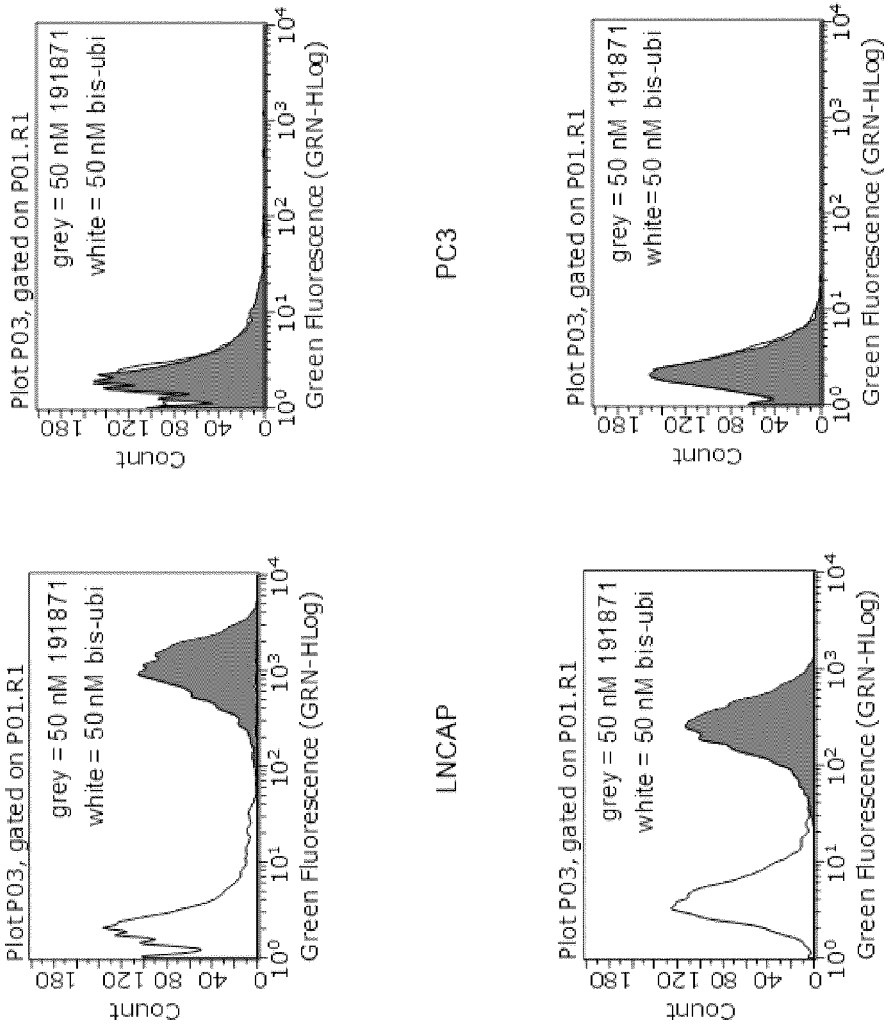
FIGURE 2. Structural and functional characteristics of PSMA binding proteins based on ubiquitin; motif KHNTW

Affilin	9a	9b	9c	9d	9e	9f	42	44	62	63	64	65	66	68	70	72	further	KD_PSMA	Thermal transition	cell binding
164667	F	E	H	P	S	F	H	Y	K	H	N	T	W	E	M	F		7.29E-09	n.d.	yes
161717	F	E	H	H	S	F	H	Y	K	H	N	T	W	E	M	F		4.00E-08	83.5	yes
161776	F	E	H	K	S	F	H	Y	K	H	N	T	W	E	M	F		4.11E-08	83	yes
162055	F	E	H	N	S	F	H	Y	K	H	N	T	W	E	M	F	(46.A>V)	3.98E-08	77.6	yes
162034	P	Q	P	P	E	F	H	Y	K	H	N	T	W	E	M	F		8.77E-08	83.5	yes
161843	P	Q	P	P	E	W	H	Y	K	H	N	T	W	E	M	F		5.17E-08	82.1	yes
161374	P	Q	P	P	E	Y	H	Y	K	H	N	T	W	E	M	F		4.35E-08	69.7	yes
162102	P	I	P	P	D	W	H	Y	K	H	N	T	W	E	M	F		6.36E-08	83.8	yes
161912	P	P	F	A	F	W	H	Y	K	H	N	T	W	E	M	F		7.78E-08	81.5	yes

FIGURE 3. Structural and functional characteristics of PSMA binding proteins based on ubiquitin; motif GXAHR and/or WTTTF

		1st moiety based on SEQ1								2nd moiety based on SEQ1										
SEQ ID NO:	Affilin	6	8	62	63	64	65	66		6	8	62	63	64	65	66	further substutiton	KD_PSMA	Thermal transition	cell binding
3	162462	R	P	G	F	A	H	R		M	Q	W	T	T	T	F		1.35E-08	78.56	yes
7	164052	R	G	G	F	A	H	R		M	Q	W	T	T	T	F		1.67E-08	71.05	yes
9	164058	R	I	G	F	A	H	R		M	Q	W	T	T	T	F		2.43E-08	72.61	yes
15	164668	R	M	G	F	A	H	R		M	Q	W	T	T	T	F		4.32E-09	67.4	yes
8	161438	R	H	S	F	A	H	R		M	R	W	T	T	T	F		2.33E-08	66	yes
19	161644	R	P	S	Y	A	H	R		M	Q	W	T	T	T	F	(51.E>A)	3.01E-08	70.5	yes
24	163970	W	P	G	F	A	H	L		M	Q	W	T	T	T	F		1.10E-08	72.63	yes
17	157424	R	P	Q	P	A	H	Q		M	Q	W	T	T	T	F	(74.R>C)	4.06E-07	75.29	yes
18	157423	R	P	Q	P	A	H	Q		M	R	W	T	T	T	F		1.67E-07	67.01	yes
23	162633	R	W	W	W	A	D	R		M	Q	W	T	T	T	F		1.35E-08	69.4	yes
10	185036	R	L	G	F	A	H	R		L	Q	W	T	P	S	I		2.41E-09	65.3	yes
12	191822	R	L	G	F	A	H	R		L	Q	W	T	P	S	I	(11.K>Q).(51.E>A)	4.38E-09	55.08	yes
4	191871	R	Q	G	W	A	H	R		H	N	W	T	E	T	I		4.61E-09	62.81	yes
21	185113	R	W	G	F	A	H	R		M	H	G	D	G	D	V		3.26E-07	82.58	n.d.
11	192444	R	L	G	F	A	H	R		L	Q	W	T	P	S	I	(33.K>T).2nd: (48.K>T)	5.35E-09	61.74	yes
16	185071	R	M	W	G	G	H	R		L	Q	W	T	P	S	I		2.19E-07	68.71	yes

FIGURE 4. Functional characterization of PSMA binding proteins on PSMA-overexpressing cells (flow cytometry)



REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2006028429 A2 **[0006]**

Non-patent literature cited in the description

- **SHEN et al.** *PLoS ONE,* 2013, vol. 8 (7), e68339 **[0006]**
- **AGGARWAL et al.** *Cancer Res,* 2006, vol. 66 (18), 9171-7 **[0006]**
- A multilingual glossary of biotechnological terms: (IUPAC Recommendations). Helvetica Chimica Acta, 1995 **[0015]**