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(54) Title: MONOClonal antibodies and single chain antibody fragments against cell-surface prostate specific membrane antigen

(57) Abstract: Isolated monoclonal antibodies or an antigen binding portion thereof which bind to prostate specific membrane antigen in its native form occurring on the surface of tumor cells characterized in that it is linked to a label or a cytotoxic agent or constructed as a part of a bispecific antibody or a recombinant diabody.

MONOCLONAL ANTIBODIES AND SINGLE CHAIN ANTIBODY FRAGMENTS AGAINST CELL-SURFACE PROSTATE SPECIFIC MEMBRANE ANTIGEN

Cancer of the prostate is the most commonly diagnosed cancer in men and the second most common cause of death in the Western civilization. No curative treatment currently exists for this tumor after progression beyond resectable boundaries. Because of the significant mortality and morbidity associated with disease progression, there is an urgent need for new targeted treatments. In contrast to cancer in other organ systems, prostate cancer represents an excellent target for antibody therapy for a number of reasons, that include i) the prostate expresses tissue specific antigens, ii) the prostate is a non-essential organ and its destruction will not harm the host, iii) the sites of metastasis are lymph nodes and bone that receive high levels of circulating antibodies, and iv) the PSA serum levels provide a means to monitor therapeutic response.

Among several candidate markers that have been identified for prostate cancer, prostate specific membrane antigen (PSMA) seems to be most prominent. This type II transmembrane glycoprotein of about 100 KD consists of a short intracellular segment (amino acids 1 - 18), a transmembrane domain (amino acids 19 - 43), and an extensive

extracellular domain (amino acids 44 - 750). PSMA may serve as a useful target for immunotherapy because it meets the following criteria: i) expression is primarily restricted to the prostate, ii) PSMA is abundantly expressed as protein at all stages of disease, iii) it is presented at the cell surface but not shed into the circulation, iv) expression is associated with enzymatic or signaling activity. PSMA is also expressed in the neovasculature of most other solid tumors, and therefore may be a target for specific anti-angiogenetic drug delivery.

Because of their target-oriented specificities, a lot of emphasis has been put on the development of monoclonal antibodies (mAbs) for diagnostic and therapeutic applications in cancer medicine. However, the *in vivo* use of mAbs is associated with serious problems, because of their size and immunogenicity. Therefore, research has focused on the development of smaller therapeutic antibodies with fewer side effects, better tumor accessibility and faster clearance rates. Genetic engineering has made it possible to construct single chain antibody fragments (scFv) which are potentially powerful tools for cancer therapy. These small antibodies are composed of the variable domains of the light chain (V_L) and the heavy chain (V_H) connected by a linker peptide. They show little immunogenicity, almost no toxic effects, an increased clearance rate, an improved uptake by the tumor and a better penetration into the tumor cells. Recombinant murine scFv can be established according to standard methods using either expression libraries from hybridomas or spleen cells of specifically immunized mice [Chowdhury et al., Mol. Immunol. 4 (1997), p. 9-20].

The first published mAb (7E11-C5) against PSMA targets at the intracellular domain of the protein and was shown to be highly prostate specific [Horoszewicz et al., Anticancer Res. 7 (1987), p. 927-935]. Also, monoclonal antibodies against the extracellular domain of PSMA have been raised after immunization with the antigen. However, there is still a discrepancy between binding to the antigen on fixed cells and histological sections on the one hand and binding to viable tumor cells on the other hand.

Prostate specific membrane antigen (PSMA) is a prostate marker that is highly expressed in normal prostate as well as in prostate cancer. Its expression is increased in prostate cancer and is found primarily in the prostate.

Prostate specific membrane antigen (PSMA) is a unique membrane bound cell protein which is over expressed manifold on prostate cancer as well as in the neovasculature of many other solid tumors, but not in the vasculature of the normal tissues. This unique expression of PSMA makes it an important marker as well as a large extracellular target of imaging agents.

PSMA can serve as target for delivery of therapeutic agents such as cytotoxins or radionuclides. PSMA has two unique enzymatic functions, folate hydrolase and NAALADase and it is found to be recycled like other membrane bound receptors through clathrin coated pits.

A radio-immuno-conjugate form of the anti-PSMA monoclonal antibody (mAb) 7E11, is commercially available as "ProstaScint®" which is currently being used to diagnose prostate cancer metastasis and recurrence. The PSMA epitope recognized by monoclonal antibody 7E11-C5.3 is located in the cytoplasmic domain of the prostate specific membrane antigen.

There are, however, also reports describing PSMA expression in non-prostatic tissues including kidney, liver and brain. A possible explanation therefore is provided by O'Keefe et al. (Prostate, 2004, February 1; 58 (2) 200-10), namely that there is a PSMA-like gene which possesses 98% identity to the PSMA gene at the nucleotide level, which is expressed in kidney and liver under the control of a different promoter to the PSMA gene.

WO 01/009192 describes the development of human monoclonal antibodies to prostate-specific membrane antigen. Human anti-PSMA monoclonal antibodies were generated by immunizing mice with purified PSMA or enriched preparations of PSMA antigen. Such purified antigen is a denatured PSMA since it has been purified by immunoabsorption after cell lysis with ionic detergents.

WO 97/35616 describes monoclonal antibodies specific for the extracellular domain of prostate-specific membrane antigen. The immunizations were performed with a C-terminal peptide or a PSMA-expressing tumor membrane preparation. The mAbs do not bind specifically to PSMA-expressing cells and can therefore not be used for diagnostic or therapeutic purposes.

Bander et al., Seminars in Oncology, 2003, pp 667-677 and US 2004/0213791 respectively disclose monoclonal antibodies directed against prostate-specific membrane antigen. Since the immunization was performed with purified antigen, the monoclonal antibodies do not have a high cell binding and no scFv could be obtained from either of these mAb.

WO 98/03873 describes the same antibodies as in US 2004/0213791 or binding portions thereof which recognize an extracellular domain of prostate-specific membrane antigen. It could not be shown that the binding portions of the antibodies do in fact bind to the antigen.

Fracasso et al., *The Prostate*, 2002, pp 9-23 describe anti-PSMA monoclonal antibodies which are chemically coupled to the ricine-A-chain. The constructs described in this article do not bind sufficiently specific to the target and have the generally described disadvantages of generation on immunotoxins.

It is one object of the present invention to provide superior means and constructs which help to differentiate with higher reliability between tumor cells and healthy cells which do express PSMA or a similar protein and PSMA-negative cells. Such constructs can be used to target more specifically prostate cancer.

It is another object to provide constructs which destroy specific prostate cancer cells which express PSMA.

Prostate-specific membrane antigen (PSMA) is an attractive target for immunotherapy of prostate cancer. However, on prostate cells PSMA is expressed with a specific tertiary and quaternary structure and antibodies elicited with isolated denatured PSMA do not efficiently recognize PSMA expressing tumor cells. Antibodies and scFv binding to denatured PSMA can be obtained after immunization with the isolated purified antigen. The present invention, however, allows the generation of high affinity antibodies and scFv against native cellular PSMA by a different immunization method which gives only a poor yield of positive clones. Only the later antibodies elicited with native PSMA may react with cell-surface PSMA and can be used as diagnostic and therapeutic tools.

Monoclonal antibodies (mAbs), single chain antibody fragments (scFv) and diabodies of the present invention were prepared according to conventional methods from mice spleen cells. However, the mice had been immunized with LNCaP cells and LNCaP cell lysate containing full-length native PSMA. In a preferred embodiment of the present invention the antigen, namely the full length native PSMA has been obtained after treatment of the cells, preferably LNCaP cells with a special lysis buffer called M-PER, mammalian protein extraction reagent which is commercially available from Pierce, Roquefort, Illinois. The M-PER buffer uses a proprietary detergent in 25 mM bicine buffer (pH 7.6). Hybridomas and scFv were screened and selected by flow cytometry on PSMA-positive LNCaP cells after absorption with PSMA-negative DU 145 prostate cells. Additionally, they were tested for reactivity with purified PSMA. Resulting monoclonal antibodies and scFv were characterized by flow cytometry on LNCaP and PSMA-transfected DU 145 and by western blot with purified glycosylated and deglycosylated PSMA. In addition, immunocytology with LNCaP cells and immunohistochemistry on paraffin sections of prostate cancer samples was prepared.

In the course of the present invention three mAbs (3/F11, 3/A12 and 3/E7) could be obtained, that were reactive with viable LNCaP cells and PSMA-transfected DU 145 cells but not with other cell lines not expressing PSMA. Binding to LNCaP cells was very strong. At saturation concentrations (100 nM) the mean PE fluorescence intensity (MFI) was between 1000 and 1600. Reactivity with purified PSMA was stronger with the native form (ELISA) than with the denatured and deglycosylated protein (western blot). Immunohistochemistry on paraffin sections was specifically positive for epithelial cells with mAb E7.

From the mAb 3/A12 two scFv, called E8 and A5, were obtained by selection of recombinant phages on LNCaP cells and purified PSMA. The sequence of scFv E8 was identical to a scFv A4, which was obtained from the B-cell library of the same mouse. ScFv E8 was strongly reactive with LNCaP cells showing a MFI of about 100 at saturation concentrations, whereas the MFI of scFv A5 was only about 40 under the same conditions. No or minimal binding was seen with other cell lines lacking PSMA expression. Binding of both scFv to purified denatured glycosylated and deglycosylated PSMA was weak. Furthermore, from mAb 3/F11 the scFv called D7 and for mAb 3/E7 the scFv called H12 was obtained.

In the present application we describe three mAb, which are different from those published by other authors with respect to high binding affinity and high staining of PSMA expressing prostate cancer cells. The antibodies 3/F11, 3/A12 and 3/E7 do not only show a strong binding activity but also internalization into LNCaP cells as shown by immunofluorescence cytology and detection with confocal laser scanning microscopy. These mAbs were obtained after immunisation with full length native PSMA, which is in contrast to different published immunisation methods.

After immunization with purified denatured PSMA mAbs were obtained which were highly specific for the antigen, but had only a limited binding to PSMA expressing LNCaP cells and also were not internalized into the cells. These control data are not shown in the present application. There are a few anti-PSMA mAbs described in literature. However, the mean fluorescence intensity values were much lower than with the antibodies of the present invention.

Similarly to the mAbs, anti-PSMA scFv were generated after immunisation with denatured and native PSMA. With the denatured PSMA we obtained scFv highly specific to the antigen, but not binding to LNCaP cells (data not shown in the present application). In contrast, with

native PSMA we obtained scFv with a high cell binding activity, but a poor binding to the isolated denatured antigen.

However, the problems identified in this and other trials with chemically linked immunotoxins are the development of antibodies against the immunotoxins, liver toxicity and vascular leak syndrome and also difficulties in producing large quantities of defined material. These problems are, at least in part, overcome by using recombinant DNA technology which makes the construction of less immunogenic and smaller immunotoxins feasible, and more easily permits the production of immunotoxins in large quantities. It is also believed that penetration into tumors should be better for small proteins than large conjugates. Therefore, recombinant immunotoxins were engineered by fusing the coding sequence of the scFv E8, H12, D7 and A5 and the toxin PE40. The central finding was that all recombinant immunotoxins effectively killed cultured prostate cancer cells in a dose dependent manner. Strong killing was found not only with the highly binding E8- with IC50 of about 0,05 nM, but also with the lower binding A5-fusion protein with IC50 of about 0,09 nM. Killing of not PSMA expressing prostate cancer cells was more than 2000-fold less. This may be traced back to residual bacterial proteins or other toxic agents in the immunotoxin preparations, because the same background could be observed in equally high concentrations with the scFv alone. The term IC50 is defined as the concentration in nM of the toxin which reduces cells proliferation to 50% of the cell proliferation without adding a toxin.

The antibodies and scFv described in this application specifically bind to native cell-surface PSMA and therefore will have value in diagnostic and therapeutic applications focusing on PSMA as a target antigen for prostate cancer.

Since PSMA is expressed on prostate cancer cells with a specific tertiary and quaternary structure, only antibodies against this cellular conformation may recognize and strongly bind to viable prostate cancer cells and PSMA-expressing tissue. Therefore, the aim of the present study was to generate such mAbs and scFv that can be used for therapeutic and diagnostic targeting of prostate cancer.

The present invention provides therefore an isolated monoclonal antibody or an antigen binding portion thereof which binds to prostate specific membrane antigen in its native form occurring on the surface of tumor cells which is linked to a label or a cytotoxic agent.

The term "isolated monoclonal antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains interconnected by disulfid bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated as V_H) and a heavy chain

constant region. The heavy chain constant region is comprised of three domains, namely CH1, CH2 and CH3. Each light chain contains a light chain variable region (V_L) and a light chain constant region (C_L). The V_H and V_L regions can be further subdivided into regions of hypervariability, which are also called complementarity determining regions (CDR) interspersed with regions that are more conserved. Those regions are also called framework regions (FR). Each V_H and V_L region is composed of three CDRs and four FRs arranged from amino terminus to carboxy terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

In Figures 13, 14 and 20, 21 the CDRs are marked by grey boxes. Those areas are important for the binding of the monoclonal antibody or the antigen binding portion thereof. The other areas are framework regions which can be replaced by other sequences, provided the three-dimensional structure which is required for binding is not disturbed. In case the structure of the construct is changed, there will be no sufficient binding to the antigen. Monoclonal antibodies derived from mouse may cause unwanted immunological side-effects due to the fact that they contain a protein from another species which may elicit antibodies. In order to overcome this problem the monoclonal antibodies or the antigen binding portions thereof may be humanized. The process of humanizing monoclonal antibodies is known to the person skilled in the art. The framework regions of a mouse mAb are replaced by the corresponding human framework regions. In order to maintain the preferred binding properties the sequences of the CDRs should be maintained as far as possible. It may be required, however, to perform some amino acid changes in order to optimise the binding properties. This can be performed by the person skilled in the art by standard proceedings. Furthermore by introducing a human framework it may be necessary to perform amino acid changes and/or deletions in order to improve the properties of the construct.

The term "antigen binding portion" of the monoclonal antibody refers to one or more fragments of such an antibody which retained the ability to specifically bind to the prostate specific membrane antigen in its native form. Examples of antigen binding portions of the antibody include a Fab fragment, a monovalent fragment consisting of the V_L , V_H , C_L and C_{H1} domains, an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfid bridge at the hinge region, an Fd fragment consisting of the V_H and C_{H1} domain, an F_v fragment consisting of the V_L and V_H domains of a single arm of an antibody, a dAb fragment which consists of a V_H domain and an isolated complementarity determining region (CDR).

The isolated monoclonal antibody or antigen binding portion thereof according to the present invention can preferably be internalized by a tumor cell if it is used for therapeutic purposes. For diagnostic purposes an internalisation may not be required.

The isolated monoclonal antibody or an antigen binding portion thereof according to the present invention binds strongly to LNCAP cells but not to cells which lack expression of prostate specific membrane antigen.

The binding of the isolated monoclonal antibody or antigen binding portion thereof is measured by PE fluorescence intensity (MFI) which is preferably equal or higher than 40 for an scFv and preferably higher than 1000 for an mAb at saturating concentrations.

The binding properties of the isolated monoclonal antibodies or an antigen binding portion thereof to the native PSMA were compared by treating LNCAP cells with increasing concentrations of the first step anti-PSMA Ab followed by incubation with the second step PE-labeled antibody. From the resulting saturation curves the antibody concentration reaching 50% saturation of PSMA sites can be read. The three mAb 3/F11, 3/A12 and 3/E7 showed a high binding activity reaching 50% saturation of PSMA sites at approximately 16 nM (3/F11), 2 nM (3/A12) and 30 nM (3/E7). With the scFv a 50% saturation of PSMA sites was found at 10 nM (E8) and 60 nM (A5).

In order to determine the binding strength the PE (phycoerythrin) fluorescence intensity (MFI) was measured. The MFI values were plotted against the antibody (or binding fragments thereof) concentration whereby the plateau value of MFI corresponds to 100% saturation with antigen. After having determined the top value (plateau corresponding to 100% saturation of antigen) the value corresponding to 50% of saturation can be easily determined. By using the graph the corresponding concentration of the antibodies or binding fragments thereof in nM can be seen.

The isolated monoclonal antibody or an antigen binding portion thereof comprises a label which may be a particle which emits radioactive radiation. This particle may be a radioactive element in a form which can be linked to the construct, preferably in the form of a complex. For example an mAb labeled with $^{111}\text{Indium}$ may be used as a radioimmuno scintigraphy agent in the detection of distant metastatic tumors in prostate cancer patients. Of course other suitable radioactive elements like ^{35}S or ^{131}I can be used.

Alternatively the isolated monoclonal antibody or antigen binding portion thereof may comprise a cytotoxic agent which is a cell toxic substance selected from the group consisting of toxins, for example taxol, cytocalasin B, gramicidin D, ethidium bromid, emetine, mitomycin, etopside, tenopside, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy antracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosteron, glucocorticoids, procain, tetracaine, lidokaine, propranolol and/or puromycin.

In a preferred embodiment of the present invention an isolated monoclonal antibody or an antigen binding portion thereof comprises a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:1 (scFv E8), SEQ ID NO:10 (scFv A5), SEQ ID NO:20 (scFv H12) and/or SEQ ID NO:22 (scFv D7). In a preferred embodiment the monoclonal antibody or antigen binding protein thereof comprises at least 25 or, more preferred, at least 35 and most preferred at least 50 consecutive amino acids of SEQ ID NO:1, SEQ ID NO:10, SEQ ID NO:20 and/or SEQ ID NO:22, respectively.

In a preferred embodiment the isolated monoclonal antibody or antigen binding portion thereof comprises at least one of the CDRs having SEQ ID NO:2 – SEQ ID NO:7 and/or SEQ ID NO:11 to 16. More preferably such construct comprises at least 3 and more preferably at least 5 of those CDR sequences. In a similar manner the CDRs can be deduced from Fig. 20 and 21 wherein the CDR sequences are designated.

It is a further aspect of the invention to provide DNA sequences which can be used for the preparation of monoclonal antibodies or binding fragments thereof. SEQ ID NO:8 and 9 relate to scFv E8 and SEQ ID NO:17 and 18 relate to scFv A5. SEQ ID NO:19 and 23 relate to scFv H12 and SEQ ID NO:21 and 24 relate to scFv D7. The sequences report the coding strand and the complementary strand thereto. SEQ ID NOS:9 and 18 are shown in the 5'→3' orientation. The polynucleotides of the present invention comprise a contiguous sequence of at least 20, preferably 50 and more preferably 75 and most preferred at least 100 nucleotides of the group consisting of SEQ ID NOS: 8, 9, 17, 18, 19, 21, 23 and 24. The sequence coding for the CDR are in particular preferred.

It is one aspect of the present invention to provide a pharmaceutical composition comprising an isolated monoclonal antibody or an antigen binding portion thereof as described in the present application. The pharmaceutical composition of the present invention comprises the monoclonal antibody or an antigen binding portion thereof together with pharmaceutically acceptable additives. Preferably such a composition is prepared for intramuscular or intraveneous injection. Alternatively the antibody may be provided in a depot formulation which allows the sustained release of the biologically active agent over a certain period of

time which may range preferably from one to six months. Such a sustained release formulation may comprise a biodegradable polymer like a polylactide or polylactide/polyglycolide copolymer which is degraded over a prolonged period of time in the human body whereby the antibody or the antigen binding portion thereof preferably having a toxine is released in a controlled manner over a certain period of time.

The isolated monoclonal antibody or an antigen binding portion thereof may be used for the preparation of a medicament for the treatment of cancer, in particular prostate cancer.

Alternatively the invention provides a diagnostic kit for the detection of tumor cells comprising an isolated monoclonal antibody or an antigen binding portion thereof. In such embodiments the label allows the detection of the construct with suitable detection devices.

The invention provides also a method for the in vitro identification of tumor cells by which the tumor cells to be identified are contacted with an isolated monoclonal antibody or an antigen binding portion thereof which carries a label which can be detected by suitable analytical devices. The label allows the diagnostic identification of tumor cells, for example in section of human tissues obtained after surgery or biopsy.

Brief Description of the Figures

Fig. 1: FACS-analysis of the mAb 3/F11, 3/A12 and 3/E7 binding to the surface of PSMA-expressing LNCaP cells at saturation concentrations

Fig. 1a-c: Antigen saturation curves of mAb 3/F11 (a), 3/A12 (b), 3/E7 (c)

Fig. 2: Immunfluorescence cytology: Binding of a) mAb 3/F11 b)mAb 3A/12 c) 3E7 to LNCaP cells. The left pictures show a control staining with 4',6-Diamidino-2-phenylindole (DAPI).

Fig. 3: Immunfluorescence cytology: Internalization of a) mAb 3/F11 b) mAb 3A/12 c) 3E7 in LNCaP cells. The left pictures show control staining with 4',6-Diamidino-2-phenylindole (DAPI).

Fig. 4: Western blot with purified PSMA and the mAbs 3/E7, and 3/A12 and 3/F11

Fig. 5: Western blot with glycosylated and deglycosylated PSMA and mAb 3/A12

Fig. 6: Immunohistochemistry of mAb 3/E7 on a paraffin section of prostate cancer

Fig. 7a/b: FACS-analysis of the scFv E8 (a), and A5 (b) on PSMA-expressing LNCaP cells at saturation concentrations

Fig. 7c/d: Antigen saturation curves of scFv E8 (c) and A5 (d)

Fig. 8: Western blot with purified PSMA and the scFv A5 and E8

Fig. 9: immunocytology of scFv E8 on LNCaP cells

Fig. 10: construct of the immunotoxin E8-P40

Fig. 11: Cytotoxic effect of recombinant immunotoxin E8-P40 on LNCaP cells

Fig. 12: Cytotoxic effect of recombinant fusion protein A5-P40 on LNCaP cells

Fig. 13: Sequence of scFv E8. DNA sequence is given as well as amino acid sequence whereby the region of the CDWs is identified by a marked area.

Fig. 14: Sequence of scFv A5. DNA sequence is given as well as amino acid sequence whereby the region of the CDWs is identified by a marked area.

Fig. 15: This Figure shows binding of the scFv A5, H12 and D7 to PSMA-negative DU145 cells (A) and PSMA-positive LNCaP cells (A5 = B, H12 = C, D7 = D). Cells were stained with the mAbs and a PE-conjugated anti-mouse IgG mAb. Histogramms represent logarithms of PE fluorescence on flow cytometer. Negative control was done with secondary antibody only.

Fig. 16: The binding of the scFv A5, H12 and D7 to PSMA-negative BOSC cells (A) and PSMA-transfected BOSC cells (A5 = B, H12 = C, D7 = D). Cells were stained with the scFv anti-c-myc mAb and PE-conjugated anti-mouse Ig. Histogramms represent logarithms of PE fluorescence on flow cytometer. Negative control was done with secondary antibody only.

Fig. 17: demonstrates the cytotoxic effect of recombinant immunotoxin HE12-PE40 on LNCaP (black) and DU cells (white).

Fig. 18: shows schematically the construction scheme of the A5-CD3 diabody.

Fig. 19: shows the cytotoxic effect of a diabody constructed from scFv A5 (A5/CD3) at different concentrations and peripheral blood lymphocytes (effect or target ratio 10:1) on LNCaP cells after 48 h incubation.

Fig. 20: shows the sequence of scFv H12. The amino acid sequence is given in the one-letter-code in the first line (corresponding to SEQ ID NO:20). The coding strand is shown on the second line (SEQ ID NO:19) and the complementary strand is shown in the third line. This sequence corresponds to SEQ ID NO:23. The CDRs are specifically designated as CDR H1, H2, H3, L1, L2 and L3. The nucleic acid sequences coding for the CDR regions are shown on a grey background.

Fig. 21: shows the sequence of scFv D7. The amino acid sequence is shown on the first line in the one letter code. This sequence corresponds to SEQ ID NO:22. The coding nucleic acid strand is shown on the first line. This sequence corresponds to SEQ ID NO:21 and the complementary strand is shown on the third line. This sequence corresponds to SEQ ID NO:24. The CDR regions H1, H2, H3, L1 and L2 are shown in the sequence. The nucleic acid sequences coding for those regions are shown on a grey background.

The present invention is further illustrated by the following examples.

Example 1a) *Preparation of PSMA*

The human prostate carcinoma cell lines LNCaP, DU 145, PC-3 and HeLa as well as the hybridoma 7E11-C5.3 (IgG1-k, PSMA) were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. LNCaP, DU 145 and HeLa were cultured in RPMI 1640 medium, PC-3 in F12 Nutrimix medium, both supplemented with penicillin (100 000 U/l), streptomycin (100 mg/l) and 10 % FCS at 37 °C in a humidified atmosphere of 5 % CO₂. For the generation of LNCaP cells expressing unglycosylated PSMA on their surface 2 µg/ml tunicamycin (ICN Biomedicals) were added to the medium for 48 h.

Fixed LNCaP cells were obtained by treatment with 4 % paraformaldehyd for 10 min at RT, and then thoroughly washing with PBS.

For preparing purified PSMA, 10⁸ LNCaP cells were washed with PBS and then lysed in PBS containing 1 % IGEPAL for 20 min at room temperature. After centrifugation at 10,000 g the supernatant was given on a 7E11-C5 affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) and PSMA was eluted with 100 mM glycine buffer pH 2,5 containing 1 % Triton X-100. After neutralisation the protein was extensively dialyzed with PBS.

For preparation of deglycosylated PSMA, 1/10 vol glycoprotein-denaturing buffer (BioLabs), was added to the solution with purified PSMA and heated for 10 min at 100 °C. Then 1/10 vol 10 % NP-40 (10 %) and 50 U PNGase per µg PSMA was added and incubated at 37 °C for 1 h.

For preparation of a LNCaP cell lysate containing full length native PSMA, cells were lysed with M-PER reagent (Pierce) for 10 min and then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant containing native full length PSMA was collected (M-PER-lysate). The high molecular fraction (100 to 600 KD) of this lysate was separated by HPLC on a Biosil 250 size exclusion column.

b) Transfection of full length PSMA into DU 145 and PC3 cells

Full length PSMA was cloned in two fragments (fragment 1 from bp 262 to the unique EcoRI restriction site at bp 1573 and fragment 2 from position 1574 to 2512) into the vector pCR3.1 (Invitrogen). Transient transfection was obtained by adding a mixture of 4 µg DNA and 10 µl Lipofectamine (Invitrogen) in 500 µl RPMI medium to 10⁶ cells according to the manufacturer's protocol. After 48 h incubation the transient transfected cells were used for testing.

Example 2

Immunization of mice

Four-month old female Balb/c mice were immunized intraperitoneally with 300 µg M-PER lysate from LNCaP cells or with the high molecular HPLC fraction of the lysate, or with 10⁶ LNCaP cells, fixed with 2 % paraformaldehyde. These preparations were mixed 1:1 with complete Freund's adjuvant. Each mouse received 4 or 5 immunizations at 2-week intervals. Four days after the last immunization spleen cells were collected and either used for the preparation of hybridomas or a B-cell library.

Example 3

Preparation of a B-cell library

The mouse spleen was washed in phosphate buffered saline (PBS), minced to small pieces, washed again in PBS and then gently homogenized in a "loose-fitting" hand homogenizer. The resulting single cell suspension was overlaid onto Ficoll (Pharmacia, Freiburg, Germany) and centrifuged at 400 g for 20 min at room temperature. Interphase B cells were isolated with CD19 microbeads according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany). 10⁶ B-cells were lysed in 350 µl of a solution consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % sodium N-lauroyslsarcosinate and 100 mM 2-mercaptoethanol.

Example 4***a) Preparation of Hybridomas***

The spleen was aseptically removed and a single cell suspension was prepared in RPMI-1640 medium without serum. The splenocytes were added to SP2/0 myeloma cells at a ratio of 10:1 and the fusion and selection was performed to established procedures [Galfre et al., *Nature* (1979), p. 131-133].

Hybridoma supernatants were tested by FACS on LNCaP and DU145 cells and by an ELISA with purified PSMA as solid phase. Monoclonal antibodies were purified using a protein G column (Pharmacia).

b) Isotype determination of the mAbs

Ig-isotypes of the anti-PSMA mAbs were determined by ELISA using either unlabelled (solid phase) or peroxidase-labeled (tracer) anti-isotype specific antibodies (Southern Biotechnology Associates, Birmingham, AL).

c) Isolation and characterization of anti-PSMA conformational monoclonal antibodies

From Balb/c mice which were immunized 5 times with the M-PER-lysate from LNCaP cells, spleen cells were fused with SP2/0 cells according to established methods. Positive hybridomas were selected by flow cytometry with LNCaP cells and ELISA on purified PSMA. By this way three positive clones were obtained. The corresponding mAbs with their isotypes were 3/F11 (IgG2a), 3/A12 (IgG1) and 3/E7 (IgG2b).

d) Characterization of mAbs

By flow cytometry it could be observed that the three mAbs and stained LNCaP cells bind very well with a percentage of positive cells ranging from 95% to 98%. The shape of the curves of fluorescence versus number of events suggesteds that PSMA is homogeneously distributed within the LNCaP cell population (Fig. 1). To evaluate the binding specificity of the anti-PSMA mAbs, PSMA-negative DU145, PC3 cells, HeLa and Jurkat cells were also stained and analyzed by flow cytometry. All three mAbs did not stain the PSMA-negative cells (percentage of positive cells ranging from 0,04% to 2%).

The binding properties of the three antibodies were compared by treating LNCaP cells with increasing concentrations of the first step anti-PSMA mAb followed by incubation with a saturating amount of a second step PE-(phycoerythin)-labeled goat antibody followed by cytofluorometry analysis. At antigen saturation concentrations [100 nM] the corrected mean PE (phycoerythin) fluorescence intensity was about 1000 for mab 3A12, and about 1400 for mAb 3F11 and about 1600 for mAb 3E7. As shown for mAb 3A12 the MFI was 5-fold lower on LNCaP cells expressing unglycosylated PSMA (grown with tunicamycin).

By immunofluorescence cytology and detection with a laser scanning confocal microscope a strong binding of the three mAbs to LNCaP cells (Fig 2) and also an internalization into these cells could be shown (Fig. 3). All mAbs were positive in an ELISA with purified PSMA as solid phase. With denatured PSMA the mAbs showed a signal at about 100 KD in western blot (Fig 4) whereas the blot with deglycosylated PSMA was weak giving a signal at about 84 KD, which corresponds to literature data (Fig. 5).

Immunohistochemistry on paraffin sections of prostate cancer was positive with mAb 3/E7 but not with mAbs 3/F11 and 3/A12 (Fig. 6). Data are summarized in Table 1.

Table 1: Characterization of 3 monoclonal antibodies against cell-surface PSMA

Hybridoma	Isotype	FACS LNCaP [MFI]*	FACS PSMA- transf.DU* [MFI]	ELISA PSMA	Blot PSMA	Blot degl. PSMA	Immunohisto- chemistry
3/F11	IgG2a	1400	105	pos	pos	(pos)	neg
3/A12	IgG1	1000	110	pos	pos	(pos)	neg
3/E7	IgG2b	1600	90	pos	pos	(pos)	pos

MFI = mean fluorescence intensity at scFv concentration reaching antigen saturation (background staining with secondary antibody alone is subtracted)

(pos) = slightly positive

From these data it is concluded that the 3 mAbs show a very strong and highly specific binding to native and denatured PSMA. Although the binding to deglycosylated PSMA is weaker, a sugar specificity can be excluded from the fact that no binding is seen to cells that do not express PSMA.

Example 5*Preparation of a scFv expression library in the phagemid pSEX*

From the B-cell library or from hybridoma cells total RNA and mRNA was isolated with silicagel-based membranes (Rneasy, Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA synthesis was performed at 42°C for 60 min in a final volume of 50 µl which contained 25 µl of denatured RNA, 10 µl 5x buffer (Promega, Heidelberg, Germany), 5 µl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP, Promega), 1,5 µl RNAsin (40 U/µl, Promega) 2,5 µl of 150 pM random hexamer primers, and 2,5 µl of AMV reverse transcriptase (10 U/µl, Promega). Then the encoding regions of the heavy-chains and the gamma and kappa chains were amplified by PCR as previously described by Orum et al. [Nucleic Acies Res. (1993), 4491-4498]. For each chain 25 separate reactions were carried out by combining 25 different constant region forward primers with one corresponding reverse primer. The amplified products for the light chains and the heavy chains were purified by agarose gel electrophoresis.

The PCR products for the light chains were digested with MluI and NotI, and ligated into the phagemid pSEX81 [Dübel et al., Gene (1993), 97-101] using a molar ratio of 1:3 (2 µg vector, 400 ng insert). The products of one ligation were used for the electroporation of 50 µl electrocompetent *E. coli* XL1 blue cells (Stratagene) according to the supplier's protocol. The bacteria were plated on nine 80 mm diameter agarose plates containing 100 µg/ml ampicillin and 0,1 M glucose (SOB-AG) of and incubated overnight at 30 °C. Bacteria were isolated by adding 3 ml 2xYT medium on each plate, scraping them off with a sterile glass spreader and pelleted at 3,000 g for 15 min. From these bacteria plasmid DNA was isolated which revealed the VI sublibrary. Then the PCR products for the heavy chain and the VI sublibrary were digested with NcoI and HindIII. Ligation was prepared at a ratio of 3:1 (2 µg sublibrary and 400 ng insert). Transformation by electroporation, plating and collection of transformed bacteria was done as described for the VI sublibrary. From nine 80 mm diameter SOB-AG plates a total of 18 ml V_HV_L library was obtained.

Example 6*Production and selection of antibody-displaying phage*a) *Production*

In the $V_H V_L$ library in phagemid pSEX the antibody genes are fused in frame with gene III, which encodes the minor surface protein gIIIp of the filamentous phage. Therefore, production of recombinant phagemid particles displaying the antibody on the surface requires infection of the phagemid-carrying bacterial cell with the replication defective phage M13KO7 [14]. M13KO7 was added to a 10 ml library culture at a multiplicity of 10. After incubation at 37°C for 90 min the cells were pelleted and resuspended in 15 ml 2xYT-medium containing 100 µg/ml ampicillin, 10 µg/ml tetracycline and 50 µg/ml kanamycin. The culture was incubated overnight at 37°C at 250 rpm, then chilled on ice and centrifuged to remove cells. The supernatant containing the phages was mixed with 1/5 volume of an aqueous solution containing 20% PEG 8,000 and 14% NaCl and incubated 1 h at 4°C. Then a centrifugation step of 30 min at 4°C und 6,200 g was added. The pellet containing the phages was resuspended in 2 ml 10 mM Tris/HCl pH 7,5, 20 mM NaCl, 2 mM EDTA pH 7,5 and used for panning.

b) *Panning to select for antigen- and cell-binding clones*

Panning on purified PSMA was done in 96 well Maxi-Sorb microtiter plates (Nunc) which were coated with a solution of purified PSMA (100 µl/well, 12 µg/ml PSMA in PBS) and blocked with 4% non-fat milk/PBS. One ml of purified recombinant phages (circa 10^{11}) were incubated in 1 ml 4% non-fat milk/PBS supplemented with 15 µl 10% Triton X100 for 15 min and then allowed to bind to 8 wells coated with PSMA for 2 h at 37°C. After 20 rounds of washing with PBS/Tween (0,1%) the bound phages were eluted with 0,1 M Glycin-Puffer pH 2,2. For panning on viable LNCaP cells phages were previously absorbed on DU 145 cells. For this procedure 1 ml (circa 10^{11}) recombinant phages were incubated in 2% non-fat milk/PBS for 15 min and then with 10^7 DU 145 cells for 1 h at room temperature on a shaker. Then the cells were centrifuged and the supernatant with non absorbed phages was incubated with 10^6 LNCaP cells for 1 h at room temperature on a shaker. After 10 washing rounds with 2% non-fat milk/PBS and 5 rounds with PBS the bound phages were eluted with 50 mM HCl with subsequent neutralization with 1 M Tris-HCl (pH 7,5).

E. coli TG1 cells were infected with the eluted phages, plated on SOB-AG plates and incubated overnight at 30°C. An aliquot of the eluate was used for titration. The selection procedure was repeated three to six times.

c) *Small scale phage rescue*

From the titration plate 96 individual colonies were isolated and each transferred into one well of a 96-deep-well microtiter plate filled with 500 µl 2xYT medium containing 100 µg/ml ampicillin and 0,1 M glucose (YT-AG) and incubated overnight at 37°C (master plate). Then 40 µl of saturated culture from each well of the master plate were transferred to the corresponding well of a new plate containing 400 µl of 2x YT-AG medium.

To each well about 1×10^{10} M13KO7 helper phages were added and incubated on a shaker for 2 hours at 37°C. Then the plate was centrifuged and the pellet suspended in 2xYT medium supplemented with 100 µg/ml ampicillin, 10 µg/ml tetracycline, and 50 µg/ml kanamycin and incubated at 29°C and 240 rpm overnight. After centrifugation the supernatant containing the rescued phagemids was removed and used for phage ELISA and flow cytometry.

d) *Phage-ELISA*

Microtiter plates were coated with purified PSMA (1,5 µg PSMA/ml PBS) overnight and then blocked with 2% non-fat milk/PBS. To each well 200 µl of rescued phagemids, preincubated 1:1 with 2% non fat-milk/PBS, were added and incubated for 2 h at room temperature. After five washing steps with PBS-Tween, bound phages were detected with 200 µl /well anti-M13 antibody conjugated to horseradish peroxidase (Pharmacia) for 2 h at room temperature. Development was carried out with 3,3',5',5'-tetramethylbenzidine as substrate.

e) *Isolation and characterization of anti-PSMA conformational scFv*

For generation of anti-PSMA conformational scFv a V_HV_L library in the phagemid pSEX was constructed from the B cell library of a mouse immunized with M-PER-lysate of LNCaP cells. This library had a complexity of 10^7 . In a similar way a V_HV_L library was prepared from the monoclonal antibody 3/A12, which was obtained from the same mouse immunized with LNCaP lysate. This V_HV_L library had a complexity of 10^5 . To isolate phages displaying cellular PSMA binding scFv on their surface, six rounds of panning were performed alternatively on LNCaP cells after absorption with DU-145 cells in polystyrene tubes and in

microtiter plates coated with 20 µg/ml purified PSMA. After three, four and six panning rounds isolated phagemid colonies were grown and phage particles were rescued by infection with M13KO7. Analysis of 800 phage clones from the B-cell library by flow cytometry with LNCaP cells and ELISA on purified PSMA showed one positive clone called E8. Out of the $V_H V_L$ library from mAb 3/A12 two positive clones were obtained after the fourth panning round called A4 and A5. By sequencing it was found that A4 was identical to E8.

The coding region of the scFv E8 and A5 were transferred from the phagemid pSEX into the expression vector pHOG, containing C-terminal c-myc and His-tags. The sequences with the corresponding CDRs are given in Fig. 13 and Fig. 14. The regions coding for the CDR's of the antigen binding portions are marked in Fig. 13 and 14. Those sequences should not be changed whereas the other parts of the sequence which are not marked can be changed. The appropriate three-dimensional structure must, however, be maintained.

The scFv E8 strongly reacted with viable LNCaP cells as measured by flow cytometry with MFI values of about 100 at saturating concentrations, whereas binding of A5 was much weaker with MFI-values of about 40 at saturating concentrations (Fig 7). In contrast, binding to purified PSMA as solid phase in an ELISA was weak for E8 and somewhat stronger for A5. A similar pattern was seen in western blots with denatured glycosylated and deglycosylated PSMA (Fig.8). By immunofluorescence cytology with LNCaP cells and detection by confocal laser microscopy a very good binding of the scFv E8 and internalization could be shown (Fig 9). Data of the scFv are summarized in Table 2.

Table 2: Characterization of 2 scFv against cell-surface PSMA

ScFv	Origin	FACS LNCaP [MFI]	FACS PSMA- transf.DU [MFI]	ELISA PSMA	Blot PSMA	Blot degl. PSMA
E8 = A4	B-cell library and mAb A12	100	70	pos	(pos)	(pos)
A5	MAb A12	40		pos	pos	(pos)

MFI = mean fluorescence intensity at scFv concentration reaching antigen saturation
(background staining with secondary antibody alone is subtracted)

(pos) = slightly positive

Example 7*ScFv expression and purification*

ScFv fragments were expressed in *E. coli* XL1-Blue (Stratagene) using the secretion vector pHOG 21 which contains the sequences for the His-6 and c-myc-tag in a C-terminal position of the scFv [Kipriganov et al., J.Immunol.Methods (1997), p. 69-77]. *E. coli* bacteria transformed with pHOG plasmids were grown overnight in 2 x YT-AG-medium, then diluted 1:20 and grown as 600 ml cultures at 37°C. When cultures reached OD 0.8, bacteria were pelleted by centrifugation at 1,500 g for 10 min and resuspended in the same volume of fresh YT medium containing 50 µg/ml ampicillin, 0.4 M sucrose and 1 mM IPTG. Then growth was continued at room temperature for 18 to 20 h. Cells were harvested by centrifugation at 5,000 g for 10 min and 4°C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 mM Tris-HCl, 20% sucrose, 1 mM EDTA pH 8.0. After a 1 h incubation on ice, the spheroblasts were centrifuged at 20,000 g at 4°C for 30 min yielding soluble periplasmic extract in the supernatant. The periplasmic extract was concentrated using Amicon YM10 membranes with a 10 kDa cut-off (Amicon, Witten, Germany) followed by thorough dialysis against 50 mM Tris-HCl, 1 M NaCl, pH 7.0.

Purification was achieved by immobilized metal affinity chromatography. This was performed using a 1 ml column of chelating Sepharose (Pharmacia) charged with Cu²⁺ and equilibrated with a buffer containing 50 mM Tris-HCl and 1 M NaCl, pH 7.0. The periplasmatic extract was loaded, washed with twenty column volumes of equilibration buffer containing 30 mM imidazole and then eluted with the same buffer containing 250 mM imidazole. Eluted material was dialyzed against PBS.

Determination of the protein content was performed with the Micro BCA Protein Reagent Kit (Pierce) according to the manufacturer's instructions.

Protein induction was obtained with IPTG and the scFv yield from a 600 ml *E. coli* XL1 culture was about 20 µg.

Example 8*Flow cytometry*

LNCaP, DU 145, and PC3 cells were freshly harvested from tissue culture flasks and a single cell suspension was prepared in PBS with 3% FCS and 0,1% NaN₃. Approximately 10⁵ cells were incubated with 50 µl of rescued phagemids, preincubated 1:1 with 2% non-fat milk/PBS, 1 h on ice. After 3 rounds of washing with PBS 25 µl/well anti-c-myc monoclonal antibody 9E10 (10 µg/ml; Becton Dickinson) or when phages were tested 25 µl/well anti-M13 antibody (10 µg/ml; Pharmacia) were added and incubated 40 min on ice. After washing 3 times with PBS the cells were incubated with 100 µl of PE-labeled goat anti-mouse IgG (Becton Dickinson) for 40 min on ice. The cells were then washed again and resuspended in 100 µl of a solution containing 1 µg/ml propidium iodide (Sigma, Deisenhofen) in PBS with 3% FCS and 0,1% NaN₃ in order to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer and the CellQuest software (Becton Dickinson Mountain View, CA).

Example 9*Immunofluorescence cytology*

LNCaP cells were grown on glass coverslips for 24 hours. For fixation, cells were treated with 2% paraformaldehyde in PBS for 30 min at RT, which does not permeabilize the cell membrane, washed with 1% BSA-PBS, quenched for 10 min in 50 mM NH₄Cl in PBS, and rinsed with 1% BSA-PBS. Primary monoclonal antibody at 4 µg/ml in 1% BSA-PBS was added and incubated for 60 min at 4°C. FITC-labeled goat anti-mouse secondary antibody (2 µg/ml; Southern Biotechnology Associates Inc. Birmingham, USA) was incubated for 30 min and washed extensively with 1% BSA-PBS. Slides were mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA).

For internalization experiments the primary antibody was incubated for 30 min at 37°C before fixation of the cells with 2% paraformaldehyde and permeabilization with 0,5 % Triton X100 in PBS.

Example 10**a) *Immunohistochemistry***

Paraffin tissue sections were first deparaffinized and then treated with 0,3% Triton X100 in PBS for antigen retrieval. Kryostat sections were fixed in cold acetone. The sections were treated 30 min at with 3% H₂O₂ and 10 % methanol for quenching of endogenous peroxidase. After blocking with 1% BSA-PBS the primary antibody was added at a concentration of 2 µg/ml and incubated for 1 h at RT. For the scFv a secondary mouse-anti-c-myc antibody was added for 1 h at RT. Then a biotinylated goat-anti-mouse antibody was incubated for 30 min at RT and finally developed with ABC-reagent (Vectastain).

b) *Western blot analysis*

Western blot analysis was performed following sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis of purified PSMA and cell lysate from LNCaP cells and transferred to polyvinylidene difluoride membranes. The blots were blocked overnight in PBS containing 5% non-fat milk and incubated with the purified mAbs or scFv at concentrations of 10 µg/ml for 1 h. Then the blots were washed 5 times with PBS-Tween (0,5%) and incubated with horseradish peroxidase conjugated goat anti-mouse IgG for 1 hour at RT. After 5 washes with PBS-Tween (0,5%) the blots were developed by using 3,3',5',5'-tetramethylbenzidine as substrate.

Example 11***Construction, expression and purification of scFv-PE40 proteins***

The toxin used in our approach was the truncated version of *Pseudomonas* exotoxin (PE40), lacking domain Ia and containing only domains Ib, II, and III [Pastan et al., J.Biol.Chem. (1989), p. 15157-15160]. The DNA with the coding region in the vector pSW200 was obtained from Prof. W. Wels, Frankfurt [Wels et al., Biotechnology (1992), p. 1128-1132]. The DNA fragment from bp position 253 to 613 encoding PE40 was amplified by PCR from plasmid pSW200. The amplified DNA was then ligated into the vector pHOG-His-scFv in a C-terminal position to the scFv using the restriction site XbaI. All cloning steps were performed according to standard methods in *E. coli* XL1 blue and the products were confirmed by sequencing.

Protein induction of the immunotoxin and purification by IMAC was the same like the scFv. The products were tested and characterized by SDS-page, western blot and flow cytometry.

Example 12

Cytotoxicity of scFv-PE40 Immunotoxins

The metabolism of the red tetrazolium salt WST to a water soluble formazan dye was determined according to the manufacturer's instructions (Boehringer). Target cells (LNCaP and DU 145 as control) were seeded at 2.5×10^4 /well of a 96-well plate and grown for 24 hours until a confluent cell layer was formed. Various dilutions of the recombinant immunotoxins in aliquots of 50 μ l/well were added and the plates were incubated for 48 hours at 37°C, 5% CO₂. After this time the cultures were pulsed with 15 μ l/well WST reagent and incubated for 90 min at 37°C, 5% CO₂. Then the spectrophotometrical absorbances of the samples were measured at 450 nm (reference 690 nm). The immunotoxin concentration required to achieve a 50% reduction in cell viability relative to that of untreated control cultures (50% inhibitory concentration = IC₅₀) was calculated.

Cytotoxicity assays (WST) with the immunotoxins E8-P40 and A5-P40 were prepared with PSMA expressing LNCaP cells and DU 145 control cells. As shown in Fig. 11 a high cytotoxic effect could be shown with the immunotoxin E8-PE40 on LNCaP cells with a IC₅₀ value of 0.05 nM. In Fig. 12 the cytotoxic effect of the immunotoxin A5-PE40 is shown with an IC₅₀ of about 0.09 nM. The cytotoxic background on not PSMA expressing DU 145 cells was 5% for the E8 construct and only 0.01% for the A5 construct evidencing a very good therapeutic window.

Example 13

Generation of the scFv H12 and D7 from mAb 3/F11 and 3/E7

From each mAb a scFv expression library in the phagemid pSEX was generated as described in Example 5.

Production and selection of antibody-displaying phage was done according to Example 6.

After six panning rounds alternatively on PSMA and LNCaP cells one specific positive clone was obtained, from mAB 3/E7, which was named H12 and one positive clone was obtained from mAB 3/F11, which was named D7. The coding region of each scFv was transferred into the expression vector pHOG-21.

ScFv expression and purification was done as described in Example 7.

Example 14

Characterization of the scFv H12 and D7

a) Flow cytometry on PSMA-positive and –negative cell lines

The scFvs H12 and D7 reacted with viable LNCaP cells as measured by flow cytometry.

From the saturation curves the antibody concentration reaching 50 % saturation of PSMA sites was determined to be approximately 120 nM (H12) and 20 nM (D7) respectively. At saturating concentrations MFI values of 70 (H12) and 40 (D7) were reached (Fig. 15).

To evaluate the PSMA binding specificity of the scFv H12 and D7, PSMA-negative prostate cancer cells of DU145 and PC3 and other PSMA negative cell lines (HeLa, MCF7, HCT15 and Jurkat) were additionally stained and analyzed by flow cytometry. All three scFv did not stain the PSMA-negative cells.

b) Flow cytometry on PSMA transfectants

To verify a PSMA-specific binding, the scFv H12 and D7 were also tested on BOSC-23 cells transfected with PSMA. Both scFv showed a concentration dependent binding to BOSC cells transfected with full-length PSMA but not to non-transfected cells (Fig. 16). Saturating conditions were reached at 100 nM (D7) and 200 nM (H12). Similar to the mAbs, MFI-values on the transfectants were lower than on LNCaP cells and showed a broad distribution, which may correspond to varying PSMA molecules on the cell surface of the former.

c) Immunofluorescence cytology

Immunofluorescence cytology was prepared as described in Example 4. After detection with a laser scanning confocal microscope a strong binding of the scFv to LNCaP cells and also an internalization into these cells was observed.

d) ELISA and Western blotting

Binding of the scFv H12 and D7 to purified PSMA in an solid phase ELISA and by Western blotting was weak.

The sequences (amino acid and nucleic acid) of H12 and D7 are given in Fig. 20 and Fig. 21.

Table 3: Characteristics of the anti-PSMA scFv H12 and D7

scFv	Original mAb	FACS on LNCaP MFI*	FACS on PSMA-transfected BOSC (MFI*)	Blot on PSMA	SEQ ID NO of nucleic acid sequence (coding strand)	SEQ ID NO of amino acid sequence	SEQ ID NO of nucleic acid sequence (complementary strand)
H12	3/E7	70	25	100 kD	19	20	23
D7	3/F11	40	24	100 kD	21	22	24

* MFI = Mean fluorescence intensity values at saturating conditions after subtraction of the background staining with an irrelevant isotype-matched control antibody or anti-mouse immunoglobulin alone.

Example 15

Construction and cytotoxicity of a H12-PE40 immunotoxin and D7-PE40 immunotoxin

Construction of the H12-PE40 and the D7-PE40 immunotoxin was similar to A5 and E8 immunotoxins described in example 11. PE-40 represents the *Pseudomonas* exotoxic fragment.

Cytotoxicity was tested as described in example 12.

The imunotoxin promoted death of LNCaP cells in a time-dependent manner; highest cytotoxic effects could be observed after 48 h incubation.

At this time IC50 values of about 200 pM were found for H12-PE40 and D7-PE40 (Fig. 17).

Additionally, cytotoxicity of H12-PE40 and D7-PE40 was tested on the PSMA-negative cell lines DU 145, PC-3, MCF7 and HCT 15. No cytotoxicity was found on these cell lines at concentrations up to 25 000 pM..

Example 16

Construction of an anti-PSMA/CD3 diabody

A bispecific diabody specific for PSMA and the CD3 chain of the T cell receptor complex was generated. The bispecific diabody was expressed in E.coli using a vector containing the dicistronic operon for cosecretion of VhCD3-VIA5 and VhA5-VICD3 scFv (Fig. 18). For the anti-A5/CD3 diabody construction the plasmids pKID19x3 and pKID 3x19 were used [Kipriyanov, Int.J.Cancer 1998, pp 763]. Bacterial periplasmatic expression and purification was similar to the scFv.

Example 17

Induction of specific cytotoxicity by diabody A5-CD3

The ability of the bispecific diabody to induce tumor cell lysis by redirecting T cell-mediated cytotoxicity was investigated using PBMC from healthy donors as effector cells. After incubation with or without IL-2 for 4 days, the cells were added to LNCaP target cells, which were seeded at 1.5×10^4 cells/well of a 96-well plate. The effector-target ratio was 10:1. Diabody was added at different concentrations. After incubation of 48 hours the cultures were pulsed with 15 μ l/well WST reagent and incubated for 90 min at 37 °C and 5 % CO₂. Then the spectrophotometrical absorbances of the samples were measured at 450 nm (reference 690 nm).

In this in vitro test the diabody appeared to be quite potent in retargeting activated and inactivated PBMC to lyse the target LNCaP cells in a concentration dependent manner (Fig. 19).

Example 18*Diabody A5-A5*

This bivalent monospecific diabody was generated similar to the A5-CD3 diabody (example 16). Bacterial periplasmatic expression and purification was similar to the scFv.

By flow cytometry a strong and specific binding of diabody A5-A5 to LNCaP cells could be shown.

Claims:

- 1) An isolated monoclonal antibody or an antigen binding portion thereof which
 - a) binds to prostate specific membrane antigen in its native form occurring on the surface of tumor cells
 - b) can be internalized by a tumor cell,
 - c) binds strongly to LNCAP cells but not or only minimally to cells which lack expression of prostate specific membrane antigen and
 - d) characterized in that it is linked to a label or a cytotoxic agent.
- 2) Isolated monoclonal antibody or antigen binding portion thereof according to claim 1 characterized in that the PE fluorescence intensity (MFI) of the mAb is higher than 1000 and of the scFv higher than 40 at antigen saturation.
- 3) Isolated monoclonal antibody or an antigen binding portion thereof according to claims 1 and 2 which show a high binding activity to LNCAP cells reaching 50% saturation of PSMA sites at concentrations between 1 nM and 120 nM.
- 4) Isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-3 characterized in that the label is a particle which emits radioactive or fluorescence radiation.
- 5) Isolated monoclonal antibody or antigen binding portion thereof according to claims 1-4 characterized in that the cytotoxic agent is a cell toxic substance selected from the group consisting of toxins, in particular taxol, cytocalasin B, gramicidin D, ethidium bromid, emetine, mitomycin, etopside, tenopside, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy antracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosteron, glycocorticoids, procain, tetracaine, lidokaine, propranolol and/or puromycin.
- 6) Isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1 to 5 characterized in that it comprises a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:1.
- 7) Isolated monoclonal antibody or antigen binding portion thereof according to claim 6, characterized in that it comprises at least one of SEQ ID NO:2 – SEQ ID NO:7.

- 8) Isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1 to 5 characterized in that it contains a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:10.
- 9) Isolated monoclonal antibody or antigen binding portion thereof according to claim 8, characterized in that it comprises at least one of SEQ ID NO:11 – SEQ ID NO:16.
- 10) Isolated monoclonal antibody or antigen binding portion thereof according to any of claims 1 to 5 characterized in that it contains a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:20.
- 11) Isolated monoclonal antibody or antigen binding portion thereof according to claim 10 characterized in that it comprises a partial amino acid sequence of at least 25 consecutive amino acids of SEQ ID NO:20.
- 12) Isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1 to 5 characterized in that it comprises a partial amino acid sequence of at least 10 consecutive amino acids of SEQ ID NO:22.
- 13) Isolated monoclonal antibody or an antigen binding portion thereof according to claim 12 characterized in that it comprises at least 25 consecutive amino acids of SEQ ID NO:20.
- 14) Pharmaceutical composition comprising an isolated monoclonal antibody or an antigen binding portion thereof according to any of the preceding claims.
- 15) Use of an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-13 for the preparation of a medicament for the treatment of cancer.
- 16) Diagnostic kit for the detection of tumor cells comprising an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-13.
- 17) A method for the in vitro identification of tumor cells characterized in that the tumor cells to be identified are contacted with an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1-13.

- 18) Use of an isolated monoclonal antibody or an antigen binding portion thereof according to any of claims 1 to 13 for the diagnostic identification of tumor cells.
- 19) Isolated polynucleotide characterized in that it comprises a contiguous sequence of at least 20 nucleotides of any sequence of the group consisting of SEQ ID NOS: 8, 9, 17, 18, 19, 21, 23 and 24.

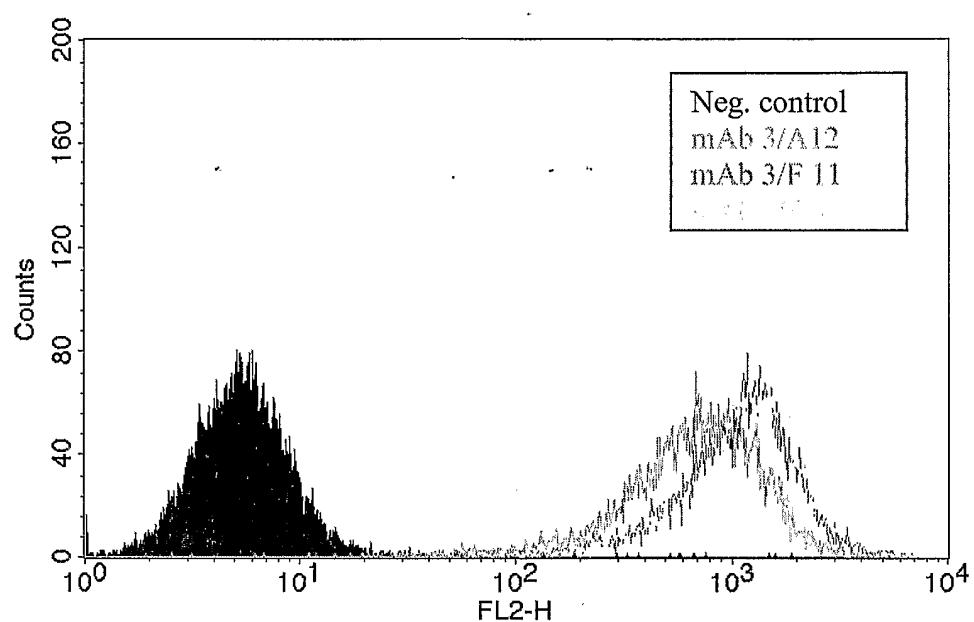


Fig 1

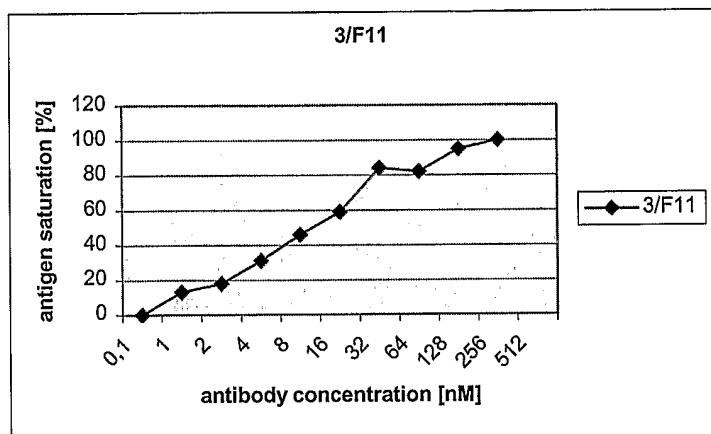


Fig 1a

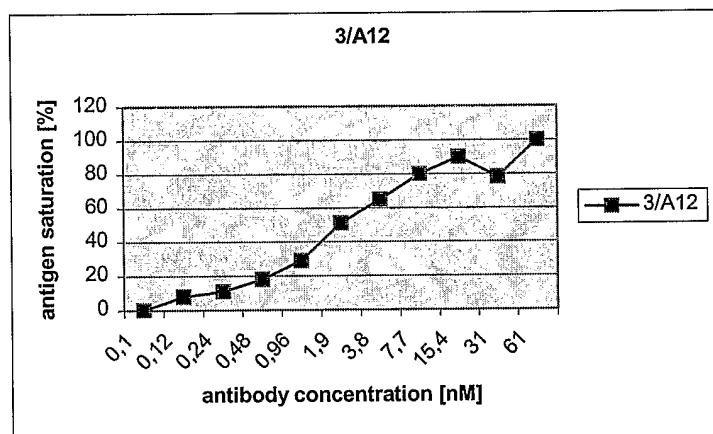


Fig 1b

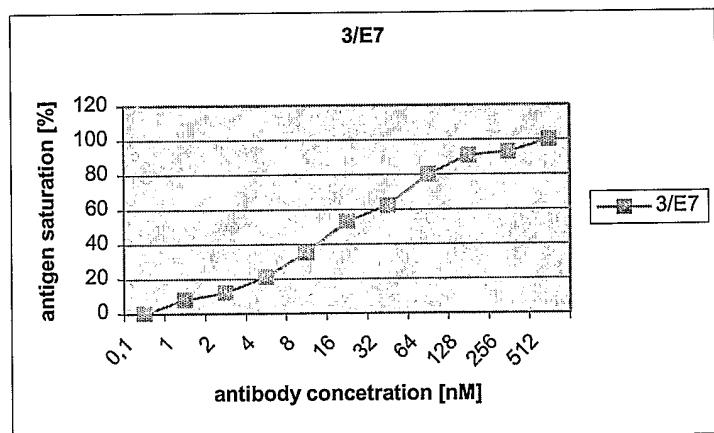
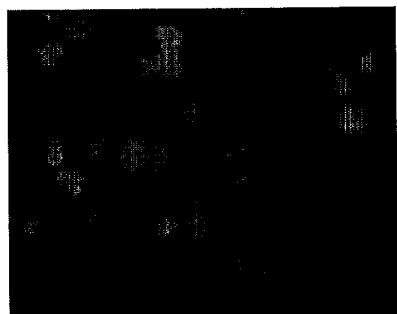


Fig 1c

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3/F11



3/A12



3/E7

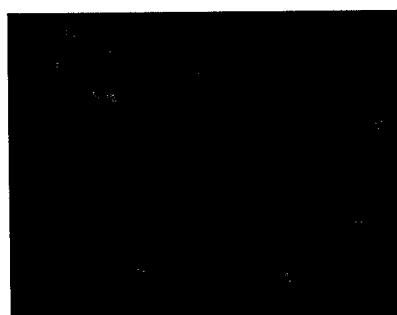
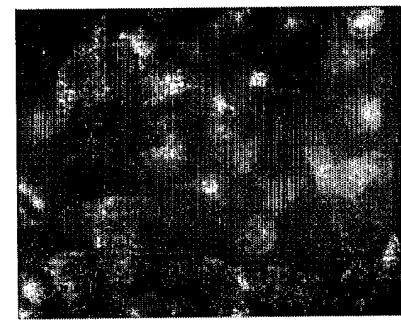
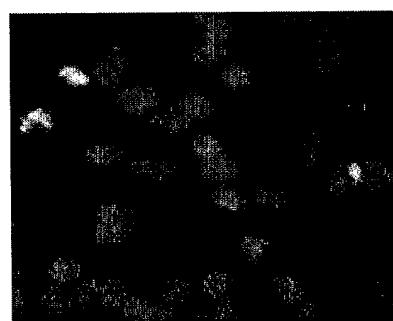


Fig 2

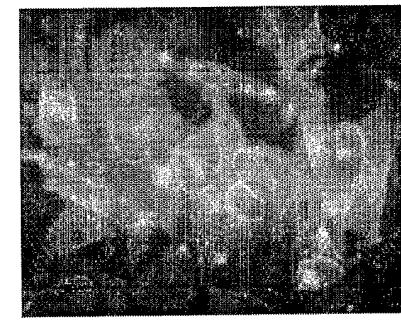
4/25



3/F11



3/A12



3/E7

Fig 3

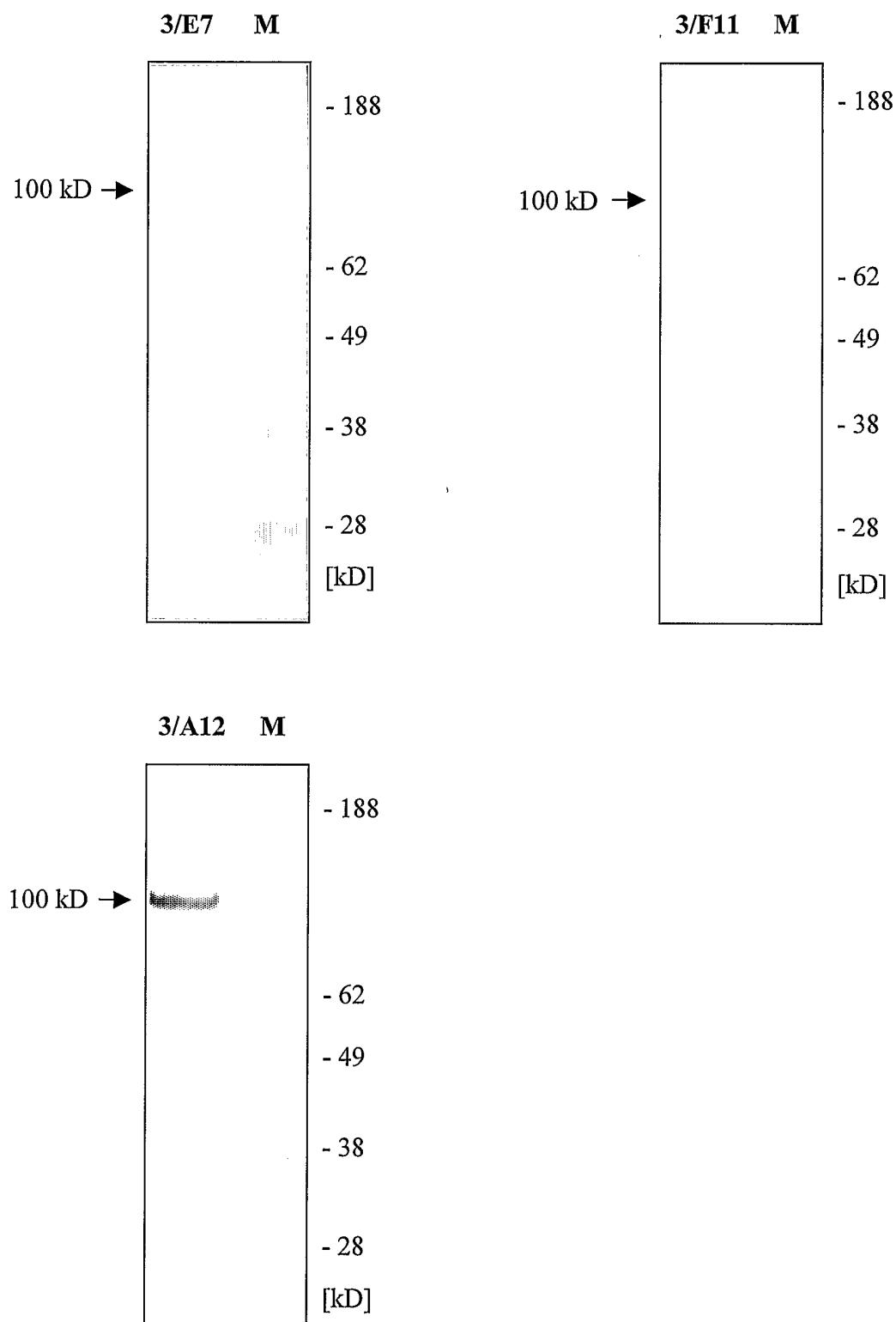


Fig 4

3/A12

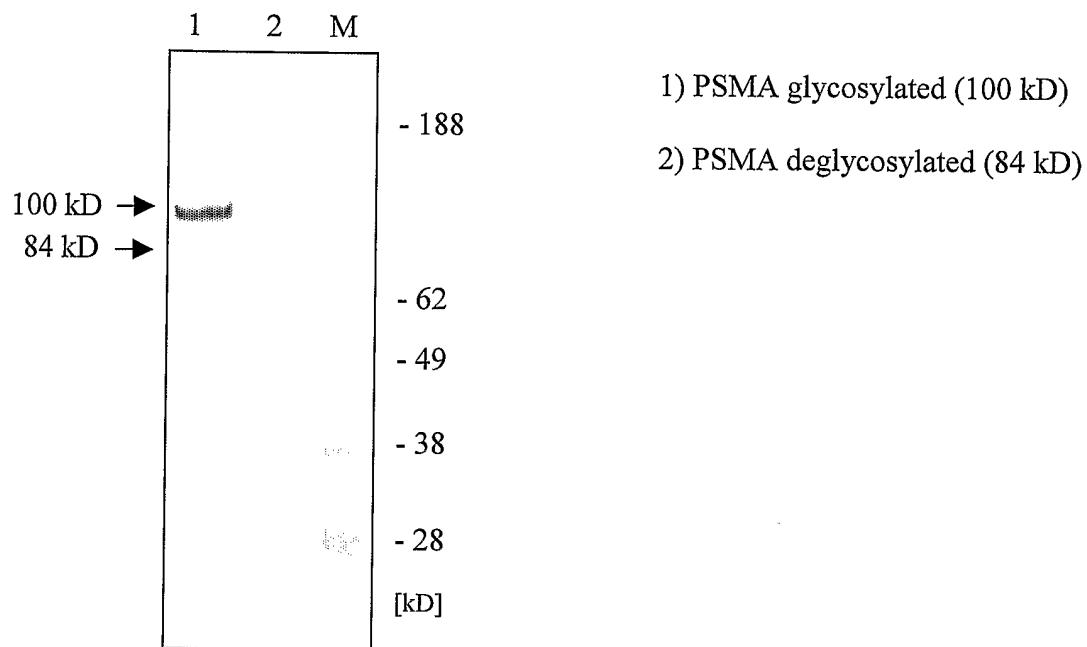


Fig 5

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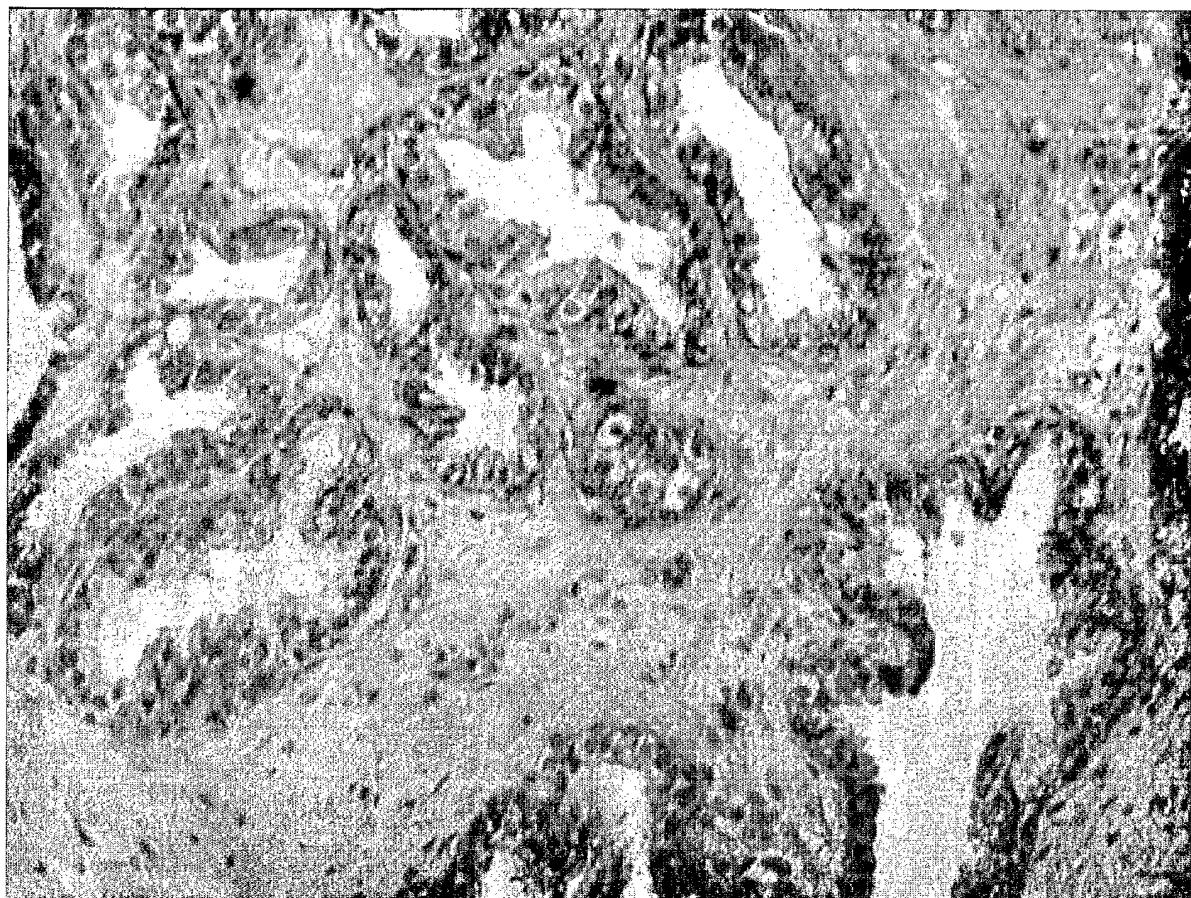


Fig 6

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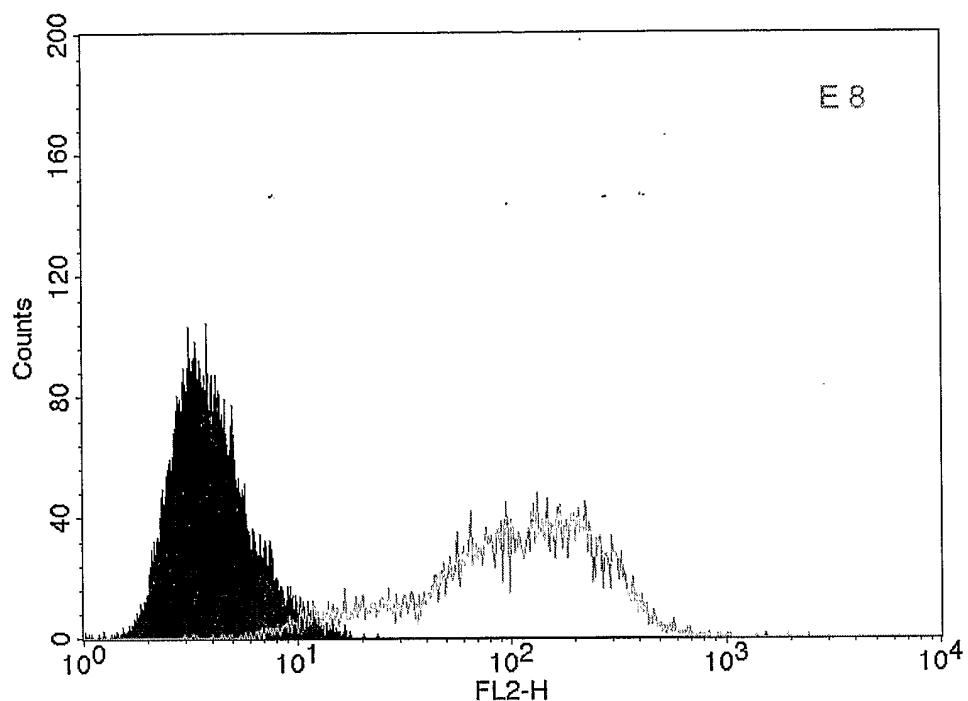


Fig 7 a

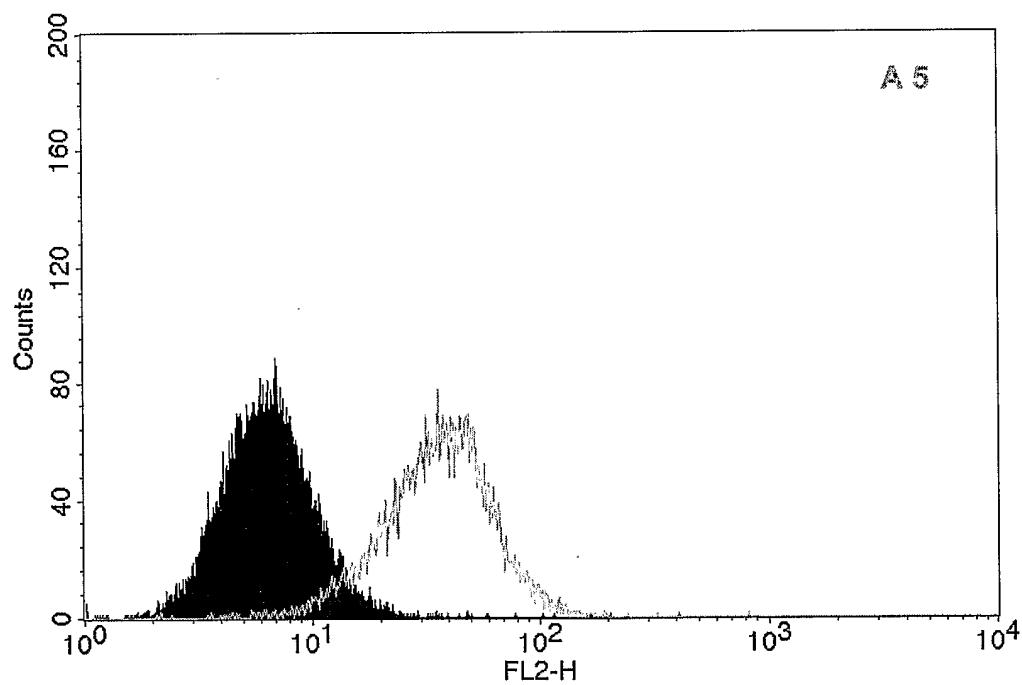


Fig 7 b

Fig 7 a,b

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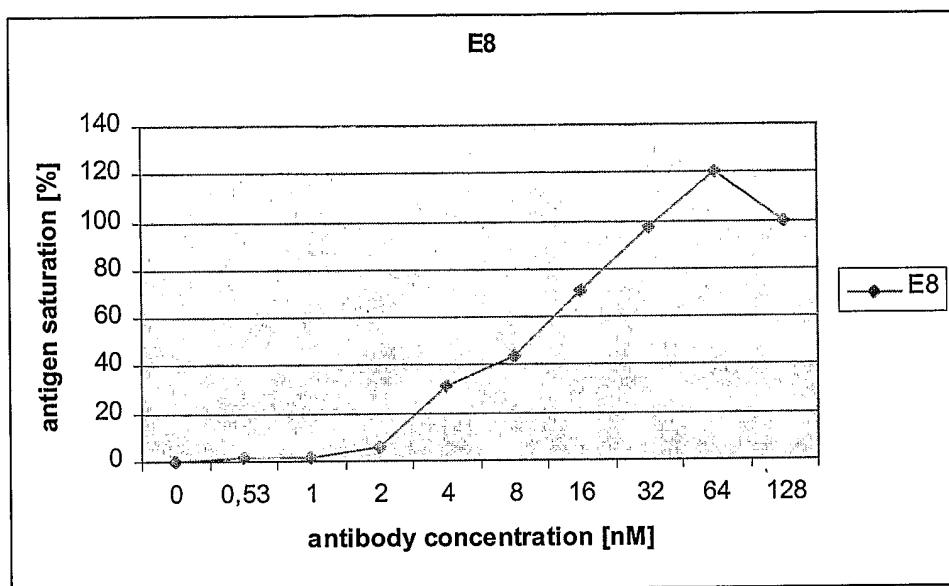


Fig 7c

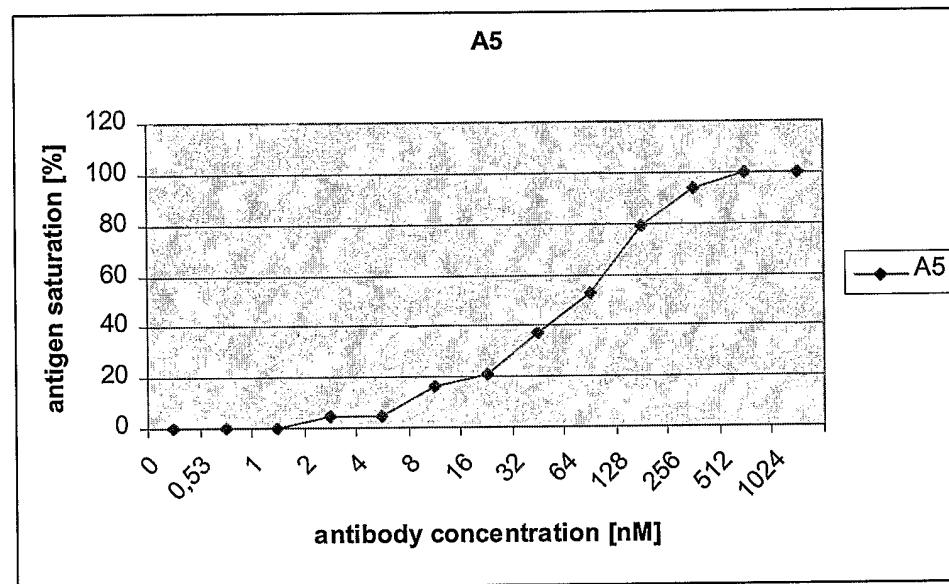


Fig 7d

Fig 7c,d

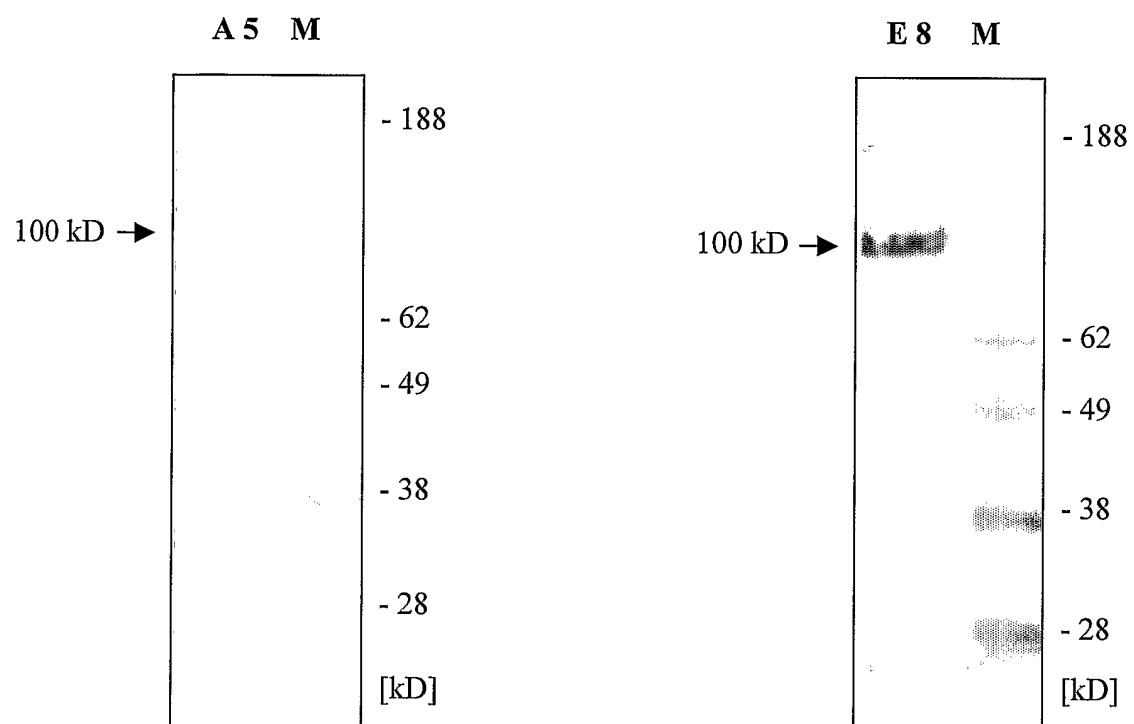


Fig 8

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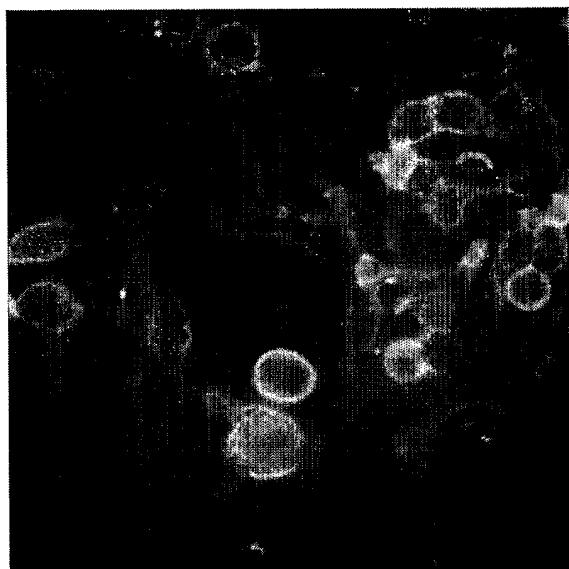
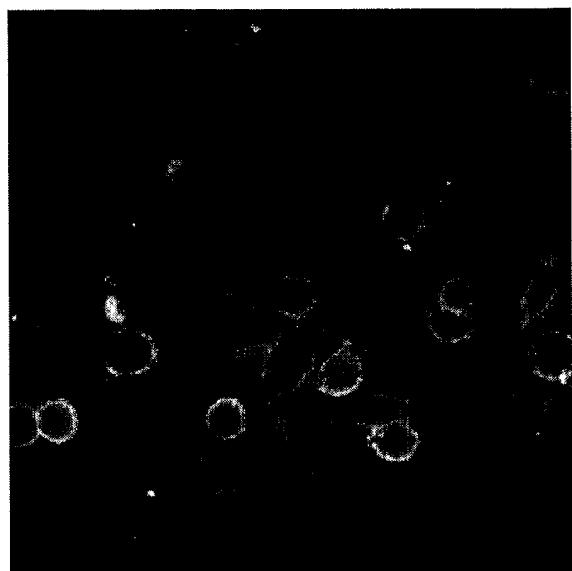


Fig 9

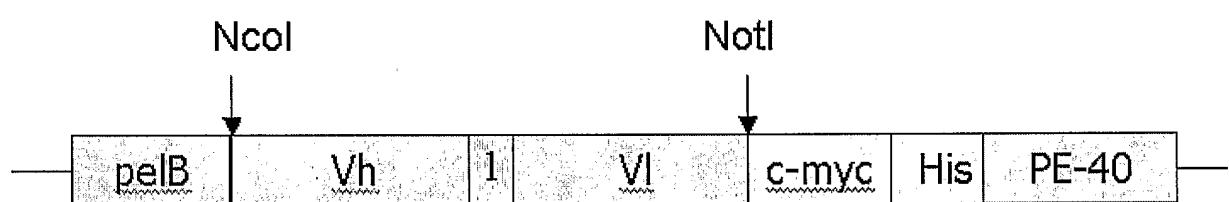


Fig 10

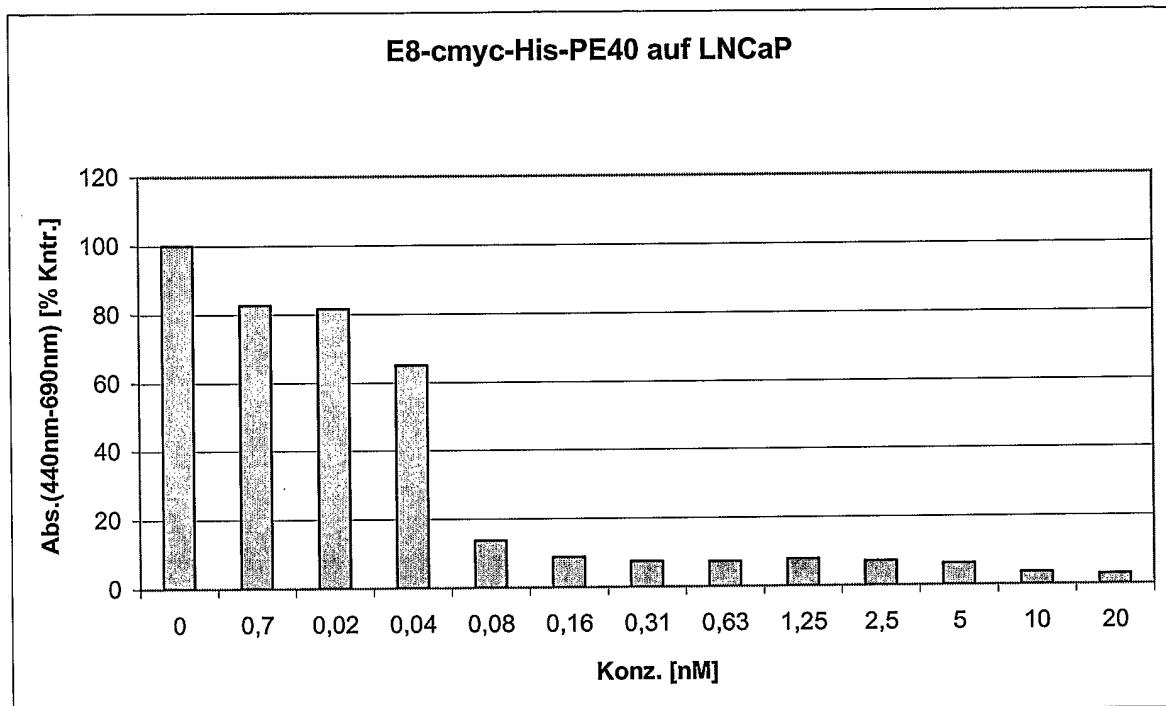


Fig 11

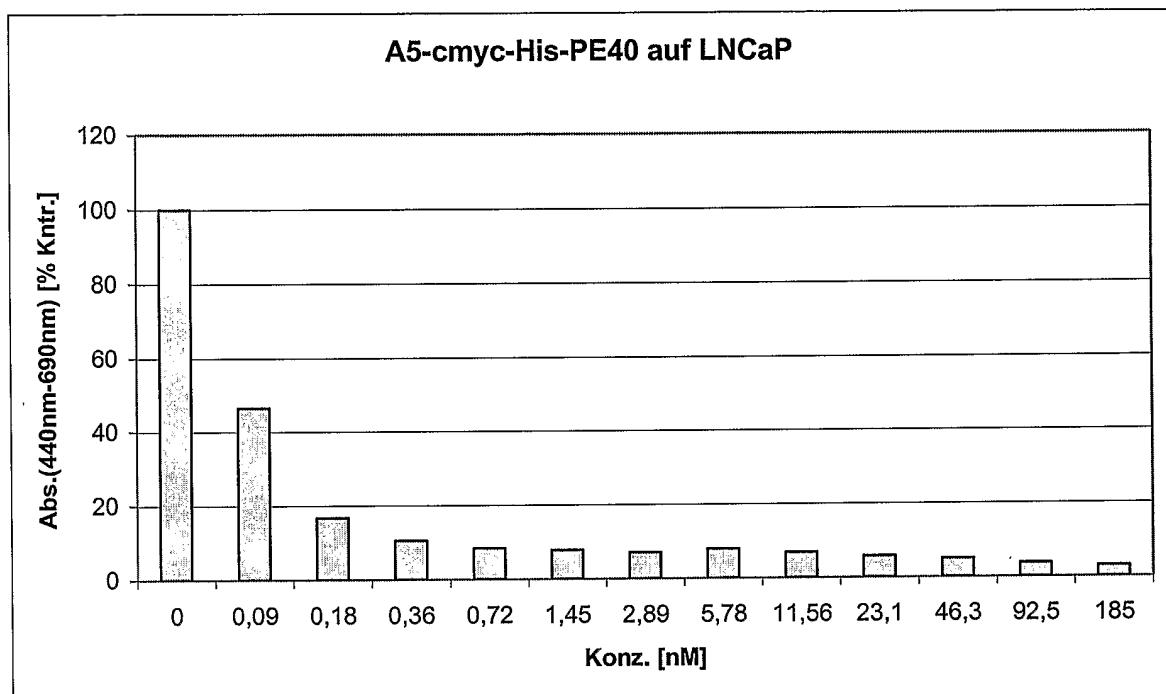


Fig. 12

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	M	A	E	V	Q	L	Q	Q	S	G	P	D	L	V	K	P	G	A											
1	ATG	GCC	GAG	GTG	CAG	CTG	CAG	CAG	TCA	GGA	CCC	GAC	CTG	GTG	AAG	CCT	GGG	GCC											
	TAC	CGG	CTC	CAC	GTC	GAC	GTC	GTC	AGT	CCT	GGG	CTG	GAC	CAC	TTC	GGA	CCC	CGG											
	S	M	K	I	S	C	K	A	S	G	Y	T	F	T	D	Y	N	M											
55	TCA	ATG	AAG	ATT	TCC	TGC	AAG	GCT	TCT	GGA	TAC	ACA	TTC	ACT	GAC	TAC	AAC	ATG											
	AGT	TAC	TTC	TAA	AGG	ACG	TTC	CGA	AGA	CCT	ATG	TGT	AAG	TGA	CTG	ATG	TTG	TAC											
	D	W	V	K	E	R	H	G	K	S	L	E	W	I	G	D	I	N											
109	GAC	TGG	GTG	AAG	GAG	AGA	CAT	GGA	AAG	AGC	CTT	GAG	TGG	ATT	GGA	GAT	ATT	AAT											
	CTG	ACC	CAC	TTC	CTC	TCT	GTA	CCT	TTC	TCG	GAA	CTC	ACC	TAA	CCT	CTA	TAA	TTA											
	P	K	N	G	V	T	I	Y	N	Q	K	F	K	G	K	A	T	L											
163	CCT	AAA	AAT	GGC	GTT	ACT	ATT	TAC	AAC	CAG	AAG	TTC	AAG	GGC	AAG	GCC	ACA	TTG											
	GGA	TTT	TTA	CCG	CAA	TGA	TAA	ATG	TTG	GTC	TTC	AAG	TTC	CCG	TTC	CGG	TGT	AAC											
	T	V	D	K	S	S	T	T	A	Y	M	E	L	R	S	L	T	S											
217	ACT	GTA	GAC	AAG	TCC	TCC	ACC	ACA	GCC	TAC	ATG	GAG	CTC	CGC	AGC	CTG	ACA	TCT											
	TGA	CAT	CTG	TTC	AGG	AGG	TGG	TGT	CGG	ATG	TAC	CTC	GAG	GCG	TCG	GAC	TGT	AGA											
	E	D	T	A	V	Y	Y	C	A	R	G	D	X	Y	G	N	Y	F											
271	GAA	GAC	ACT	GCA	GTC	TAT	TAT	TGT	GCA	AGA	GGG	GAC	TMC	TAT	GGT	AAC	TAC	TTT											
	CTT	CTG	TGA	CGT	CAG	ATA	ATA	ACA	CGT	TCT	CCC	CTG	AKG	ATA	CCA	TTG	ATG	AAA											
	D	Y	W	G	Q	G	T	S	L	T	V	S	S	A	K	T	T	P											
325	GAC	TAC	TGG	GGC	CAA	GGC	ACC	AGT	CTC	ACA	GTC	TCC	TCA	GCC	AAA	ACG	ACM	CCC											
	CTG	ATG	ACC	CCG	GTT	CCG	TGG	TCA	GAG	TGT	CAG	AGG	AGT	CGG	TTT	TGC	TGK	GGG											
	YOL	epitope	100.0%																										
	~~~~~																												
	K	L	E	E	G	E	F	S	E	A	R	V	D	I	Q	M	T	Q											
379	AAG	CTT	GAA	GAA	GGT	GAA	TTT	TCA	GAA	GCA	CGC	GTA	GAC	ATT	CAG	ATG	ACA	CAG											
	TTC	GAA	CTT	CTT	CCA	CTT	AAA	AGT	CTT	CGT	GCG	CAT	CTG	TAA	GTC	TAC	TGT	GTC											
	S	P	A	S	L	S	V	S	V	G	E	T	V	T	I	T	C	R											
433	TCT	CCA	GCC	TCC	CTA	TCT	GTA	TCT	GTG	GGG	GAA	ACT	GTC	ACC	ATC	ACA	TGT	CGA											
	AGA	GGT	CGG	AGG	GAT	AGA	CAT	AGA	CAC	CCT	CTT	TGA	CAG	TGG	TAG	TGT	ACA	GCT											
	T	S	E	N	I	Y	S	N	L	A	W	Y	Q	Q	K	Q	G	K											
487	ACA	AGT	GAG	AAT	ATT	TAC	AGT	AAT	TAA	GCA	TGG	TAT	CAG	CAG	AAA	CAG	GGA	AAA											
	TGT	TCA	CTC	TTC	TAA	ATG	TCA	TAA	AGT	CGT	ACC	ATA	GTC	TTC	GTC	CCT	TTT												
	S	P	Q	L	L	V	Y	T	A	T	N	L	A	D	G	V	P	S											
541	TCT	CCT	CAG	CTC	CTG	GTC	TAT	ACT	GCA	ACA	AAC	TTA	GCA	GAT	GGT	GTG	CCC	TCA											
	AGA	GGA	GTC	GAG	GAC	CAG	ATA	TGA	CGT	TGT	TTG	AAT	CGT	CTA	CCA	CAC	GGG	AGT											
	R	F	S	G	S	G	S	G	T	Q	Y	S	L	K	I	N	S	L											
595	AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGC	ACA	CAG	TAT	TCC	CTC	AAG	ATC	AAC	AGC	CTG											
	TCC	AAG	TCA	CCG	TCA	CCT	AGT	CCG	TGT	GTC	ATA	AGG	GAG	TTC	TAG	TTG	TCG	GAC											
	Q	S	D	D	S	G	T	Y	Y	C	Q	H	F	W	G	T	P	Y											
649	CAG	TCT	GAT	GAT	TCT	GGG	ACT	TAT	TAC	TGT	GAA	CAT	TTT	TGG	GGT	ACT	CGG	TAC											
	GTC	AGA	CTA	CTA	AGA	CCC	TGA	ATA	ATG	ACA	GTT	GTA	AAA	ACC	CCA	TGA	GGG	ATG											
	T	F	G	G	T	K	L	E	I	K	R	A	D	A	A	A	A												
703	ACG	TTC	GGG	GGG	GGG	ACC	AAG	CTG	GAA	ATA	AAA	CGG	GCT	GAT	GCT	GCG	GCC												
	TGG	AAG	CCT	CCC	CCC	CCC	TGG	TTC	GAC	CTT	TAT	TTT	GCC	CGA	CTA	CGA	CGC	CGG											

Sequence of scFv E8

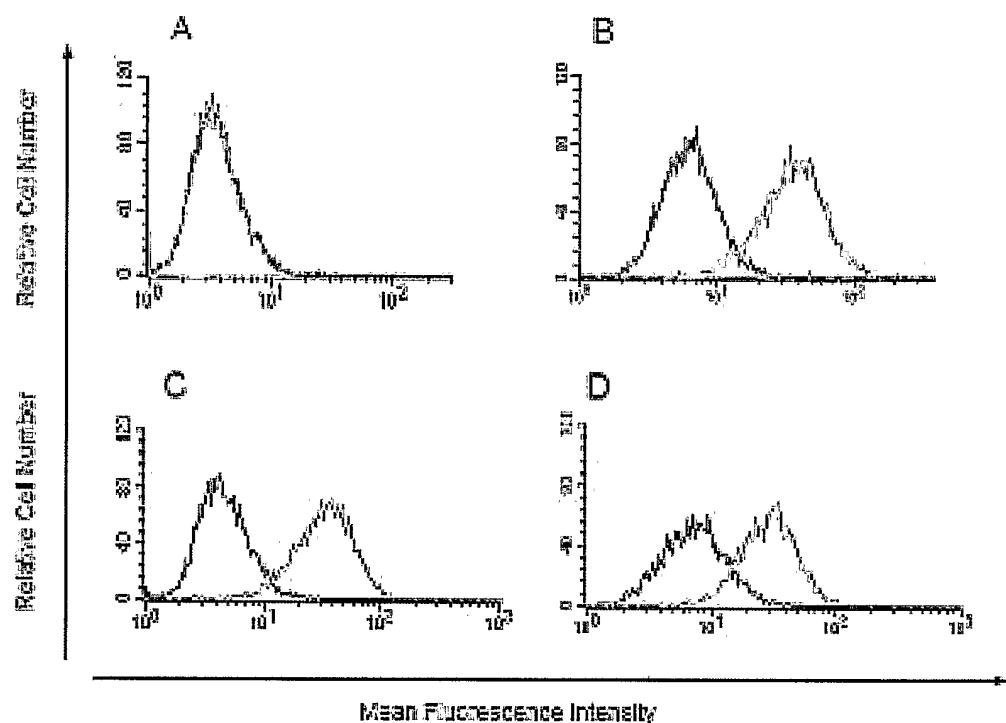
Fig 13

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1	ATG	GCC	GAC	GTG	AAG	TTG	GTG	GAG	TCT	GGG	GGA	GGA	GGC	TTA	GTG	AAG	CCT	GGA	GAG
	TAC	CGG	CTG	CAC	TTC	AAC	CAC	CTC	AGA	CCC	CCT	CCG	AAT	CAC	TTC	GGA	CCT	CTC	
	S	L	K	L	S	C	I	A	S	G	F	T	F	S	D	Y	Y	M	
55	TCC	CTG	AAA	CTC	TCC	TGT	ATA	GCC	TCT	GGA	TTC	ACT	TTC	AGT	GAC	TAT	TAT	ATG	
	AGG	GAC	TTT	GAG	AGG	ACA	TAT	CGG	AGA	CCT	AAG	TGA	AAG	TCA	CTG	ATA	ATA	TAC	
	Y	W	V	R	Q	T	P	E	K	R	L	E	W	V	A	I	I	S	
109	TAT	TGG	GTT	CGC	CAG	ACT	CCG	GAA	AAG	AGG	CTG	GAG	TGG	GTC	GCA	ATC	ATT	AGT	
	ATA	ACC	CAA	GCG	GTC	TGA	GTC	CTT	TTC	TCC	GAC	CTC	ACC	CAG	CGT	TAG	TAA	TCA	
	D	G	G	Y	Y	T	Y	Y	S	D	I	I	K	G	R	F	T	I	
163	GAT	GGT	GGT	TAT	TAT	ACC	TAC	TAT	TCA	GAC	ATT	ATC	AAG	GGG	CGA	TTC	ACC	ATC	
	CTA	CCA	CCA	ATA	ATA	TGG	ATG	ATA	AGT	CTG	TAA	AGT	TTC	CCC	GCT	AAG	TGG	TAG	
	S	R	D	N	A	K	N	N	L	Y	L	Q	M	S	S	L	K	S	
217	TCC	AGA	GAC	AAT	GCC	AAG	AAC	AAC	CTG	TAC	CTC	CAA	ATG	AGC	AGT	CTG	AAG	TCT	
	AGG	TCT	CTG	TTA	CGG	TTC	TTG	TTG	GAC	ATG	GAG	GTT	TAC	TCG	TCA	GAC	TTC	AGA	
	E	D	T	A	M	Y	Y	C	T	R	G	F	P	L	L	R	H	G	
271	GAG	GAC	ACA	GCC	ATG	TAT	TAC	TAT	TCA	AGA	GGT	TTT	CCT	CTA	CTA	CGG	CAC	GGG	
	CTC	CTG	TGT	CGG	TAC	ATA	ATG	ACA	TGT	TCT	CCA	AAA	GGA	GAT	GAT	GCC	GTG	CCC	
	A	M	D	Y	W	G	L	G	T	S	V	T	V	S	S	T	K	T	
325	GCT	ATG	GAC	TAC	TGG	GGT	CTT	GGA	ACC	TCA	GTC	ACC	GTC	TCC	TCA	ACC	AAA	ACG	
	CGA	TAC	CTG	ATG	ACC	CCA	GAA	CCT	TGG	AGT	CAG	TGG	CAG	AGG	AGT	TGG	TTT	TGC	
	YOL	epitope	100.0%																
	-----																		
	T	P	K	L	E	E	G	E	F	S	E	A	R	V	D	I	Q	M	
379	ACA	CCC	AAG	CTT	GAA	GAA	GGT	GAA	TTT	TCA	GAA	GCA	CGC	GTA	GAC	ATT	CAG	ATG	
	TGT	GGG	TTC	GAA	CTT	CTT	CCA	CTT	AAA	AGT	CTT	CGT	GCG	CAT	CTG	TAA	GTC	TAC	
	T	Q	S	P	K	F	M	S	T	S	V	G	D	R	V	S	V	T	
433	ACC	CAG	TCT	CCA	AAA	TTC	ATG	TCC	ACA	TCG	GTA	GGA	GAC	AGG	GTC	AGC	GTC	ACC	
	TGG	GTC	AGA	GGT	TTT	AAG	TAC	AGG	TGT	AGC	CAT	CCT	CTG	TCC	CAG	TCG	CAG	TGG	
	C	K	A	S	Q	N	V	D	T	N	V	A	W	Y	Q	Q	K	P	
487	TGC	AAG	GCC	AGT	CAG	AAT	GTC	GAT	ACT	AAT	GTA	GCG	TGG	TAT	CAA	CAG	AAA	CCA	
	ACG	TTC	CGG	TCA	GTC	TTA	CAA	CTA	TGA	TTA	GAT	CGG	ACC	ATA	GTT	GTC	TTT	GGT	
	G	Q	S	P	K	A	L	I	Y	S	A	S	Y	R	Y	S	D	V	
541	GGA	CAA	TCT	CCT	AAA	GCA	CTG	ATT	TAC	TCC	GCA	TCC	TAC	CGG	TAC	AGT	GAC	GTC	
	CCT	GTT	AGA	GGA	TTT	CGT	GAC	TAA	ATG	AGC	CGT	AGG	ATG	GCC	ATG	TCA	CTG	CAG	
	P	D	R	F	T	G	S	E	S	G	T	D	F	T	L	T	I	S	
595	CCT	GAT	CGC	TTC	ACA	GGC	AGT	GAA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	
	GGA	CTA	GCG	AAG	TGT	CCG	TCA	CTT	AGA	CCC	TGT	CTA	AAG	TGA	GAG	TGG	TAG	TCG	
	N	V	Q	S	E	D	L	A	E	Y	F	C	Q	Q	Y	D	S	Y	
649	AAT	GTG	CAG	TCT	GAA	GAC	TTG	GCA	GAG	TAT	TTC	TGT	CAC	CAA	TAT	GAC	AGC	TAT	
	TTA	CAC	GTC	AGA	CTT	CTG	AAC	CGT	CTC	ATA	AAG	ACA	GTC	GTT	ATA	CTG	TGG	ATA	
	P	Y	T	F	G	G	G	T	K	L	E	I	K	R	A	D	A	A	
703	CCA	TAC	AGC	TTC	GGG	GGG	GGG	ACC	AAG	CTG	GAA	ATA	AAA	CGG	GCT	GAT	GCT	GCG	
	GGT	ATG	TGG	AAG	CCT	CCC	CCC	TGG	TTG	GAC	CTT	TAT	TTT	GCC	CGA	CTA	CGA	CGC	
	A																		
757	GCC																		
	CGG																		

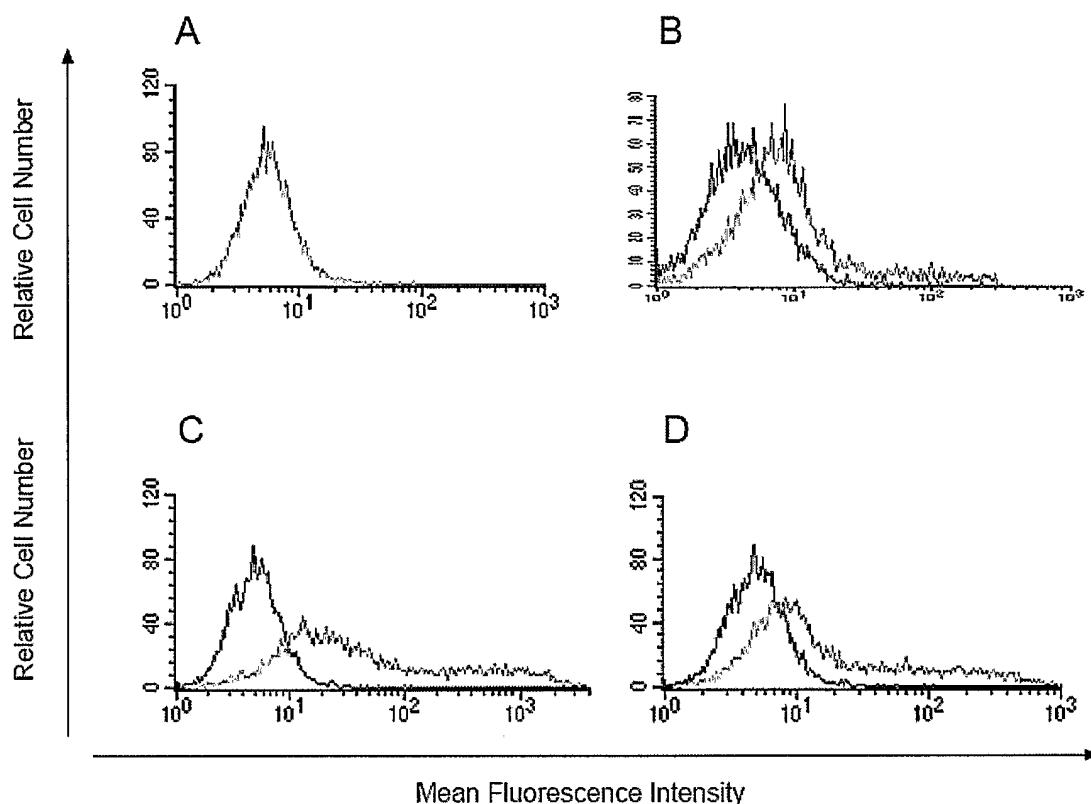
Sequence of scFv A5

Fig. 14

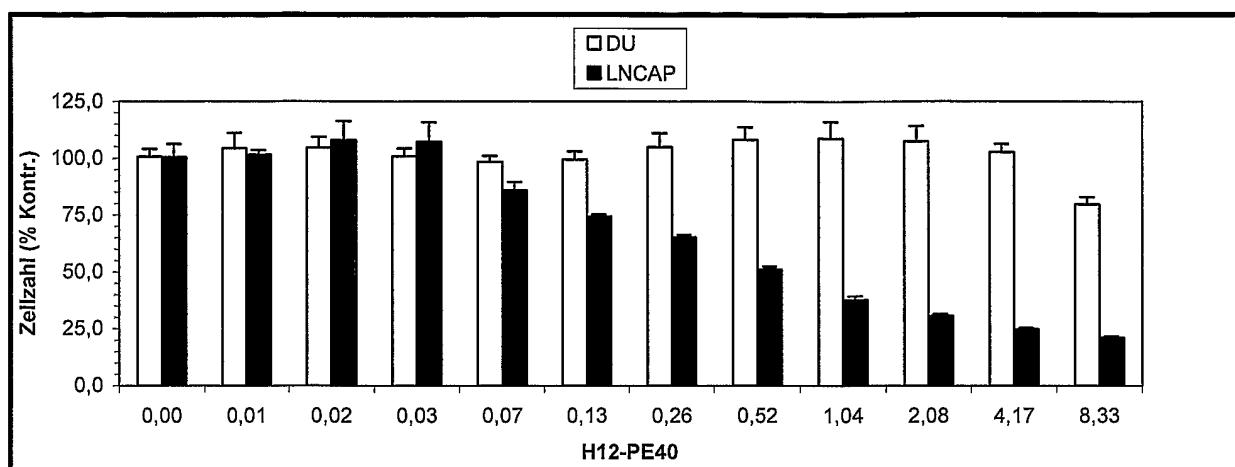


**Fig. 15**

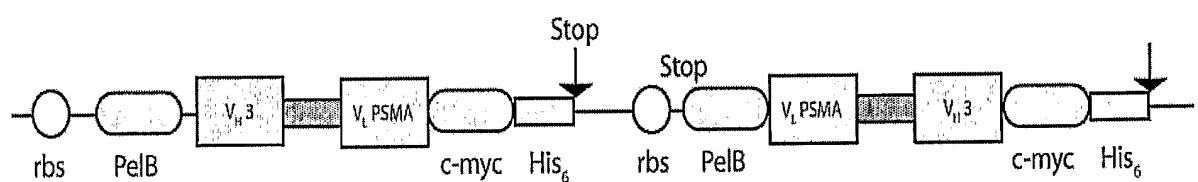
Binding of the scFv A5, H12 and D7 to PSMA-negative DU 145 cells (A) and PSMA-positive LNCaP cells (A5=B, H12=C, D7=D). Cells were stained with the mAbs and a PE-conjugated anti-mouse IgG mAb. Histograms represent logarithms of PE fluorescence on flow cytometer. Negative control with secondary antibody only.

**Fig 16**

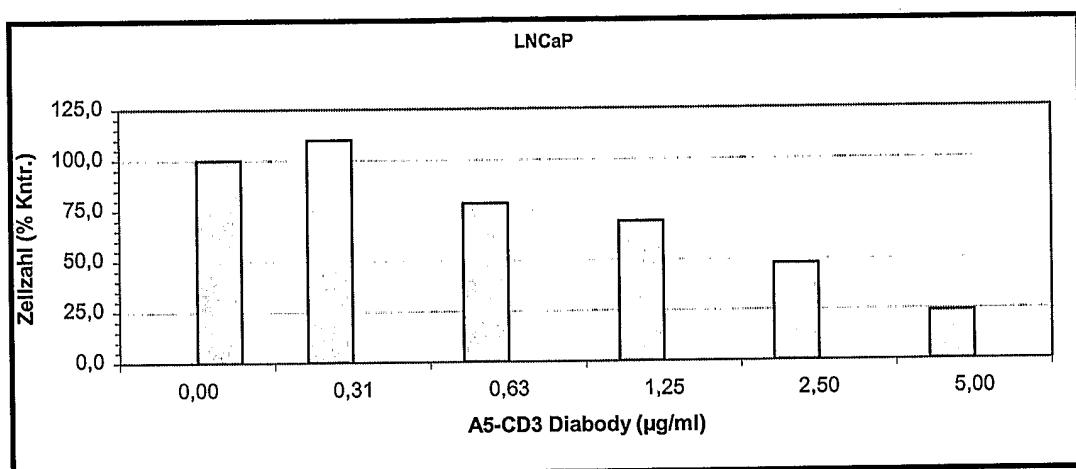
Binding of the scFv A5, H12 and D7 to PSMA-negative BOSC cells (A) and PSMA-transfected BOSC cells (A5=B, H12=C, D7=D). Cells were stained with the scFv, anti-c-myc mAb and PE-conjugated anti-mouse Ig. Histograms represent logarithms of PE fluorescence on flow cytometer. Negative control with secondary antibody only.

**Fig. 17**

Cytotoxic effect of recombinant immunotoxin H12-PE40 on LNCaP (black) and DU cells (white)

**Fig. 18**

Construction scheme of the A5-CD3 diabody

**Fig. 19**

Cytotoxic effect of diabody A5/CD3 at different concentrations and peripheral blood lymphocytes (effector target ratio 10:1) on LNCaP cells after 48 h incubation

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+1 M A R F S S S S S L D L N W Y S L G L Q X  
 1 ATG GCG AGG TTC AGC TCC AGC AGT CTG GAT CTG AAC TGG TAT AGC CTG GGG CTT CAG NTG  
 TAC CGC TCC AAG TCG AGG TCG TCA GAC CTA GAC TTG ACC ATA TCG GAC CCC GAA GTC NAC

## CDR-H1

+1 K L S C K A S G Y T F T Y F D I N W L R  
 61 AAA TTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA [TAC TTT GAC ATA AAC TGG TTG AGA  
 TTT AAC AGG ACG TTC CGA AGA CCG ATG TGG AAG TGT ATG AAA CTG TAT TTG ACC AAC TCT

## CDR-H2

+1 Q R P E Q G L E W I G V I S P G D G N T  
 121 CAG AGG CCT GAA CAG GGA CTT GAG TGG ATT GGA [GTG ATT TCT CCT GGA GAT GGC AAT ACA  
 GTC TCC GGA CTT GTC CCT GAA CTC ACC TAA CCT [CAC TAA AGA GGA CCT CTA CCG TTA TGT

+1 N Y N E N F K G K A T L T I D K S S T T  
 181 AAC TAC AAT GAG AAC TTC AAG GGC AAG GCC ACA CTG ACT ATA GAT AAA TCC TCC ACC ACA  
 TTG ATG TTA CTC TTG AAG TTC CCG TTC CGG TGT GAC TGA TAT CTA TTT AGG AGG TGG TGT

+1 A Y I Q L S R L T S E D S A V Y F C A R  
 241 GCC TAC ATT CAG CTT AGC AGG CTG ACA TCT GAG GAC TCT GCT GTC TAT TTC TGT GCA AGA  
 CGG ATG TAA GTC GAA TCG TCC GAC TGT AGA CTC CTG AGA CGA CAG ATA AAG ACA CGT TCT

CDR-H3

+1 D G N F P Y Y A M D S W G Q G T S V T V  
 301 GAT GGC AAC TTC CCT TAC TAT GCT ATG GAC TCA TGG GGT CAA GGA ACC TCA GTC ACC GTC  
 CTA CCG TTG AAG GGA ATG ATA CGA TAC CTG ACT ACC CCA GTT CCT TGG AGT CAG TGG CAG

## YOL-epitope

+1 S S A K T T P K L E E G E F S E A R V D  
 361 TCC TCA GCC AAA ACG ACA CCC AAG CTT GAA GAA GGT GAA TTT TCA GAA GCA CGC GTA GAC  
 AGG AGT CGG TTT TGC TGT GGG TTC GAA CTT CTT CCA CTT AAA AGT CTT CGT GCG CAT CTG

+1 I V M T Q I P L S L P V I L G D Q A S I  
 421 ATT GTG ATG ACC CAG ATT CCA CTC TCC CTG CCT GTC ATT CTT GGA GAT CAA GCC TCC ATC  
 TAA CAC TAC TGG GTC TAA GGT GAG AGG GAC GGA CAG TAA GAA CCT CTA GTT CGG AGG TAG

## CDR-L1

+1 S C R S S Q S L V Y S N G N T Y L H W F  
 481 TCT TGC AGA TCT AGT CAG AGC CTT GTC TAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TTC  
 AGA ACG TCT AGA TCA GTC TCG GAA CAT ATG TCA TTA CCT TTG TGG ATA AAT GTA ACC AAG

## CDR-L2

+1 L Q K P G Q S P K L L I Y N V S N L F S  
 541 CTG CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC [AAT GTT TCC AAC CTA TTT TCT  
 GAC GTC TTC GGT CCG GTC AGA GGT TTC GAG GAC TAG ATG TTA CAA AGG TTG GAT AAA AGA

Fig. 20 a

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+1 G V P D R F S G S G S G T D F T L K I S  
601 GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACT GAT TTC ACA CTC AAG ATC AGC  
CCC CAG GGT CTG TCC AAG TCA CCG TCA CCT AGT CCC TGA CTA AAG TGT GAG TTC TAG TCG

CDR-L3

+1 R V E A E D L G I Y F C S Q S T H V P T  
661 AGA GTG GAG GCT GAG GAT CTG GGA ATT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCC ACG  
TCT CAC CTC CGA CTC CTA GAC CCT TAA ATA AAG AGC AGA GTT TCA TGT GTA CAA GGG TGC

+1 F G G G T K L E I K R A D A A A A G S  
721 TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCG GCC GCT GGA TCC  
AAG CCT CCC CCC TGG TTC GAC CTT TAT TTT GCC CGA CTA CGA CGC CGG CGA CCT AGG

Sequence of scFv H12

Fig. 20 b

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M A Q V Q L Q Q S G A E L V E P G A S V  
 1 ATG GCC CAG GTG CAG CTG CAG CAG TCT GGG GCT GAA CTG GTA GAG CCT GGG GCT TCA GTG  
 TAC CGG GTC CAC GTC GAC CCC CGA CTT GAC CAT CTC GGA CCC CGA AGT CAC

## CDR-H1

+1 K L S C K A S G Y T F T Y F D I N W L R  
 61 AAA CTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA TAC TTT GAC ATA AAC TGG TTG AGA  
 TTT GAC AGG ACG TTC CGA AGA CCG ATG TGG AAG TGT ATG AAA CTG TAT TTG ACC AAC TCT

## CDR-H2

+1 Q R P E Q G L E W I G G I S P G D G N T  
 121 CAG AGG CCT GAA CAG GGA CTT GAG TGG ATT GGA GGG ATT TCT CCT GGA GAT GGT AAT ACA  
 GTC TCC GGA CTT GTC CCT GAA CTC ACC TAA CCT CCC TAA AGA GGA CCT CTA CCA TTA TGT

+1 N Y N E N F K G K A T L T I D K S S T T  
 181 AAC TAC AAT GAG AAC TTC AAG GGC AAG GCC ACA CTG ACT ATA GAC AAA TCC TCC ACC ACA  
 TTG ATG TTA CTC TTG AAG TTC CCG TTC CGG TGT GAC TGA TAT CTG TTT AGG AGG TGG TGT

+1 A Y I Q L S R L T S E D S A V Y F C A R  
 241 GCC TAC ATT CAG CTC AGC AGG CTG ACA TCT GAG GAC TCT GCT GTC TAT TTC TGT GCA AGA  
 CGG ATG TAA GTC GAG TCG TCC GAC TGT AGA CTC CTG AGA CGA CAG ATA AAG ACA CGT TCT

## CDR-H3

+1 D G N F P Y Y A M D S W G Q G T S V T V  
 301 GAT GGC AAC TTC CCT TAC TAT GCT ATG GAC TCA TGG GGT CAA GGA ACC TCA GTC ACC GTC  
 CTA CCG TTG AAG GGA ATG ATA CGA TAC CTG AGT ACC CCA GTT CCT TGG AGT CAG TGG CAG

## YOL-epitope

+1 S S A K T T P K L E E G E F S E A R V D  
 361 TCC TCA GCC AAA ACG ACA CCC AAG CTT GAA GAA GGT GAA TTT TCA GAA GCA CGC GTA GAC  
 AGG AGT CGG TTT TGC TGT GGG TTC GAA CTT CCA CTT AAA AGT CTT CGT GCG CAT CTG

+1 I E L T Q S P L S L P V I L G D Q A S I  
 421 ATT GAG CTC ACC CAA TCT CCA CTC TCC CTG CCT GTC ATT CTT GGA GAT CAA GCC TCC ATC  
 TAA CTC GAG TGG GTT AGA GGT GAG AGG GAC GGA CAG TAA GAA CCT CTA GTT CGG AGG TAG

## CDR-L1

+1 S C R S S Q S L V H S N G N T Y L H W F  
 481 TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TTT  
 AGA ACG TCT AGA TCA GTC TCG GAA CAT GTG TCA TTA CCT TTG TGG ATA AAT GTA ACC AAA

## CDR-L2

+1 L Q K P G Q S P K L L I Y T V S N R F S  
 541 CTG CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC ACA GTT TCC AAC CGA TTT TCT  
 GAC GTC TTC GGT CCG GTC AGA GGT TTC GAG GAC TAG ATG TGT CAA AGG TTG GCT AAA AGA

Fig. 21 a

25/25

+1 G V P D R F S G S G S G T D F T L K I S  
601 GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC  
CCC CAG GGT CTG TCC AAG TCA CCG TCA CCT AGT CCC TGT CTA AAG TGT GAG TTC TAG TCG

CDR-L3

~~~~~  
+1 R V E A E D L G V Y F C S Q S T H V P T
661 AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACC CAT GTT CCC ACG
TCT CAC CTC CGA CTC CTA GAC CCT CAA ATA AAG ACG AGA GTT TCA TGG GTA CAA GGC TGC

+1 F G G G T K L E I K R A D A A A A G S
721 TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCG GCC GCT GGA TCC
AAG CCT CCC CCC TGG TTC GAC CTT TAT TTT GCC CGA CTA CGA CGC CGG CGA CCT AGG

Sequence of scFv D7

Fig. 21 b

SEQUENCE LISTING

<110> Universitaetsklinikum Freiburg
<120> Monoclonal Antibodies and Single Chain Antibody Fragments against
Cell-Surface Prostate Specific Membrane Antigen as Diagnostic
and Therapeutic Tools for Prostate Cancer

<130> ZEE20050222c

<150> EP 05011536.9
<151> 2005-05-27

<160> 24

<170> PatentIn version 3.2

<210> 1
<211> 251
<212> PRT
<213> Artificial

<220>
<223> scFv E8

<220>
<221> MISC\_FEATURE
<222> (103)..(103)
<223> Xaa means Tyr or Ser

<400> 1

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1 5 10 15

Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asp Tyr Asn Met Asp Trp Val Lys Glu Arg His Gly Lys Ser Leu Glu
35 40 45

Trp Ile Gly Asp Ile Asn Pro Lys Asn Gly Val Thr Ile Tyr Asn Gln
50 55 60

Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Thr Thr
65 70 75 80

Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr
85 90 95

Tyr Cys Ala Arg Gly Asp Xaa Tyr Gly Asn Tyr Phe Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Ser Leu Thr Val Ser Ser Ala Lys Thr Thr Pro Lys Leu
115 120 125

Glu Glu Gly Glu Phe Ser Glu Ala Arg Val Asp Ile Gln Met Thr Gln
130 135 140

Ser Pro Ala Ser Leu Ser Val Ser Val Gly Glu Thr Val Thr Ile Thr
145 150 155 160

Cys Arg Thr Ser Glu Asn Ile Tyr Ser Asn Leu Ala Trp Tyr Gln Gln
165 170 175

Lys Gln Gly Lys Ser Pro Gln Leu Leu Val Tyr Thr Ala Thr Asn Leu
180 185 190

Ala Asp Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln
195 200 205

Tyr Ser Leu Lys Ile Asn Ser Leu Gln Ser Asp Asp Ser Gly Thr Tyr
210 215 220

Tyr Cys Gln His Phe Trp Gly Thr Pro Tyr Thr Phe Gly Gly Gly Thr
225 230 235 240

Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala
245 250

<210> 2

<211> 10

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 2

Gly Tyr Thr Phe Thr Asp Tyr Asn Met Asp
1 5 10

<210> 3

<211> 11

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 3

Gly Asp Ile Asn Pro Lys Asn Gly Val Thr Ile
1 5 10

<210> 4

<211> 11

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<220>

<221> MISC\_FEATURE

<222> (4)..(4)

<223> Xaa means Tyr or Ser

<400> 4

| | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Gly | Asp | Xaa | Tyr | Gly | Asn | Tyr | Phe | Asp | Tyr |
| 1 | | | | 5 | | | | | 10 | |

<210> 5

<211> 11

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 5

| | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Thr | Ser | Glu | Asn | Ile | Tyr | Ser | Asn | Leu | Ala |
| 1 | | | | 5 | | | | | 10 | |

<210> 6

<211> 7

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 6

| | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|
| Thr | Ala | Thr | Asn | Leu | Ala | Asp |
| 1 | | | 5 | | | |

<210> 7

<211> 9

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 7

| | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln | His | Phe | Trp | Gly | Thr | Pro | Tyr | Thr |
| 1 | | | | 5 | | | | |

<210> 8

<211> 753

<212> DNA

<213> Artificial

<220>

<223> scFv E8

<400> 8

atggccgagg tgcagctgca gcagtcagga cccgacctgg tgaagcctgg ggcctcaatg 60

aagatttcct gcaaggcttc tggatacaca ttcactgact acaacatgga ctgggtgaag 120

gagagacatg gaaagagcct tgagtggatt ggagatatta atcctaaaaa tggcgttact 180

atttacaacc agaagttcaa gggcaaggcc acattgactg tagacaagtc ctccaccaca 240

gcctacatgg agctccgcag cctgacatct gaagacactg cagtctatta ttgtgcaaga 300

ggggactmct atggtaacta ctttgactac tggggccaag gcaccagtct cacagtctcc 360

| | | | | | | |
|------------|-------------|------------|------------|------------|-------------|-----|
| tcagccaaaa | cgacmcccaa | gcttgaagaa | ggtgaatttt | cagaagcacg | cgttagacatt | 420 |
| cagatgacac | agtctccagc | ctccctatct | gtatctgtgg | gagaaactgt | caccatcaca | 480 |
| tgtcgaacaa | gtgagaatat | ttacagtaat | ttagcatggt | atcagcagaa | acagggaaaa | 540 |
| tctcctcagc | tcctggtcta | tactgcaaca | aacttagcag | atggtgtgcc | ctcaagggttc | 600 |
| agtggcagtg | gatcaggcac | acagtattcc | ctcaagatca | acagcctgca | gtctgatgat | 660 |
| tctgggactt | attactgtca | acattttgg | ggtactccgt | acacgttcgg | aggggggacc | 720 |
| aagctggaaa | taaaaacgggc | tgatgctgcg | gcc | | | 753 |

<210> 9

<211> 753

<212> DNA

<213> Artificial

<220>

<223> reverse and complement of SEQ ID NO:8

<400> 9

| | | | | | | |
|------------|------------|-------------|------------|------------|-------------|-----|
| ggccgcagca | tcagcccgtt | ttatccag | cttggtcccc | cctccgaacg | tgtacggagt | 60 |
| accccaaaaa | tgttgacagt | aataagtccc | agaatcatca | gactgcaggc | tgttgatctt | 120 |
| gagggaatac | tgtgtgcctg | atccactgcc | actgaacctt | gagggcacac | catctgctaa | 180 |
| gtttgttgca | gtatagacca | ggagctgagg | agattttccc | tgtttctgct | gataccatgc | 240 |
| taaattactg | taaatattct | cacttggtcg | acatgtgatg | gtgacagttt | ctcccacaga | 300 |
| tacagatagg | gaggctggag | actgtgtcat | ctgaatgtct | acgcgtgctt | ctgaaaattc | 360 |
| accttcttca | agcttggkg | tcgtttggc | tgaggagact | gtgagactgg | tgccttggcc | 420 |
| ccagtagtca | aagtagttac | catagkagtc | ccctcttgca | caataataga | ctgcagtgtc | 480 |
| ttcagatgtc | aggctgcgga | gctccatgt | ggctgtggtg | gaggacttgt | ctacagtcaa | 540 |
| tgtggccttg | cccttgaact | tctggttgt | aatagtaacg | ccatttttag | gattaatatc | 600 |
| tccaatccac | tcaaggctct | ttccatgtct | ctcccttacc | cagtccatgt | tgttagtcagt | 660 |
| aatgtgtat | ccagaagcct | tgcagggaaat | cttcatttag | gccccaggct | tcaccaggtc | 720 |
| gggtcctgac | tgctgcagct | gcacctcggc | cat | | | 753 |

<210> 10

<211> 253

<212> PRT

<213> Artificial

<220>

<223> scFv A5

<400> 10

| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ala | Asp | Val | Lys | Leu | Val | Glu | Ser | Gly | Gly | Gly | Leu | Val | Lys | Pro |
| 1 | | | | 5 | | | | 10 | | | | | 15 | | |

| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Glu | Ser | Leu | Lys | Leu | Ser | Cys | Ile | Ala | Ser | Gly | Phe | Thr | Phe | Ser |
| 20 | | | | | | 25 | | | | | 30 | | | | |

Asp Tyr Tyr Met Tyr Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu
35 40 45

Trp Val Ala Ile Ile Ser Asp Gly Gly Tyr Tyr Thr Tyr Ser Asp
50 55 60

Ile Ile Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Asn
65 70 75 80

Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met Tyr
85 90 95

Tyr Cys Thr Arg Gly Phe Pro Leu Leu Arg His Gly Ala Met Asp Tyr
100 105 110

Trp Gly Leu Gly Thr Ser Val Thr Val Ser Ser Thr Lys Thr Thr Pro
115 120 125

Lys Leu Glu Glu Gly Glu Phe Ser Glu Ala Arg Val Asp Ile Gln Met
130 135 140

Thr Gln Ser Pro Lys Phe Met Ser Thr Ser Val Gly Asp Arg Val Ser
145 150 155 160

Val Thr Cys Lys Ala Ser Gln Asn Val Asp Thr Asn Val Ala Trp Tyr
165 170 175

Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile Tyr Ser Ala Ser
180 185 190

Tyr Arg Tyr Ser Asp Val Pro Asp Arg Phe Thr Gly Ser Glu Ser Gly
195 200 205

Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser Glu Asp Leu Ala
210 215 220

Glu Tyr Phe Cys Gln Gln Tyr Asp Ser Tyr Pro Tyr Thr Phe Gly Gly
225 230 235 240

Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala
245 250

<210> 11

<211> 9

<212> PRT

<213> Artificial

<220>

<223> CDR sequence

<400> 11

Gly Phe Thr Phe Ser Asp Tyr Tyr Met
1 5

<210> 12
<211> 7
<212> PRT
<213> Artificial

<220>
<223> CDR sequence

<400> 12

Ile Ile Ser Asp Gly Gly Tyr
1 5

<210> 13
<211> 12
<212> PRT
<213> Artificial

<220>
<223> CDR sequence

<400> 13

Gly Phe Pro Leu Leu Arg His Gly Ala Met Asp Tyr
1 5 10

<210> 14
<211> 11
<212> PRT
<213> Artificial

<220>
<223> CDR sequence

<400> 14

Lys Ala Ser Gln Asn Val Asp Thr Asn Val Ala
1 5 10

<210> 15
<211> 7
<212> PRT
<213> Artificial

<220>
<223> CDR sequence

<400> 15

Ser Ala Ser Tyr Arg Tyr Ser
1 5

<210> 16
<211> 9
<212> PRT
<213> Artificial

<220>
<223> CDR sequence

<400> 16

Gln Gln Tyr Asp Ser Tyr Pro Tyr Thr

1 5

<210> 17
 <211> 759
 <212> DNA
 <213> Artificial

 <220>
 <223> scFv A5

 <400> 17
 atggccgacg tgaagtttgtt ggagtctggg ggaggccttag tgaaggctgg agagtccctg 60
 aaactctcct gtatacgccctc tggattcaact ttcagtgact attatatgtt ttgggttcgc 120
 cagactccgg aaaagaggct ggagtgggtc gcaatcatta gtgatggtgg ttattataacc 180
 tactattcag acattatcaa ggggcgattc accatctcca gagacaatgc caagaacaac 240
 ctgtacctcc aaatgagcag tctgaagtct gaggacacag ccatgttatta ctgtacaaga 300
 ggttttcctc tactacggca cggggctatg gactactggg gtcttggAAC ctcagtcacc 360
 gtctcctcaa ccaaaacgac acccaagctt gaagaaggtt aattttcaga agcacgcgtt 420
 gacattcaga tgacccagtc tccaaaattt atgtccacat cggtaggaga cagggtcagc 480
 gtcacctgca aggccagtca gaatgtggat actaatgttag cctggtatca acagaaaccca 540
 ggacaatctc ctaaaggactt gatttactcg gcatcctacc ggtacagtga cgtccctgtt 600
 cgcttcacag gcagtgaatc tgggacagat ttcaactctca ccatcagcaa tgtcagtct 660
 gaagacttgg cagagtattt ctgtcagcaa tatgacagct atccatacac gttcggaggg 720
 gggaccaagc tggaaataaa acgggctgtat gctgcggcc 759

<210> 18
 <211> 759
 <212> DNA
 <213> Artificial

<220>
 <223> reverse and complement of SEQ ID NO:17

<400> 18
 ggccgcagca tcagcccggtt ttatttccag cttggcccc cctccgaacg tgtatggata 60
 gctgtcatat tgctgacaga aatactctgc caagtcttca gactgcacat tgctgtatgg 120
 gagagtgaaa tctgtcccgat attcaactgccc tggtaagcga tcagggacgt cactgtaccg 180
 gtaggatgcc gagtaaatca gtgcttttagg agattgtcct ggtttctgtt gataccaggc 240
 tacatttagta tccacattct gactggccctt gcaggtgacg ctgaccctgt ctcctaccga 300
 tgtggacatg aattttggag actgggtcat ctgaatgtct acgcgtgctt ctgaaaattt 360
 accttcttca agcttgggtg tcgttttgt tgaggagacg gtgactgagg ttccaagacc 420
 ccagtagtcc atagccccgt gccgttag agaaaaacct cttgtacagt aatacatggc 480
 tgtgtcctca gacttcagac tgctcatttgg gaggtacagg ttgttcttgg cattgtctct 540
 ggagatggtg aatcgccccct tgataatgtc tgaatagtag gtataataac caccatca 600
 aatgattgca acccaactcca gcctcttttc cggagtctgg cgaacccaaat acatataata 660

gtcactgaaa gtgaatccag aggctataca ggagagttc agggactctc caggcttcac 720
 taagcctccc ccagactcca ccaacttcac gtcggccat 759

<210> 19
 <211> 777
 <212> DNA
 <213> artificial
 <220>
 <223> scFv H12

<220>
 <221> misc\_feature
 <222> (58)..(58)
 <223> n is a, c, g, or t
 <400> 19
 atggcgaggt tcagctccag cagtctggat ctgaactggc atagcctggg gcttcagntg 60
 aaattgtcct gcaaggcttc tggctacacc ttcacatact ttgacataaa ctggttgaga 120
 cagaggcctg aacagggact tgagtggatt ggagtgattt ctcctggaga tggcaataca 180
 aactacaatg agaacttcaa gggcaaggcc acactgacta tagataaatac ctccaccaca 240
 gcctacattc agcttagcag gctgacatct gaggactctg ctgtctattt ctgtgcaaga 300
 gatggcaact tcccttacta tgctatggac tcatgggtc aaggaacctc agtcaccgtc 360
 tcctcagcca aaacgacacc caagcttcaa gaaggtgaat tttcagaagc acgcgtagac 420
 attgtatga cccagattcc actctccctg cctgtcattc ttggagatca agcctccatc 480
 tcttgcagat ctagtcagag ccttgtatac agtaatggaa acacctattt acattggttc 540
 ctgcagaagc caggccagtc tccaaagctc ctgatctaca atgtttccaa cctatatttct 600
 ggggtcccaag acaggttcag tggcagtggaa tcagggactg atttcacact caagatcagc 660
 agagtggagg ctgaggatct gggaaattat ttctgctctc aaagtacaca tggcccacg 720
 ttcggagggg ggaccaagct ggaaataaaa cgggctgatg ctgcggccgc tggatcc 777

<210> 20
 <211> 259
 <212> PRT
 <213> artificial
 <220>
 <223> scFv H12

<220>
 <221> MISC\_FEATURE
 <222> (20)..(20)
 <223> The 'xaa' at location 20 stands for Met, Val, or Leu.

<400> 20

Met Ala Arg Phe Ser Ser Ser Ser Leu Asp Leu Asn Trp Tyr Ser Leu
 1 5 10 15

Gly Leu Gln Xaa Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr

20

25

30

Tyr Phe Asp Ile Asn Trp Leu Arg Gln Arg Pro Glu Gln Gly Leu Glu
35 40 45

Trp Ile Gly Val Ile Ser Pro Gly Asp Gly Asn Thr Asn Tyr Asn Glu
50 55 60

Asn Phe Lys Gly Lys Ala Thr Leu Thr Ile Asp Lys Ser Ser Thr Thr
65 70 75 80

Ala Tyr Ile Gln Leu Ser Arg Leu Thr Ser Glu Asp Ser Ala Val Tyr
85 90 95

Phe Cys Ala Arg Asp Gly Asn Phe Pro Tyr Tyr Ala Met Asp Ser Trp
100 105 110

Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Pro Lys
115 120 125

Leu Glu Glu Gly Glu Phe Ser Glu Ala Arg Val Asp Ile Val Met Thr
130 135 140

Gln Ile Pro Leu Ser Leu Pro Val Ile Leu Gly Asp Gln Ala Ser Ile
145 150 155 160

Ser Cys Arg Ser Ser Gln Ser Leu Val Tyr Ser Asn Gly Asn Thr Tyr
165 170 175

Leu His Trp Phe Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
180 185 190

Tyr Asn Val Ser Asn Leu Phe Ser Gly Val Pro Asp Arg Phe Ser Gly
195 200 205

Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala
210 215 220

Glu Asp Leu Gly Ile Tyr Phe Cys Ser Gln Ser Thr His Val Pro Thr
225 230 235 240

Phe Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala
245 250 255

Ala Gly Ser

<210> 21

<211> 777

<212> DNA

<213> artificial

<220>

<223> scFv D7

| | | |
|--|---|----|
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| aaactgtcct gcaaggcttc tggctacacc ttcacatact ttgacataaaa ctggttgaga | 120 | |
| cagaggcctg aacagggact tgagtggatt ggagggattt ctcctggaga tggtaataca | 180 | |
| aactacaatg agaacttcaa gggcaaggcc acactgacta tagacaaatc ctccaccaca | 240 | |
| gcctacattc agctcagcag gctgacatct gaggactctg ctgtctattt ctgtgcaaga | 300 | |
| gatggcaact tcccttacta tgctatggac tcatgggtc aaggaacctc agtcaccgtc | 360 | |
| tcctcagcca aaacgacacc caagcttcaa gaaggtaat tttcagaagc acgcgttagac | 420 | |
| attgagctca cccaatctcc actctccctg cctgtcattc ttggagatca agcctccatc | 480 | |
| tcttcagat ctagtcagag cttgtacac agtaatggaa acacctattt acattggttt | 540 | |
| ctgcagaagc caggccagtc tccaaagctc ctgatctaca cagttccaa ccgattttct | 600 | |
| ggggtcccag acaggttcag tggcagtggc tcagggacag atttcacact caagatcagc | 660 | |
| agagtggagg ctgaggatct gggagtttat ttctgctctc aaagtaccca tggatcccacg | 720 | |
| ttcggagggg ggaccaagct ggaaataaaaa cgggctgatg ctgcggccgc tggatcc | 777 | |

<210> 22

<211> 259

<212> PRT

<213> artificial

<220>

<223> scFv D7

<400> 22

| | | | |
|---|---|----|----|
| Met Ala Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Glu Pro | | | |
| 1 | 5 | 10 | 15 |

| | | |
|---|----|----|
| Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr | | |
| 20 | 25 | 30 |

| | | |
|---|----|----|
| Tyr Phe Asp Ile Asn Trp Leu Arg Gln Arg Pro Glu Gln Gly Leu Glu | | |
| 35 | 40 | 45 |

| | | |
|---|----|----|
| Trp Ile Gly Gly Ile Ser Pro Gly Asp Gly Asn Thr Asn Tyr Asn Glu | | |
| 50 | 55 | 60 |

| | | | |
|---|----|----|----|
| Asn Phe Lys Gly Lys Ala Thr Leu Thr Ile Asp Lys Ser Ser Thr Thr | | | |
| 65 | 70 | 75 | 80 |

| | | |
|---|----|----|
| Ala Tyr Ile Gln Leu Ser Arg Leu Thr Ser Glu Asp Ser Ala Val Tyr | | |
| 85 | 90 | 95 |

| | | |
|---|-----|-----|
| Phe Cys Ala Arg Asp Gly Asn Phe Pro Tyr Tyr Ala Met Asp Ser Trp | | |
| 100 | 105 | 110 |

| | |
|---|--|
| Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Pro Lys | |
|---|--|

115

120

125

Leu Glu Glu Gly Glu Phe Ser Glu Ala Arg Val Asp Ile Glu Leu Thr
 130 135 140

Gln Ser Pro Leu Ser Leu Pro Val Ile Leu Gly Asp Gln Ala Ser Ile
 145 150 155 160

Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr
 165 170 175

Leu His Trp Phe Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
 180 185 190

Tyr Thr Val Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly
 195 200 205

Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala
 210 215 220

Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro Thr
 225 230 235 240

Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala
 245 250 255

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/001917

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/13 A61K47/48 C07K16/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

16 May 2006

Date of mailing of the international search report

01/06/2006

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Authorized officer

Renggli, J

INTERNATIONAL SEARCH REPORT

 International application No
 PCT/EP2006/001917

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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PCT/EP2006/001917 |
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/001917

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 18 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the Invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2006/001917

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