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PROSTATE STEM CELL ANTIGEN (PSCA): A PUTATIVE TARGET FOR IMMUNOTHERAPY AND DIAGNOSIS IN PROSTATE, PANCREATIC AND BLADDER CARCINOMA

S.S.D. MED04

Coordinatore: Prof. Cristiano Chiamulera

Tutor: Dott. Giulio Fracasso

Dottoranda: Dott.ssa Giorgia Cremonese

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Riassunto

L'immunoterapia basata sull'utilizzo di anticorpi non coniugati, coniugati a tossine o radiomarcati, che riconoscono antigeni associati a tumore, è promettente per la cura di tumori solidi o ematici. Un possibile target per l'immunoterapia potrebbe essere il prostate stem cell antigen (PSCA), un antigene appartenente alla famiglia delle "GPI-anchored protein". Il PSCA è un antigene di superficie espresso a bassi livelli nel tessuto prostatico sano ed over espresso nel tumore prostatico, pancreatico e della vescica. L'espressione di PSCA è inoltre correlata positivamente a "Gleason score" e allo stadio della patologia nel tumore prostatico.

Il presente lavoro di tesi descrive la generazione e caratterizzazione di un anticorpo monoclonale murino anti PSCA (mAb), ottenuta tramite la tecnologia dell'ibridoma, e del suo frammento anticorpale a singola catena (scFv), generato clonando la regione variabile della catena leggera (V_L) e della catena pesante (V_H) nel vettore di espressione pHEN-2. Tramite citofluorimetria è stato dimostrato che l'anticorpo monoclonale possiede una buona affinità e specificità di legame all'antigene. Il potenziale diagnostico dell'anticorpo è stato dimostrato tramite Western Blot su lisati di tessuti neoplastici di prostrata e pancreas, in cui l'anticorpo è in grado di legare l'antigene denaturato e glicosilato, e tramite ELISA, in cui l'anticorpo si lega all'antigene espresso da cellule precedentemente fissate. Il potenziale terapeutico dell'anticorpo è stato valutato tramite saggio di proliferazione: l'anticorpo da solo non è in grado di indurre morte cellulare tramite un meccanismo diretto, mentre in seguito a coniugazione chimica con la catena A della ricina (RTA) rivela effetto citotossico su cellule PC-3 hPSCA con IC_{50} (concentrazione in grado di inibire la massima proliferazione cellulare del 50%) pari a $1.3x10^{-9}$, valore 100 volte più piccolo di quello ottenuto con la sola tossina RTA.

Il frammento scFv è stato prodotto nel ceppo batterico *E. Coli*. Mediante analisi citofluorimetrica su cellule PSCA positive e saggio immunoenzimatico sull'antigene ricombinante è stato verificato che il frammento anticorpale mantiene le stesse caratteristiche di specificità di legame all'antigene dell'anticorpo monoclonale parentale, ma possiede affinità minore. Quando l' scFv viene reso bivalente, tramite il

cross-linking dei monomeri utilizzando un anticorpo anti-myc, l'affinità raggiunge quasi quella dell'anticorpo parentale.

Successivamente l'scFv è stato unito attraverso fusione genetica al dominio enzimatico della tossina batterica *Pseudomonas aeruginosa* exotoxin A (PE40). L'immunotossina risultante è espressa nel ceppo batterico *E. Coli* e si accumula nei corpi d'inclusione. L'analisi citofluorimetrica su cellule PSCA positive fatta utilizzando i corpi d'inclusione rinaturati e contenenti l'immunotossina di fusione ha confermato che l'interazione tra l'scFv e l'antigene viene conservata in seguito alla fusione con la tossina PE40.

L'effetto citotossico dell'immunotossina purificata scFv-PE40 verrà valutata prima *in vitro* su linee cellulari PSCA positive e negative e poi in modelli *in vivo* che permetteranno di valutare anche eventuali effetti collaterali.

Abstract

Antibody-based therapy using unconjugated, toxin-conjugated or radiolabeled immunoglobulins recognizing tumor-associated antigens has proven beneficial for solid and hematolymphoid neoplasms. A suitable target could be prostate stem cell antigen (PSCA), a member of the "GPI-anchored protein". PSCA is a cell surface-antigen expressed at low levels in normal prostate tissue and over expressed in prostate, pancreatic and bladder carcinomas. Moreover PSCA expression is positively correlated with Gleason score and with pathologic stage in prostate cancer.

The present thesis describes the generation and characterization of the murine anti PSCA monoclonal antibody (mAb), obtained by hybridoma technology, and its fragment single chain (scFv), generated by cloning the variable heavy (V_H) and light (V_L) chain sequences in the expression vector pHEN-2. The mAb showed the ability to recognize with good affinity and specificity the native PSCA by flow cytometry. The diagnostic potential of the mAb was demonstrated by Western Blot performed with prostate and pancreatic neoplastic tissue lysates, showing the binding to denaturated and glycosylated PSCA, and by ELISA performed with fixed cells. The mAb was also assessed for its possible use in the therapeutic approach: the cell-proliferation assay demonstrated that the antibody alone is not able to induce cell death through a direct

mechanism, while when it is conjugated to the ricin A chain toxin (RTA) by chemical linkage it can poison PC-3 hPSCA cells with an IC_{50} (i.e. concentration inhibiting 50% of the maximal cell proliferation) of $1.3x10^{-9}$ M, value 100 fold lower than the IC_{50} of the RTA toxin alone.

The scFv was produced in *E. Coli* bacteria; flow cytometric analysis on PSCA-positive cells and immunoenzymatic assay on the recombinant antigen proved that the antibody fragment maintains the binding specificity of the parental monoclonal antibody. The affinity of the scFv is lower than the affinity of mAb but it is partially recovered making the scFv divalent by cross-linking the scFv monomers via an antibody-mediated myc-Tag interaction.

To create a fusion immunotoxin (IT) the scFv was later genetically fused to the enzymatic domain of *Pseudomonas aeruginosa* exotoxin A (PE40). The resulting IT was expressed in *E. Coli* bacteria and it is accumulated in the inclusion bodies. The flow cytometric analysis on PSCA-positive cells performed with the whole refolded inclusion bodies extract containing the fusion IT confirmed that the interaction of scFv with the PSCA is preserved after fusion to PE40.

The efficacy of purified scFv-PE40 will be analyse *in vitro* on positive and negative cell lines and subsequently *in vivo* models which also will be useful to study the side effects of this new drug.

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Abbreviations used

Ab/Abs	Antibody/antibodies
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
h	hour
H_2O	Water
IB	Inclusion Bodies
Ig	Immunoglobulin
IHC	Immunohistochemistry
IPTG	isopropyl-beta-D-thiogalactopyranoside
IT /ITs	Immunotoxin(s)
mAb	Monoclonal antibody
MFI	Medium Fluorescence Intensity
o.n.	overnight
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PE	*Pseudomonas aeruginosa* Exotoxin A
PE40	Truncated form of *Pseudomonas aeruginosa* Exotoxin A
PSCA	Prostate Stem Cell Antigen
PSMA	Prostate Specific Membrane Antigen
rpm	Revolutions per minute
RT	Room temperature
RTA	Ricin A Chain
scFv	Single-chain Fragment variable
SDS	Sodium dodecyl sulfate
SPDP	N-Succinimidyl-3-(2-Pyridylthio)Propionate
WT	Wild type
V_H	Heavy chain variable domain
V_L or V_κ	Light chain variable domain
^{3}HTdr	Triziate-Thymidine

INTRODUCTION

Conventional cancer therapies

The first description of a disease resembling cancer is found in an Egyptian papyri and dates back to approximately 1600 bc. Nevertheless, until the nineteenth century, when anaesthesia, improved techniques and histological control made surgery more efficient, cancer was more or less regarded as incurable. Radiation therapy became the next treatment available for cancer. In fact, the invention of the linear accelerator in the first half of the twentieth century advanced radiotherapy from a palliative method to a cancer treatment with a curative intent. However, both surgery and radiation were in most cases not sufficient to control metastastic cancer. Moreover, the increase in the incidence of cancer stressed the need to find other forms of therapy for this disease.

The introduction of nitrogen mustards in the 1940s can be considered the origin of antineoplastic chemotherapy targeting all tumour cells [Papac, 2001]. Agents that inhibit cancer growth (chemotherapy) were generally cell toxic with severe side effects. Further classes of cell toxic agents were discovered during the 1950-70s. Gradually, chemotherapy has been introduced in various tumor forms, as palliative treatment to relieve symptoms and increase the quality of life in late stages of the disease, or in conjunction with surgery and/or radiotherapy, in order to increase cure rates.

In general, there has been a trend towards using combinations of chemotherapy agents with different mechanisms of action to achieve maximal effect.

Nevertheless, these strategies usually lack specificity of action towards tumor cells, which accounts for the variety of possible side effects inherent in these types of therapies.

Radiotherapy, consisting in directing ionizing radiations on the tumor mass to damage the cancerous cells and in particular their nucleic acids, can lead to DNA mutations in the by-stander normal cells [Suit *et al.*, 2007]. The selectivity of the cytotoxic agents used in chemotherapy, for instance, is based on the higher liability of rapidly proliferating cells to the toxic effect of these drugs; the major drawback of this approach is the toxicity towards healthy tissues with a rapid cell turnover [Schrama *et al.*, 2006].

Some indolent malignancies, due to their slow progress, respond poorly to conventional treatments. Besides, tumor cells can develop a resistance to chemotherapy drugs,

hindering their mechanism of action or promoting their expulsion out of the cell before they can act [Perez-Tomas, 2006; O'Connor, 2007].

As a consequence, the need for an improved efficacy in cancer therapies has been increasingly felt in the last few years, placing the focus of research on the development of new drugs that combine power of action and selective targeting of cancerous cells. An ever clearer understanding of the biochemical events that are implicated in the onset and progression of many malignancies has allowed the designing of tumor-specific therapies that directly target the molecules involved in the development of tumors or that selectively deliver the drug to cancerous cells. The opportunity of specifically affecting a tumor is provided by the presence of molecular targets being selectively expressed on the surface of cancerous cells: these are the tumor marker antigens [Schrama *et al.*, 2006].

Rising of immunotherapy

The body has many defence mechanisms against intrusion/infection. One cornerstone in this defence system is antibodies, large molecules with the ability to bind to proteins (antigens). As early as the beginning of the 20th century, scientists suggested the potential for using the body's own defence systems treating cancer. In the 1968, Porter and Edelman managed to identify and structurally characterize a class of serum proteins that could bind a virtually unlimited variety of molecular targets with high specificity: the immunoglobulins. Few years later, in 1975, immunoglobulins became available as tools for research and clinical purposes thanks to the development of the hybridoma technology by Köhler e Milstein, which allowed the custom generation and large-scale production of monoclonal antibodies (mAbs) of murine origin [Köhler and Milstein, 1975; Schrama *et al*., 2006].

Since the early 80's more than 200 mAbs with anti-cancer potential have been evaluated in clinical trials by academic and private institutions. Although the first attempts failed to provide a clear evidence of beneficial effects on patients, antibody-based drugs (immunotherapeutics) have by now become important elements in the treatment of an increasing number of human malignancies. To date twelve mAbs for cancer treatment have been approved and marketed, with the first one, an antibody for the therapy of

colorectal cancer, approved in Germany in 1995 (eventually withdrawn from the market) [Reichert *et al.*, 2005; Reichert and Valge-Archer, 2007].

Antibody-based targeted therapy

The antibody-based targeted therapy of cancer comprises different strategies that can be divided in two main categories: direct and indirect.

Direct strategy

The direct approach implies the binding of unconjugated antibody to a specific cell-surface antigen. This can lead to: an altered signal transduction pathway, the triggering of a natural immune response (ADCC: Antibody Dependent Cellular Cytotoxicity, CDC: Complement Dependent Cytotoxicity) or the induction of apoptosis in the malignant cell (Fig. 1) [Adams and Weiner, 2005; Sharkey and Goldenberg, 2006].

- **Antibody dependent cellular cytotoxicity (ADCC)**

  It occurs when antibodies bind to antigens on a tumor cell and their Fc portions engage Fc receptors (FcR) on the surface of immune effector cells. This kind of mechanism favours recruitment of immune cells to the tumor site and allows eliciting of an immune-mediated tumor-specific cytotoxicity.

- **Complement dependent cytotoxicity (CDC)**

  Binding of an antibody on the target cell surface exposes its binding sites for protein that initiate the complement cascade, ultimately triggering the release of chemotactic factors and the formation of a membrane attack complex which promote target cell lysis.

- **Signal transduction interference**

  Some of the most common tumor-specific targets are growth factor receptors which are frequently overexpressed or deregulated on tumor cells. Their overexpression or abnormal activity promotes tumor cell growth and insensitivity to chemoterapeutic agents. For this reason they are good targets for immunotherpeutic approaches.

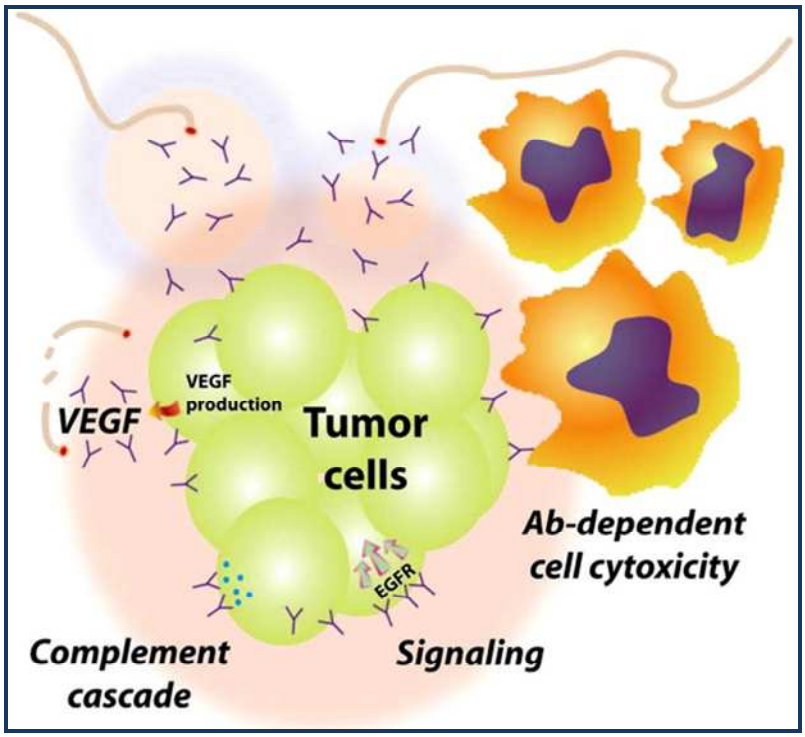

Figure 1. Mechanisms of action for unconjugated antibodies. Traditional mechanisms of action include antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Both require the Fc-portion of the IgG to activate effector cell binding or complement proteins that can lead to the death of the cells. However, antibodies that bind to growth factor receptors on the surface of the tumor cell (such as EGFR) can block critical functions and send signals that will lead to the cell's death. Alternatively, antibodies can bind to substances produced by tumors, such as VEGF, that are essential for stimulating new blood vessel formation, thereby inhibiting new tumor growth. (Modified from Sharkey and Goldenberg, 2008).

Unconjugated antibodies (i.e. immunoglobulins not coupled to any effector molecule) can be used in a direct approach, but they are not always able to induce the killing of target cells.This is because malignant cells can be very resistant to the induction of apoptosis and the immune system of the patient can be defective in triggering responses like ADCC or CDC.

Indirect strategy

The indirect strategy enhances the efficacy of antitumor antibodies by arming them by covalent linkage to effector molecules (Fig. 2) [Schrama *et al*., 2006].

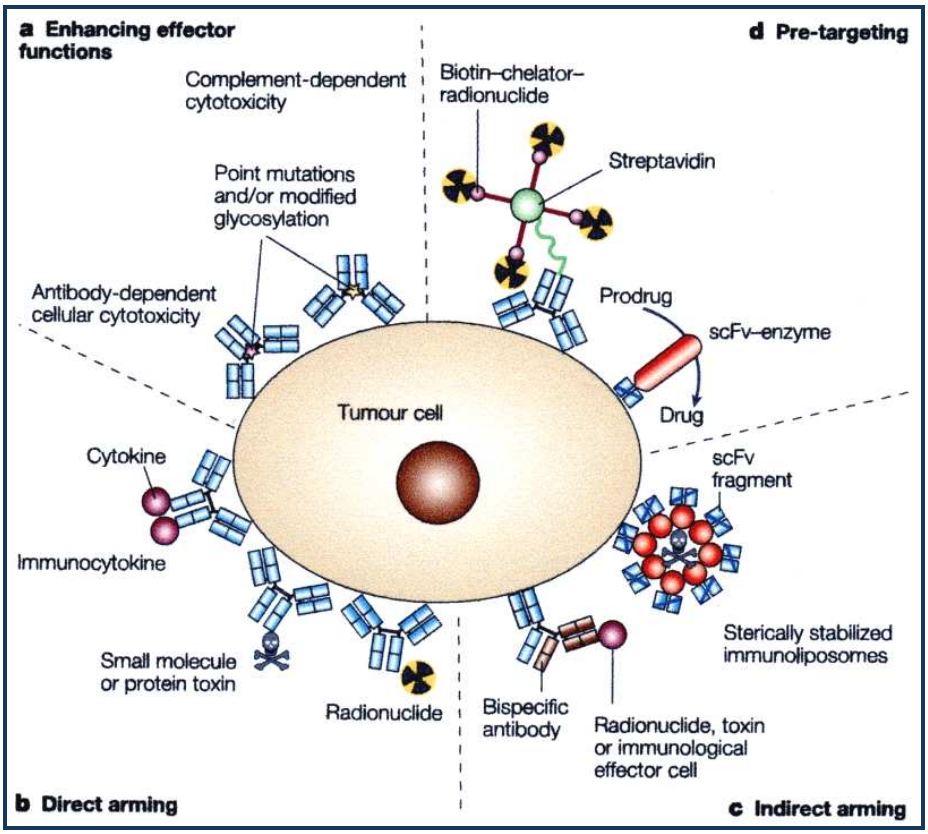

Figure 2. Strategies for enhancing the potency of antitumour antibodies. (**a**) Enhancing effector functions involve improving antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity by means of site-directed mutations or manipulation of antibody glycosylation. (**b**) Direct arming of antibodies entails their covalent linkage to killing machinery, such as radionuclides or toxins (for example, small molecules or proteins). Alternatively, arming antibodies with cytokines is intended to create high intratumour concentrations of cytokines to stimulate the antitumour immune response (T cells, B cells or natural killer cells), while avoiding the toxicities associated with systemic cytokine delivery. (**c**) Indirect arming of antibodies can be achieved by attaching engineered antibody fragments to the surface of liposomes loaded with drugs or toxins for tumour-specific delivery. Bispecific antibodies that bind to two different antigens can be preloaded with the cytotoxic machinery before administration (indirect arming) or alternatively pre-targeted to the tumour before delivery of the cytotoxic payload. (**d**) Pre-targeting strategies aim for the selective delivery of radionuclides to tumours or selective intratumour activation of prodrugs, thereby diminishing the systemic toxicities of these cytotoxic agents. For prodrug pretargeting, an antibody-fragment–enzyme fusion protein is typically allowed to localize to a tumour and be cleared from the system. A prodrug is then administered and ideally converted to an active drug solely within the tumour. For radionuclide pre-targeting, an antibody–streptavidin conjugate is allowed to accrue within a tumour and is then used to capture a biotin–chelator–radionuclide complex. scFv, single-chain variable fragment (Modified from Carter, 2001).

The cytotoxic power of antibodies can be greatly augmented by chemical conjugation or genetic fusion with effector molecules like toxins, drugs, cytokines or enzymes [Pastan *et al.*, 2006]. Another option is the conjugation to compounds containing radionuclides like ^{90}Y and ^{131}I [Wu and Senter, 2005]. These immunotherapeutic agents derive their killing ability from the toxic moiety and their selective binding from the antibody to which the toxic agent is coupled [Pastan *et al.*, 2006]. In addition to the function of targeted delivery, the antibody often grants an enhanced half-life on the toxic moiety attached to it [DiJoseph *et al.*, 2005].

Among the twelve monoclonal antibodies currently marketed for use in immunotherapy (Table 1), eight are naked antibodies, three are labelled with radionuclides and one is chemically conjugated to a toxic moiety, calicheamicin [Reichert and Valge-Archer, 2007; Reichert *et al.*, 2005].

Generic name (trade name)	Description	First approval indication	Year (country) of first approval
Unconjugated mAbs			
Edrecolomab (Panorex)	Murine, IgG2a, anti-EpCAM	Colorectal cancer	1995 (Germany)*
Rituximab (Rituxan)	Chimeric, IgG1, anti-CD20	Non-Hodgkin's lymphoma	1997 (United States)
Trastuzumab (Herceptin)	Humanized, IgG1, anti-HER2	Breast cancer	1998 (United States)
Alemtuzumab (Campath)	Humanized, IgG1, anti-CD52	Chronic lymphocytic leukemia	2001
Cetuximab (Erbitux)	Chimeric, IgG1, anti-EGF receptor	Colorectal cancer	2004
Bevacizumab (Avastin)	Humanized, IgG1, anti-VEGF	Colorectal cancer Non small-cell lung cancer Advanced Breast cancer	2004 2006 2008 (United States)
Nimotuzumab (TheraCIM)	Humanized, IgG1, anti-EGF receptor	head/neck epithelial cancer	2005 (China)
Panitumumab (Vectibix)	Human, IgG2, anti-EGF receptor	Colorectal cancer	2006 (United States)
Immunoconjugates			
Gemtuzumab ozogamicin (Mylotarg)	Humanized, IgG4 k, anti-CD33, immunotoxin (calicheamicin)	Acute myelogenous leukemia (AML)	2000 (United States)
Ibritumomab tiuxetan (Zevalin)	Murine, IgG1, anti-CD20, radiolabelled (Y-90)	Non-Hodgkin's lymphoma	2002 (United States)
^{131}ITositumomab (Bexxar)	Murine, IgG2a, anti-CD20, radiolabelled (I-131)	Non-Hodgkin's lymphoma	2003 (United States)
^{131}I ch-TNT	Chimeric, IgG1k, anti-DNA associated antigens, radiolabelled (I-131)	Lung cancer	2003 (China)

Table 1. Monoclonal antibodies approved for cancer therapy (updated from Reichert and Valge-Archer, 2007; Sharkey *et al.*, 2008). *Panorex was subsequently withdrawn from market. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; VEGF, vascular endothelial growth factor.

Tumor antigens

One of the distinguishing events occurring during the neoplastic transformation of a cell is the abnormal expression of some surface molecules like proteins or glycoproteins acting as receptors or adhesive elements. These "tumor markers" are classified as:

- **Tumor Specific Antigens** (**TSAs**), that are relatively rare. They could arise from point mutations in oncogenes or tumor suppressor genes such as *ras* in pancreatic and colorectal cancer [Gjertsen and Gaudernack, 1998] or *p53* in lung cancer [Yanuck *et al*., 1993]. Moreover these antigens could derive from virally induced tumors, which constitute almost 20% of all human cancer [Stewart and Kleihues, 2003].
- **Tumor Associated Antigens** (**TAAs)**, more common. These antigens are shared by the tumor, where they are overexpressed, and the tissue from which the tumor originates, where they are expressed at lower level. This group comprises differentiation antigens and has mainly been found in melanomas and melanocytes, e.g. Melan-A [Coulie *et al.*, 1994] and tyrosinase [Brichard *et al*., 1993] but also in other epithelial tissues such as prostate specific antigen (PSA) found in prostate.
- **Oncofetal proteins** (CEA, alphafetoprotein) are normally expressed transiently in embryonic tissues, down-regulated in normal adult tissues, and re-expressed in tumor tissues.

These types of molecules, whether specific of cancerous cells or associated with the transition to neoplastic phenotype, represent the potential targets of cancer immunotherapy.

On each malignant cell there are multiple distinct sites that may become target antigens for a therapeutic antibody, which allows to fight cancer on many fronts, thus diminishing the chances of "escape" by the tumor [Urban and Schreiber, 1992]. Within a population of neoplastic cells there might be individual cells in which a particular target antigen is lacking or its expression is repressed; many experimental evidences indicate that targeting different surface proteins remarkably improves the therapeutic efficacy of the treatment [Flavell *et al.*, 1997].

An ideal target antigen, displayed on the surface of a tumor cell, must be accessible, comparatively over-expressed on malignat cells, uniformly distributed; it should not be secreted or shed after binding to ligands and, depending on the mechanism of action of the drug, it should or should not be internalized. For instance, in the case of a therapeutic mAb whose activity is mediated by ADCC or CDC, the antigen-antibody complex must not be internalized into the cell, otherwise the Fc portion of the antibody will be unable to recruit other elements of the immune system. On the contrary, endocytosis is necessary for delivering the full cytotoxic potential of most immunotoxins (Fig. 3): the target in this case must undergo ligand-induced internalization or should alternatively possess an inherently high frequency of turn-over, thus promoting the efficient transfer of the toxic domain into the cell [Reichert and Valge-Archer, 2007].

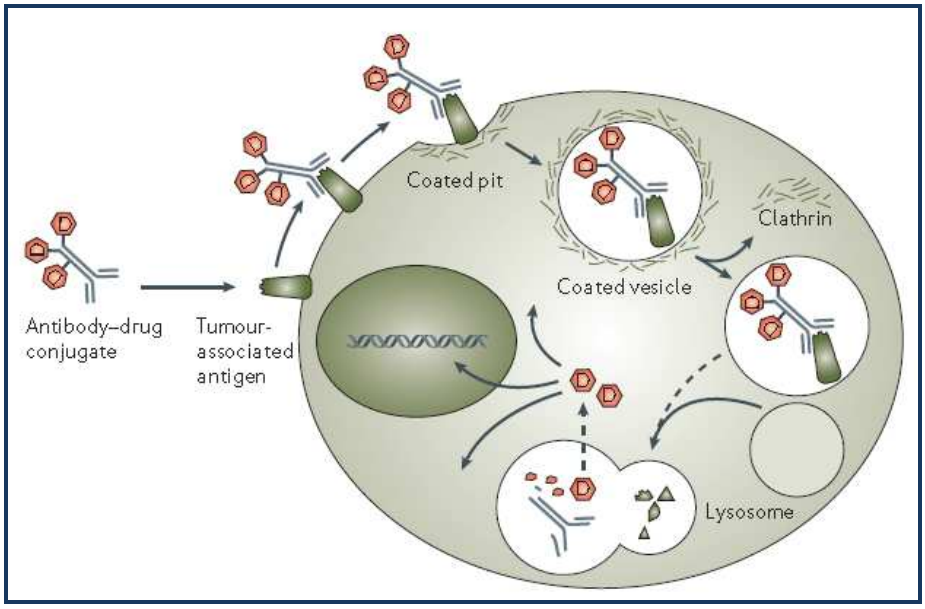

Figure 3. Internalization of antibody–drug conjugates. To regain their cytotoxic activity, the cytotoxic agent has to be cleaved from the chemo-immunoconjugate. Uptake of antibodies predominantly occurs via the clathrin-mediated endocytosis pathway. After binding the respective antigen associated with coated pits, antibody–drug conjugates will be readily endocytosed, from where they transit through several stages of transport and endosomal vesicles and finally end up in a lysosome. There, linkers and antibody will be cleaved releasing the cytotoxic agent which — after exit from the lysosomal compartment — exerts its cytotoxic effect. (Modified from Schrama *et al.*, 2006).

Monoclonal antibodies

The antibodies used in cancer diagnosis and therapy are derived from the IgG isotype. An IgG isotype antibody consists of two antigen-binding fragments (Fabs), which are connected via a flexible region (the *hinge*) to a constant (Fc) region (Fig. 4). This structure comprises two pairs of polypeptide chains, each pair containing a heavy and a light chain of dissimilar sizes. Both heavy and light chains are folded into immunoglobulin domains. The 'variable domains' in the amino-terminal part of the molecule are the domains that identify and bind antigens; the rest of the molecule is composed of 'constant domains' that vary among immunoglobulin classes. The Fc portion of the immunoglobulin serves to bind a variety of effector molecules of the immune system, as well as molecules that establish the biodistribution of the antibody [Trail *et al.*, 2003]. In nature, an antibody's function is to recognize an antigen and, by cross reaction with other immune proteins, to initiate an immunological response. This response should direct the removal of the antigen or the cell bearing the antigen as a result of the antigen/antibody recognition.

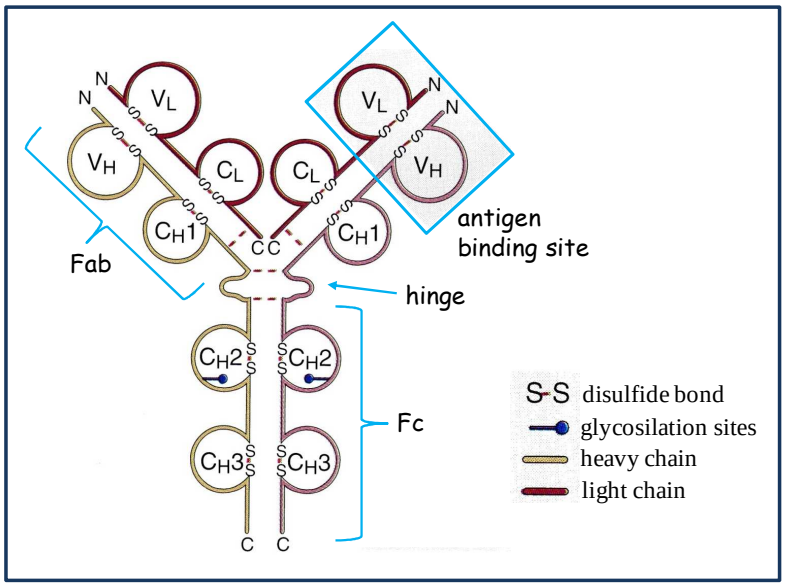

Figure 4. Schematic diagram of immunoglobulin structure. Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region that differs between antibodies of distinct clonality. The constant region (C) exhibit a limited variability that defines the two light-chain subtypes (k e λ) and the five heavy-chain subclasses (α, γ, δ, ε, μ). The molecule represented is specifically an IgG, with a heavy chain comprising three constant domains (one of them, C_H2, glycosilated) and a proline-rich *hinge* region connecting C_H2 and C_H1.

Monoclonal antibodies (mAbs) were originally developed by Köhler and Milstein in 1975 when they successfully fused an antibody-producing B cell with an immortalized myeloma cell line resulting in a hybridoma [Köhler and Milstein, 1976]. These hybridoma cells could then be cloned and screened for large-scale production of mAb [Brekke and Sandlie, 2003]. Many therapeutic antibodies currently under clinical evaluation were initially derived by immunization of mice and classic hybridoma technology. A major factor limiting repeated dosing of fully murine mAbs in humans was that the Fc region of the immunoglobulin structure was murine in origin [Mirik *et al.*, 2004]. After intravenous administration of these mAbs, patients developed human–antimouse antibodies (HAMAs) that were shown to complex with murine mAbs upon repeat administration and in some cases led to acute anaphylactic or chronic serum sickness-like illness [McLaughlin *et al.*, 1998; Kim, 2003].

Molecular engineering techniques have overcome this obstacle by creating chimeras by fusing murine variable domains, responsible for the binding activity, with human

constant domains [Neuberger *et al.*, 1985] leading to the development of a new generation of therapeutic candidates [Reichert *et al.*, 2005] (Fig. 5). These chimeric antibodies are 70% human and possess a fully human Fc portion, which makes them considerably less immunogenic in humans and allows them to interact with human effector cells and the complement cascade. With the development of antibody engineering techniques, it became possible to decrease even further the murine part of mAbs by replacing the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest, using an approach called complementarity-determining region grafting [Jones *et al.*, 1986]. These antibodies, called 'humanized', are 85–90% human and are even less immunogenic than chimeric antibodies (Fig. 5). Most of the approved mAbs in current use are either chimeric or humanized (Table 1).

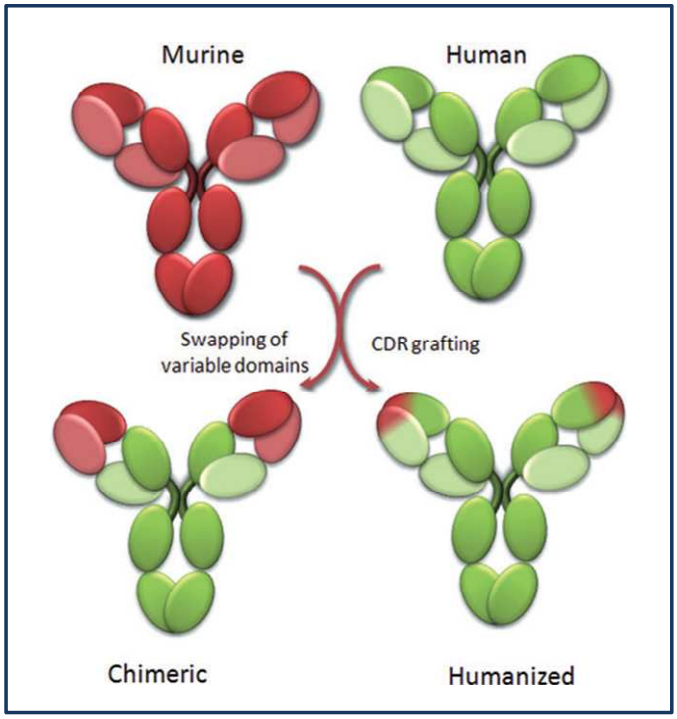

Figure 5. Chimeric and humanized antibodies. Murine sequences are depicted in red and human sequences in green, using light colours for light chain and dark colours for heavy chains. (Modified from Chames *et al.*, 2009)

Monoclonal antibody fragments

Many studies have demonstrated that it is possible to produce various antibody fragments that retain the binding activity of the full-length molecule (Fig. 6) and to use these new formats in certain specific applications [Holliger and Hudson, 2005]. The large size of antibodies limits tumor penetration, and their long serum half-life is not suitable for applications such as radioimmunotherapy or imaging as it would lead to irradiation of healthy tissues and high background respectively. Antibody fragments, such as $F(ab)_2$ and Fab fragments generated only by proteolytic cleavage, although they lack an effector function, can be an attractive alternative as they are rapidly eliminated by renal clearance. To compensate for these shortcomings, several groups have proposed new engineered antibody formats.

A major breakthrough in the technology of antibody engineering was the derivation of single-chain molecules (scFv fragments). These molecules were obtained by joining the variable heavy (V_H) and light (V_L) domains from an antibody with a flexible linker, which allowed the reconstitution of the original V_H/V_L association (Fig. 6). In this way, an antibody fragment was produced in the form of a single-chain molecule, and its antigenic specificity was retained [Bird *et al*., 1988]. Because of their reduced size (28 kDa), recombinant scFv fragments demonstrated a high tumor penetration and a very rapid plasma clearance, features that make them ideal tools for radioimmunodiagnostic purposes [Yokota *et al.,* 1992]. These antibody molecules potentially can be used not only for tumor diagnosis (imaging) but also for the development of chimeric proteins in which the recombinant antigen-binding domain is fused to a cytotoxic agent, such as a cytotoxic drug, radioisotope [Begent *et al*., 1996; Adams *et al.,* 1998], or a protein toxin [Reiter and Pastan, 1998; Kreitman, 1999].

It has been shown that alteration of the V-domain orientation of scFv as well as linker length between V-domains leads to creation of new forms of Fv modules with different size, flexibility and valency suited for in vivo imaging and therapy. This led to the design and expression of dimers, trimers or larger aggregates of antibody fragments [Todorovska *et al.*, 2001; Le Gall *et al*., 1999]. A number of multivalent scFv-based structures have been engineered, including dimeric (minibodies or diabodies), trimeric (trivalent or trispecific) or tetrameric (tetravalent or tetraspecific) conjugates [Hudson, 1999; Hudson and Kortt, 1999; Bauer *et al.,* 1999] (Fig. 6). Generally, in comparison to

the intact antibodies, the recombinant antibodies demonstrate lower retention time in non-target tissues, rapid blood clearance, lower quantitative tumor retention and a better tumor penetration due to their small size [Yokota *et al.*, 1992; Raag and Whitlow, 1995; Milenic *et al.*, 1991].

Bispecific antibodies have the ability to bind two different antigens and have been used to crosslink various cells and molecules, although they have primarily been used to redirect effector cells to target cells. For example, single-chain Fv fragments derived from monoclonal antibodies against a variety of tumor-associated antigens, such as CD19, CD20, and HER2/neu, were combined with scFv directed against CD3 or CD16 to recruit effector T-cells or NK cells [Peipp *et al.*, 2002; Xiong *et al.*, 2002]. Methods for generating bispecific antibodies tend to bias the production toward heterodimers, that is, towards two specificities instead of one.

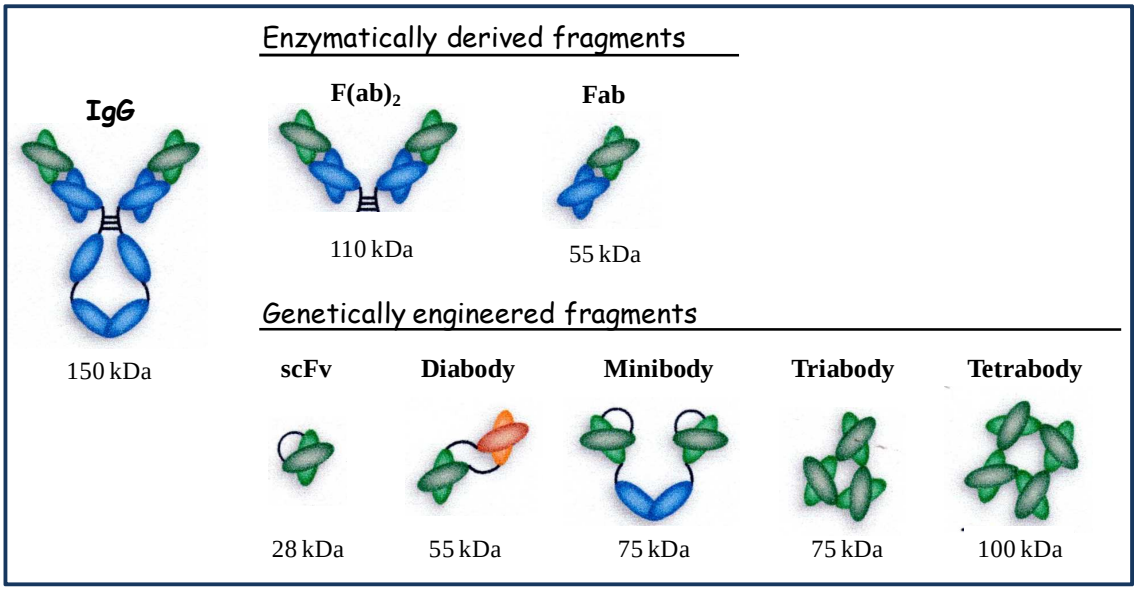

Figure 6. Schematic representation of different antibody format. The domain-based structure of immunoglobulins could be manipulated to yield a wide repertoire of antibody formats from 28 kDa to 100 kDa in size. The orange colour symbolizes different specificity. (Modified from Holliger and Hudson, 2005).

Immunotoxins

Immunotoxins (ITs) employed in cancer therapy comprise a toxic, usually proteinaceous, portion linked to a binding domain that delivers the complex to the target cell [Kreitman, 2006]. ITs interact with a surface target antigen, gain access into the malignant cell by endocytosis, the toxic subunit is released and kills the cell; the most powerful ITs that have been developed so far are based on bacterial or plant toxins [Pastan *et al.*, 2007].

First-generation ITs were obtained by chemically conjugating one whole toxin to a mAb (Fig. 7a); these ITs often showed no tumoral efficacy in animal models because they lacked specificity, were unstable or not homogeneous in composition. Information gathered from structural studies on toxins have defined the functional domains of these proteins, thereby allowing a more rational design of novel ITs. Replacement of the toxin natural binding domain with an antibody-derived domain led to the development of second-generation ITs (Fig. 7b) . As for first-generation ITs, the binding domain and the toxic portion are chemically linked, with the persistent problem of heterogeneous composition of the final immunoconjugate [Pastan *et al.*, 2007; Pastan, 2003].

Third-generation immunotoxins are based on the principles of protein engineering and exploit recombinant DNA technology: the domain with toxic activity alone is genetically fused to a defined binding domain that targets a tumor surface antigen [Kreitman, 2006; Pastan *et al.*, 2007] (Fig. 7c).

For therapeutic efficacy, the conjugates have to be internalized into the cell upon binding to their respective ligands. The cell-killing potency of immunotoxins depends on several biochemical properties, such as antigen-binding affinity, internalization rate, intracellular processing and intrinsic toxin-domain potency [Hexham *et al.*, 2001].

In the last 10-15 years several recombinant immunotoxins have been evaluated in clinical trials (Table 2).

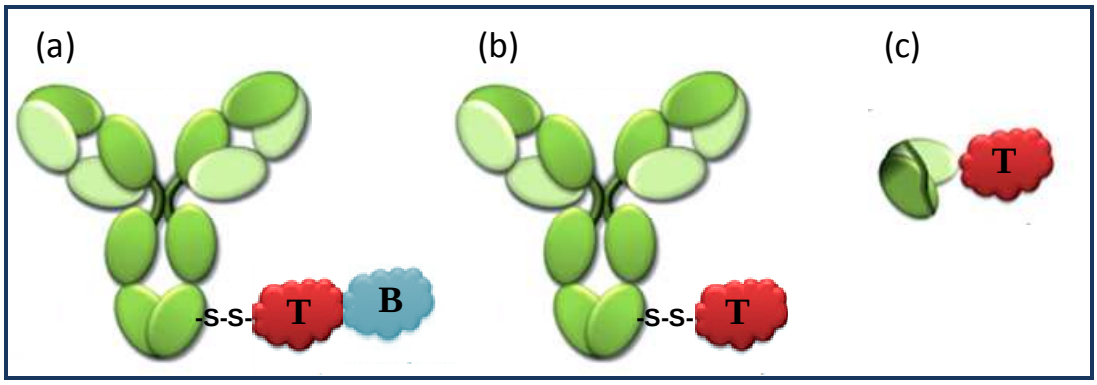

Figure 7. Schematic diagram of immunotoxins. (**a**) First-generation immunotoxins: the whole toxin (T, toxic domain, and B, binding domain) is chemically conjugated to mAb by disulphide bond. (**b**) Second-generation immunotoxins: only the toxic domain is chemically conjugated to mAb by a disulphide bond. (**c**) Third-generation immunotoxins: only the toxic domain is genetically fused to the antibody domain. (Modified from Chames *et al*., 2009).

Agent	Target antigen	Binding domain	Toxic domain	Diseases
DAB_{389}IL2 (Ontak)	IL2R	IL2	DAB_{389}	CTCL, CLL, NHL
BL22	CD22	dsFv	PE38	HCL, CLL, NHL
LMB2	CD25	scFv	PE38	NHL, Leukemias
DT_{388}-GMCSF	GMCSFR	GMCSF	DT_{388}	AML
B3(Fv)-PE38	Le^y	scFv	PE38	Adenocarcinomas
B3(dsFv)-PE38	Le^y	dsFv	PE38	Adenocarcinomas
TP40	EGFR	TGFa	PE40	Bladder Cancer, CIS
TP38	EGFR	TGFa	PE38	Glioblastoma
BR96(scFv)-PE40	Le^y	scFv	PE40	Adenocarcinomas
erb38	erbB2	dsFv	PE38	Breast cancer
NBI-3001	IL4R	IL4(38-37)	PE38KDEL	Glioma
IL13-PE38QQR	IL13R	IL13	PE38QQR	Glioblastomas
SS1(dsFv)-PE38	Mesothelin	dsFv	PE38	Mesothelioma, ovarian and pancreatic cancers
DAB_{389}EGF	EGFR	EGF	DAB_{389}	Carcinoma
TP-38	EGFR	TGFa	PE38	Glioblastomas

Table 2. Completed and ongoing recombinant immunotoxin clinical trials as of 2006 (modified from Kreitman, 2006 and Pastan *et al*, 2006)**.** Abbreviations: IL2, interleukin 2; IL2R, IL2 receptor; GMCSF, granulocyte-macrophage colony-stimulating factor; GMCSFR, GMCSF receptor; Le^y, Lewis Y antigen; EGF, epidermal growth factor; EGFR, EGF receptor; TGFα, transforming growth factor α; IL4, interleukin 4; IL4R, IL4 receptor; IL13, interleukin 13; IL13R, IL13 receptor; IL4(38-37), circularly permuted IL4; DT, diphteria toxin; DAB_{389} and DT_{388}, truncated DT; PE, *Pseudomonas exotoxin* A; PE38, PE40, PE38KDEL and PE38QQR are all truncated versions of PE.

Binding domain

In the design of an IT a variety of binding domains can be used to selectively deliver the drug to the intended cell target; besides monoclonal antibodies and fragments thence derived, other small proteins are appropriate to fulfil this function, e.g. growth factors and cytokines. Such molecules impart specificity to the IT by virtue of the higher expression level of some receptors for growth factors and cytokines on tumor cells. Interleukin 2 (IL-2) and the Granulocyte-Macrophage Colony Stimulating Factor (GMCSF), for instance, have been employed in the construction of immunotherapeutic agents to direct toxic molecules towards leukemia or lymphoma cells [Frankel *et al.*, 2002; Olsen *et al.*, 2001].

However, due to their remarkable molecular versatility and to the possibility of being raised against virtually any target, antibodies are probably the best candidates as binding domains in ITs design [Kreitman, 2006].

Toxic domain

In the design of a therapeutic IT, protein toxins proved the best candidates as cytotoxic domains: due to their polypeptidic structure they are intrinsically flexible and usually show a high stability, resisting high temperatures and proteolysis. Moreover, in most cases the protein toxin is an enzyme with a fast turnover rate, and it is capable of killing both dividing and resting cells and therefore potentially able to kill tumors that are not actively growing at the time of treatment and that would scarcely respond to traditional chemotherapy [Vitetta *et al*., 1993]. The major disadvantage inherent in the employment of a protein toxin of non-human origin lies in its likely immunogenicity [Pastan *et al*., 2006].

Small, pharmachologically active molecules like vinblastine and chalicheamicin have been also studied as putative toxic moieties in ITs. However, they are often unable to reach the target cells at concentrations sufficient to produce a therapeutic effect and, contrary to protein toxins, they have no enzymatic activity. Another drawback is that they can be attached to the binding domain of an IT only through chemical conjugation [Reichert and Valge-Archer, 2007].

The toxins that best fit the role of effector domain in an immunotoxin are the ones whose catalytic activity interferes with such conserved mechanisms as protein synthesis

that take place in most cell types. Some of the most potent ITs are based on toxins of bacterial or plant origin. Once these ITs reach their target site, a single molecule is capable of killing the targeted cell [Pastan *et al*., 2007; Kreitman, 2006].

Plant toxins

Toxins of plant origin have been studied as potential drugs for cancer therapy; RIPs (Ribosome-Inactivating Proteins) are toxins that can be found stored in the seeds of some plant species and they represent the class of plant toxins most widely used for the purpose of immunotoxin construction.

RIPs (e.g. ricin, abrin) are heterodimeric proteins of molecular mass of approximately 60-65 kDa, consisting of an enzimatically active A-chain (A = active) connected by a disulfide bond to a B-chain (B = binding) (Fig. 8). The A-chain prevents the association of elongation factors 1 and 2 (eEF1 and eEF2) with the 60S ribosome subunit, by means of the specific removal of an adenine from the sugar-phosphate scaffold in 28S rRNA. This puts a halt to protein synthesis and the cell rapidly dies [Kreitman, 2006; Fabbrini *et al*., 2003]. The B-chain has the properties of a lectine specific for terminal galactose and N-acetylgalactosamine and binds to galactosyl-terminated receptors on the cell surface, thus allowing the toxin internalization. Following endocytosis, the whole toxin is sorted to endosomes, lysosomes and finally the Golgi complex; thence ricin passes to the endoplasmic reticulum by a retrograde routing, where the disulphide bridge that holds together the two chains (A and B) forming the toxin is reduced, thus allowing the release of the catalytically active A chain, which is eventually retrotranslocated to the cytosol and exerts its toxic effect on ribosomes [Fabbrini *et al*., 2003; Bagga *et al*., 2003].

Because the enzymatic domain alone is involved in the intoxication of the cell, plant RIPs are reduced and the A-chain (e.g. RTA) is exploited for the creation of ITs (Fig. 8) [Kreitman, 2006].

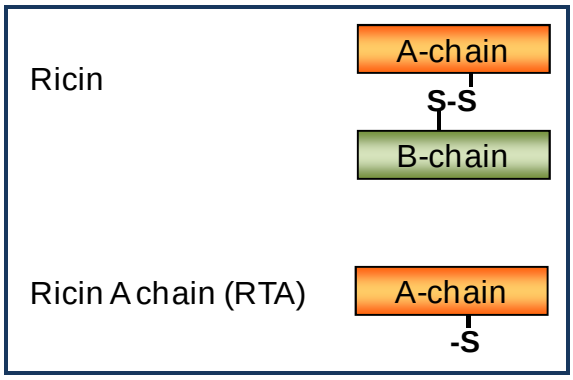

Figure 8. Plant toxins. Whole plant toxins contain activity (A) and (B) binding domains disulfide-bonded together. Reduction of disulfide bond results in ricin A chain (RTA), which has reduced toxicity. (Modified from Kreitman, 2006)

Bacterial toxins

Pseudomonas aeruginosa Exotoxin A (PE) and *Corynebacterium diphtheriae* toxin (DT) are the bacterial toxins more widely exploited for immunotherapy. They are both protein toxins that kill eukaryotic cells by catalysing the transfer of the ADP-ribose group from a NAD^+ molecule to a modified residue of histidine, also called diphthamide, which is present in the eukaryotic elongation factor 2 (eEF2), which determines the inhibition of protein synthesis. In spite of their having a common mechanism of action and similar size (66 kDa for PE and 60 kDa for DT), the two proteins do not show a high degree of sequence homology [Pastan and FitzGerald, 1989].

Differently from ricin, both PE and DT are macromolecules constituted by a single polypeptide chain organized in three functional portions: a binding domain mediating the interaction with the cell surface, a domain implicated in the translocation of the toxin into the cytosol and another one which is responsible for the ADP-ribosylating enzymatic activity (Fig. 9). In *Pseudomonas* exotoxin A, the catalytic domain is located at the C-terminus, whereas it is at the N-terminal end of DT [Kreitman, 2006].

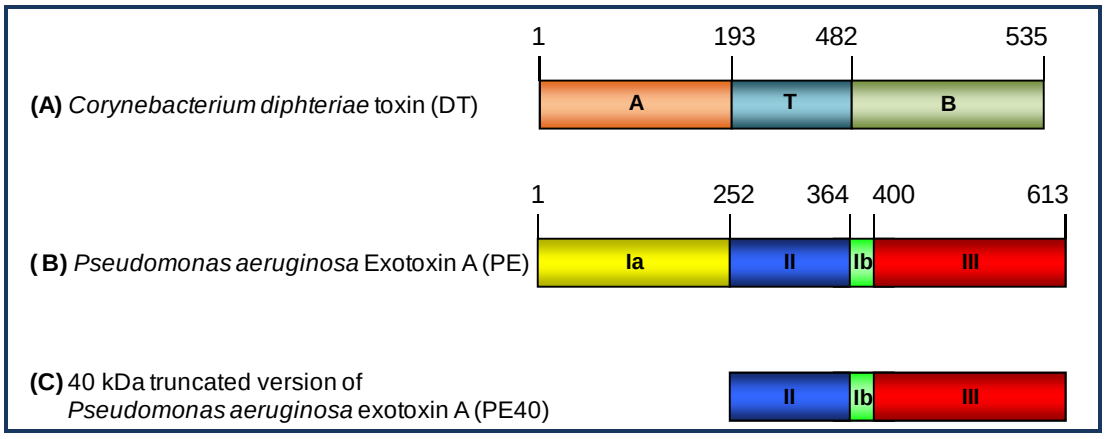

Figure 9. Schematic structure of bacterial toxins. (A) DT is 535 amino acids in length and is composed of the enzymatic A domain (amino acids 1-193) and the binding B domain (amino acids 482-535). The translocation (T) domain is located in between. **(B)** PE is a single-chain 613 amino acids protein composed of the binding domain (amino acids 1-252 form the subdomain Ia; amino acids 365-404 form the subdomain Ib), translocating domain (domain II, amino acids 253-364), and enzymatic domain (domain III, amino acids 400-613). **(C)** PE40 is the 40 kDa truncated version of PE; it laks only in the binding domain Ia.

After entering the cell, the two toxins follow distinct intracellular pathways.

- **DT**

  Before being internalized, DT is proteolitically processed at a specific site within the translocation domain; the two deriving subunits are held together via a disulphide bridge. The toxin eventually joins the precursor of the heparin-binding EGF-like growth factor and is endocytosed. The low pH inside the endosomes causes the unfolding of the toxin and the reduction of the disulphide linking the two subunits. At this stage the two domains form a hairpin structure that generates a channel spanning the endosome membrane, through which the enzymatic domain is translocated to the cytosol: here an ADP-ribose group from a NAD^+ molecule is trapped in the active site of DT and finally transferred to eEF2 [Kreitman, 2006; Potala *et al.*, 2008].

- **PE**

  As shown in Fig. 10, PE binds to CD91, also called alpha2-macroglobilin receptor/low density lipoprotein receptor-related protein, and then is endocytosed. In the acidic endosome environment PE dissociates from CD91,

undergoes a conformational change, and is cleaved by the protease furin between residues R-279 and G-280 [Ogata *et al.,* 1992]. This results in an N-terminal fragment of 28 kD and a C-terminal fragment of 37 kD, the latter being composed of a portion of domain II, domain Ib, and all of the enzymatic active domain III [Ogata *et al*., 1990; Wedekind *et al*., 2001]. Both fragments are still connected by the disulfide bond between C-265 and C-287, encompassing the furin cleavage site. It is speculated that under the mildly acidic conditions in the endosomes, there is an unfolding event, possibly aided by the binding of chaperone proteins. This leads to a surface exposure of the disulfide bond with subsequent reduction which is followed by a release of the C-terminal 37-kD fragment [McKee and FitzGerald, 1999]. The enzymatic active 37-kD fragment travels via late endosomes in a Rab9-dependent manner to the trans-Golgi network (TGN). There, it can bind in a pH-dependent manner to the KDEL receptor via its C-terminal KDEL-like sequence REDL (aa 609–612) and is transported to the endoplasmic reticulum (ER) [Kreitman and Pastan, 1995; Jackson *et al*., 1999]. The KDEL receptor cycles between the TGN and the ER via Golgi cisternae with the help of the tyrosine kinase Src [Miesenbock and Rothman, 1995; Bard *et al.,* 2003] and is responsible for recycling proteins bearing the C-terminal amino acid code KDEL to the ER [Wilson *et al.*, 1993]. Alternatively, the 37-kD fragment of PE, which has bound to DRM (detergent resistant microdamain) at the cell surface, can directly reach the TGN from early endosomes in a pathway independent of the small GTPase Rab9 and presumably reaches the endoplasmic reticulum (ER) in a lipid-dependent sorting pathway, controlled by Rab6 [White *et al.,* 1999; Smith *et al.,* 2006]. Sequences in the translocation domain II induce the dislocation of the 37-kD fragment from the ER to the cytosol [Ogata *et al.,* 1990; Theuer *et al*., 1993]. Once the enzymatic subunit of PE has reached the cytosol, it catalyzes the ADP ribosylation of its target protein, the eukaryotic elongation factor-2 (eEF-2) [Iglewski *et al.*, 1977].

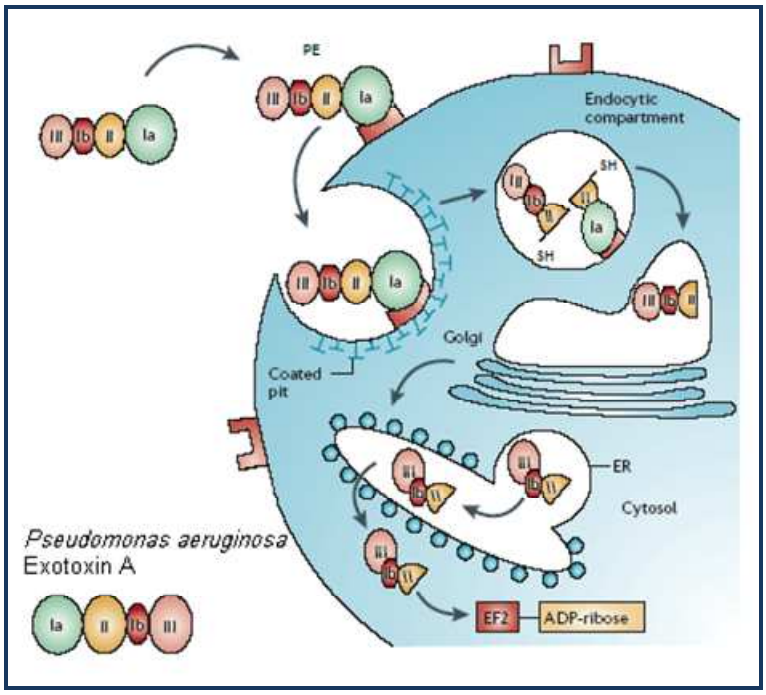

Figure 10. Proposed mechanism of cell intoxication by *Pseudomonas aeruginosa* exotoxin A. The toxin binds the receptor and is eventually internalized through clathrin-coated vesicles. Inside endosomes, the protein is cleaved into two fragments, the C-terminal one being likely transported to the ER, whence the enzymatic domain of the toxin is expelled into the cytosol and ADP-ribosylates eEF2. (Modified from Pastan, I. *et al.*, 2006).

PE is a preferred molecule for the construction of immunotoxins, because its high toxic potential is well documented and its cytotoxic pathways are well understood. For immunotoxin design, the receptor binding domain Ia (aa 1–252) of PE is removed and replaced by a ligand, which specifically binds to a tumor-associated antigen. The truncated form of PE (aa 253–613) is then known as PE40 to reflect its molecular weight of 40 kDa [Wolf and Elsässer-Beile, 2009].

A novel tumor associated antigen: prostate stem cell antigen (PSCA)

The PSCA gene was originally identified through an analysis of genes up-regulated in the human prostate cancer LAPC-4 xenograft model [Reiter *et al.*, 1998]. The PSCA gene is located on chromosome 8q24.2 and encodes a 123 amino acid cell surface protein with 30% homology to stem cell antigen type 2 (SCA-2), an immature

lymphocyte cell surface marker. It is because of this relatively weak homology that PSCA was named inaccurately since it is not a marker for a stem cell population nor is it exclusively expressed in the prostate. PSCA bears multiple N-glycosylation sites and, like SCA-2, it is a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI) anchored surface proteins. Additionally, a murine PSCA (mPSCA) homologue has been identified with 70% homology to human PSCA at the nucleotide and amino acid levels, which is located on chromosome 15 [Reiter *et al.*, 1998].

The function of PSCA in normal cellular processes or carcinogenesis is currently unknown, however PSCA homologues shed light on a variety of possible roles. It has been previously shown that GPI-anchored proteins, like PSCA, play a role in T cell activation [Presky *et al.*, 1990]. Additionally, proteins in the Thy-1 family have been reported to function in T cell activation and proliferation, stem cell survival, and cytokine and growth factor responses [Rege and Hagood, 2006]. Furthermore, the family of Ly-6 genes has been associated with carcinogenesis [Witz, 2000; Treister *et al.*, 1998], cellular activation [Malek *et al.*, 1986] and cell adhesion of tumor cells [Eshel *et al.*, 2000]. While these studies point to potential roles of PSCA, no definitive function has been attributed to the protein either in normal cellular processes or abnormal tumorigenesis.

PSCA expression in normal tissues

With any potential immunotherapeutic target it is necessary to assess the normal tissue expression. Ideal targets show overexpression on target cells with limited or no expression on normal tissues, thereby reducing the risk of damage to normal tissues. PSCA messenger RNA (mRNA) expression in normal human tissues was found to be predominantly expressed in prostate, with a lower expression in placenta and a very low (1% of the level in prostate) amount in kidney and small intestine [Reiter *et al.*, 1998, Cunha *et al.*, 2006]. In situ hybridizations of normal human prostate sections found PSCA mRNA expression limited to the subjacent basal cells, with little or no staining of secretory luminal cells [Reiter *et al.*, 1998]. In contrast, a separate study demonstrated that PSCA expression was localized to the secretory epithelial cells, with no expression in the basal cells or prostatic stroma [Ross *et al.*, 2002]. This apparent disagreement in PSCA mRNA localization may be due in part to the relatively short half-life of mRNA

in tissues combined with the sampling error inherent in small sectional biopsies within a larger tissue. To clarify this discrepancy, another study analyzed PSCA protein in the prostate, a much more stable form of expression, using monoclonal antibodies (mAbs) and found PSCA protein to be present in both the basal and secretory epithelial cell layers, along with the neuroendocrine cells of the prostate [Gu *et al*., 2000]. Additionally, in this study PSCA protein expression was demonstrated in the placenta, the bladder, the neuroendocrine cells of the stomach and colon, and weakly in the kidneys excluding the glomeruli.

PSCA expression in cancer

In the initial study on PSCA expression in human prostate cancer, 102 of 126 (81%) primary human prostate tumors specimens along with seven of nine (78%) residual tumors removed after androgen ablation therapy stained strongly for PSCA using mRNA in situ hybridization [Reiter *et al.,* 1998]. Analysis by immunohistochemistry (IHC) using an anti-PSCA mAb demonstrated 105 of 112 (94%) prostate tumors positive for PSCA, of which 21% stained very strongly and 63% stained moderately [Gu *et al*., 2000]. Importantly, this study also revealed a significant correlation between PSCA expression and Gleason score, pathologic tumor stage and progression to androgen-independence. A later study also significantly correlated high PSCA intensity with seminal vesicle invasion and capsular involvement [Han *et al*., 2004]. An additional study showed a higher percentage of metastatic prostate cancer cases staining positive for PSCA mRNA compared with nonmalignant prostate disease and organ-confined prostate cancer [Ross *et al.*, 2002]. Specifically, prostate cancer metastases to bone marrow, lymph node and liver stained positively for PSCA expression, with bone marrow metastases staining with comparatively higher intensity [Gu *et al*., 2000; Lam *et al*., 2005].

Whether overexpression of PSCA in humans is causative of prostate carcinogenesis remains unknown. Studies have shown chromosome 8q to be commonly amplified in metastatic and recurrent prostate carcinoma and associated with a poor prognosis [Visakorpi *et al.*, 1995, Sato *et al*., 1999]. Additionally, PSCA is located close to the c-myc oncogene, and therefore PSCA expression may be increased incidentally with amplification of other genes shown to be up-regulated in prostate cancer [Jenkins *et al*.,

1997, Qian *et al*., 1995]. These results also suggest that PSCA is a useful marker of MYC amplification on prostate cancer cells, allowing better characterization of each tumor [Jalkut and Reiter, 2002].

Of interest, subsequent to the identification of PSCA as a prostate tumor associated protein, several other tumors were evaluated for PSCA expression including pancreatic adenocarcinoma [Argani *et al*., 2001; Iacobuzio-Donahue *et al*., 2002; Wente *et al*., 2005], transitional cell carcinoma [Elsamman *et al*., 2006; Amara *et al*., 2001], renal cell carcinoma [Elsamman *et al*., 2006] and diffuse-type gastric cancer [The Study Group of Millennium Genome Project for Cancer, 2008]. For pancreatic adenocarcinoma, overexpression of PSCA protein was established via IHC in 36 of 60 (60%) primary tumors analyzed, while adjacent normal pancreas had no expression in 59 of 60 samples [Argani *et al*., 2001]. Additional analysis of pancreatic cancer cell lines confirmed the cell surface expression of PSCA, making it a suitable target for immunotherapy [Wente *et al*., 2005]. When human transitional cell carcinoma (TCC) was studied, PSCA was expressed in a majority of TCC and a higher level of PSCA expression correlated with increasing tumor grade [Amara *et al*., 2001] and was an independent predictor of disease recurrence [Elsamman *et al*., 2006]. In addition, PSCA mRNA expression was increased in renal cell carcinoma and the gene expression level correlated with histological grade, pathological stage and prognosis [Elsamman *et al*., 2006]. Finally, in an interesting recent article, polymorphism of the PSCA gene was shown to influence susceptibility to diffuse-type gastric cancer [The Study Group of Millennium Genome Project for Cancer, 2008].

AIM OF THE RESEARCH

The PhD thesis here presented is focused on the generation of anti PSCA (prostate stem cell antigen) monoclonal antibody and its fragments as potential tools for diagnosis and therapy of carcinomas expressing the tumor associated antigen PSCA.

The objectives of this project are:

- generation of anti PSCA monoclonal antibody (mAb) and its fragment single chain (scFv);
- characterization of their binding properties on both the purified recombinant PSCA and cells that express it on their cell surface;
- evaluation of the diagnostic potential of mAb;
- generation of chemical and genetically fused immunotoxins (IT);
- evaluation of the selective toxicity of these IT on target cells;
- generation of a cellular model of polarized epithelial tissue to study the efficacy of IT in polarized cells.

MATERIALS AND METHODS

Escherichia Coli strains and growth media

- BL21 (λDE3) and BL21 (λDE3) pLysS: growth in LB medium.
  BL21 (λDE3) pLysS are grown in medium containing 34 μg/ml chloramphenicol to maintain the selection of pLysS plasmid;
- HB2151: growth in 2X YT medium.

LB-*medium*

Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	10 g/l

2X YT-*medium*

Tryptone	16 g/l
Yeast Extract	10 g/l
NaCl	5 g/l

SOC-*medium*

Tryptone	20 g/l
Yeast Extract	5 g/l
NaCl	0.5 g/l
KCl	2.5 mM
$MgCl_2$	20 mM
Glucose	20 mM

Plasmid vectors

- pET30a (5422 bp): prokaryotic expression vector.
  The plasmid enabled the production of a tagged recombinant protein exploiting a 6-His Tag on C-terminal and N-terminal tails. This vector carries a gene for kanamycine resistance.

- pET11d (5674 bp): prokaryotic expression vector.
  The plasmid carries a β-lactamase gene for ampicillin resistance.
- pHEN2 (4522 bp): phagemid vector derived from pUC119.
  Upstream of the cloning site a sequence coding for the pelB signal peptide deriving from the gene coding for the pectate lyase of *Erwinia carotovora*) leads the periplasmic secretion of a polypeptide coded by an in-frame sequence in the multi-cloning site; downstream of the polylinker there is the sequence for a c-myc peptide tag. The plasmid carries a β-lactamase gene for ampicillin resistance.

Preparation and transformation of competent *E. Coli* cells

The strains of *Escherichia coli* utilized for the molecular cloning and for protein expression are made competent to transformation by plasmid DNA following treatment with RF1/RF2 solutions (Tab. 1).
A single colony is picked and used to start a 10 ml culture and grown o.n. at 37 °C with shaking. Few (1-2) ml of the overnight culture is then used to start a 50 ml culture then grown to OD_{600} = 0.3-0.4 at 25°C with shaking.
The bacterial culture is then kept on ice for 10 minutes, after which it is centrifuged at 5000 rpm, 15 minutes at 4 °C. Always keeping the cells at ice temperature, they are recovered and resuspended in 18.6 ml of sterile, ice-cold RF1 solution. After 15 minutes of incubation on ice the suspension is centrifuged at 5000 rpm, 15 minutes at 4 °C. The supernatant is removed and the bacterial pellet is resuspended in 4.5 ml of a sterile, ice-cold RF2 solution. Cells are finally dispensed in aliquots, frozen in dry ice and kept at -80 °C.

Table 1. RF1/RF2 solutions

RF1 solution	Concentration
RbCl	100 mM
$MnCl_2$	50 mM
CH_3COOK	30 mM
$CaCl_2$	10 mM
Glycerol	12%
Adjust to pH 5.8 with glacial acetic acid	
RF2 solution	
MOPS	10 mM
RbCl	10 mM
$CaCl_2$	75 mM
Glycerol	12%
Adjust to pH 6.8 with NaOH	

A 30 µl aliquot of competent cells are thawed on ice and mixed into a sterile tube with 5-10 ng of plasmid DNA; in case of transformation with a ligation, 4 µl of the reaction are used. The mixture is gently flicked and kept on ice for 30 minutes. Afterwards a heat shock is carried out by dipping the tube for 45 seconds in a 42°C water bath, followed by 2 minute incubation on ice. Eighty µl of SOC medium (without antibiotics) is then added to the tube which is then incubated 1 hour at 37 °C with shaking. The total amount of transformation is then plated on agar plates supplemented with the appropriate selective agent. Plates are incubated inverted at 37 °C and grown o.n.

Human cell lines

- SW780: human cell line of urinary bladder carcinoma;
- PC-3: human cell line of prostate carcinoma established from the bone marrow metastasis;
- LNCaP: human cell line of prostate carcinoma established from lymph node metastasis;

- DU145: human cell line of prostate carcinoma;
- HEK 293: human embryonic kidney cell line;
- MDCK: canine cell line of kidney;
- Anti PSCA hybridoma: the hybridoma clone was derived from spleen lymphocytes of a mouse previously immunized with recombinant hPSCA; the myeloma cell line used in the fusion was NS-1.

All cell lines are grown in flasks at 37 °C, 5% CO_2, using the following medium: RPMI 1640 medium (with 40 mg/l folic acid, 2 g/l $NaHCO_3$) (Biochromag) and DMEM (with 1000 or 4500 mg/l glucose) (Sigma) supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-Glutamine, 10 mM Hepes and penicillin-streptomycin 100 U/ml.
All supplements are added into the medium after sterilization through 0.22 μm filters.

Transfection of human cell lines

hPSCA transfected PC-3, LNCaP, HEK 293 cell lines were obtained by transfection with the linearized plasmid hPSCA-pcDNA3.1 according to the following protocol: cells are plated in a 6 wells plate and grown until 50% confluent. Five μl of lipids are added to 250 μl of OPTIMEM and 2.5 μg of DNA to 250 μl of OPTIMEM. The two solutions are combined to form DNA complex and incubated for 30 minutes at room temperature. Cell medium is replaced with 500 μl of fresh medium plus 500 μl of DNA complex. The following day new fresh medium is added to the cells. To select transfected cells the antibiotic is added to the medium two days after transfection.
hPSCA transfected MDCK cell line was obtained by transfection using the circular plasmid hPSCA-pcDNA3.1 according to the following protocol: cells are plated in a 10 cm plate and grown until 30% confluent. Medium is replaced with growth medium plus Polybrene (30 μg) and DNA (1-10 μg). Cells are incubated for 10 hours at 37°C, 5% CO_2 with gentle rocking every 2 hours. DMSO is diluted in growth medium and added for 5 min at room temperature to the DNA/Polybrene solution so that DMSO final concentration is 30%. The medium is aspirated; cells are washed twice with PBS and incubated in growth medium alone.

The selections of transfected cells were performed using G418 antibiotic at different concentrations for each cell line.

Molecular biology techniques

RNA extraction from anti PSCA hybridoma cells

Six x 10^6 cells of the anti-PSCA hybridoma are collected by centrifugation (5 minutes, 150 x *g*, 5 °C) and washed in physiologic solution. The cell pellet is then homogenized by resuspension in 1 ml of Trizol (*TRIzol Reagent* – Life Technologies, Gibco BRL) and incubated five minutes at room temperature. Two hundred µl of chloroform are added and, after a vigorous mix, incubated 3 minutes at RT. The aqueous phase containing the RNA is separated from the organic phase after centrifugation at 10000 rpm for 15 minutes at 4°C in minifuge. RNA is precipitated by addition of 500 µl isopropanol, followed by mixing and 10 minutes incubation at 4 °C. After a wash in 75% ethanol, the RNA pellet is air-dried and finally resuspended in 25 µl RNAse-free H_2O. Total RNA is quantified by spectrophotometry: 1 unit at OD_{260} corresponds to an RNA concentration of 40 µg/ml.

cDNA synthesis

Reverse transcription to cDNA is obtained using *SuperScript™ First-Strand Synthesis System for RT-PCR* kit (Invitrogen) following the manufacturer's instructions.

Before performing the amplification of the fragments of interest, the quality of the cDNA is assessed through a polymerase-chain-reaction (PCR) test with a couple of primers designed for the amplification of ribonucleoprotein S15.

Cycling programme:

98°C	30	sec	
98°C	10	sec	30 cycles
56°C	30	sec	
72°C	45	sec	
72°C	7	min	
4°C		end	

1 µl of cDNA is used as a template in a 25 µl reaction including a couple of primers (S15 fw and S15 rev) at a final concentration of 0.2 µM each, dNTPs (Invitrogen) at a concentration of 0.2 mM each, 1 unit of Phusion DNA polymerase (Finnzyme) and 2.5 µl

of 10x buffer (Finnzyme) providing a final Mg^{2+} concentration of 2 mM.

Amplification of the sequences coding for variable domains of heavy and light chains

To select the appropriate couple of primers for the amplification of fragments coding for the variable regions of heavy and light chains (V_H and V_κ, respectively) of the anti-PSCA hybridoma clone, a panel of 25 forward and 4 reverse primers are considered for each variable domain (25 V_H forward primers and 4 J_H reverse primers; 25 V_κ forward primers and 4 J_κ reverse primers). Forward primers were designed based on highly conserved sequences at the 5'-end of DNA fragments for V_H and V_L domains from several families of murine immunoglobulins; reverse primers were instead inferred from the J regions located at the 3'-end of V_H and V_L DNA regions.

Each forward primer is tested in a PCR reaction that includes a mix of the four reverse primers. Once the best forward primer has been thus selected, it is used in four individual PCR reactions, each with a single reverse primer. The forward/reverse primers pair identified as the most appropriate for amplification are then re-designed as modified versions with suitable restriction enzymes.

The sequence coding for the V_κ region of our anti-PSCA monoclonal antibody was PCR-amplified using the primers 5' V_κ 318 (*Apal I*) and 3' J_κ 491 (*Not* I), which introduce Apal I and Not I restriction sites, respectively; the V_H fragment was amplified with primers 5' V_H 348 (*Sfi* I) and 3' J_H 354 (*Xho* I), introducing Sfi I and Xho I sites.

0.4 µl of cDNA is used as a template in each 20 µl PCR reaction, along with 0.4 µM of each primer, 0.2 mM of each dNTP (Invitrogen), 0.4 µl of Phusion DNA polymerase (Finnzyme) and 2 µl 10 x buffer, providing a final Mg^{2+} concentration of 2 mM.

Cycling programme:

85°C	2	min	
94°C	2	min	
94°C	1	min	↕ 30 cycles
55°C	1	min	
72°C	1	min	
72°C	10	min	
4°C		end	

Amplification of the sequence coding for the whole scFv

The sequence coding for the whole scFv antibody is amplified using primers LMB3 and pHENSeq from the pHEN2-based construct containing the variable regions of our anti-PSCA mAb.

The 50 µl PCR reaction is assembled using 3 ng of template plasmid construct, each primer at a 0.4 µM concentration, each dNTP (Invitrogen) at 0.2 mM, 1 µl of Phusion DNA polymerase (Finnzyme) and 5 µl of 10x buffer, providing a final Mg^{2+} concentration of 2 mM.

Cycling programme:

98°C	30	sec	
98°C	10	sec	30cycles
55°C	30	sec	
72°C	50	sec	
72°C	10	min	
4°C		end	

DNA digestion with restriction enzymes

Enzymatic digestion of DNA was performed according to the indications provided by the manufacturers. Typically, in each reaction 5-10 units of enzyme are used to cut 1 µg of DNA. Reactions are usually incubated for 2 hours at the recommended temperature. In Tab. 2 are shown the restriction enzymes used.

Table 2. Restriction enzymes used.

	Manufacturer	Stock concentration
Nco I	TaKaRa	10 U/µl
Not I	TaKaRa	10 U/µl
Sfi I	New England	10 U/µl
Xho I	Roche	10 U/µl
Hind III	Roche	10 U/µl
Apal I	New England	10 U/µl

Ligation

Vectors and inserts for the preparation of all constructs were purified after agarose gel electrophoresis using the *QIAEX II Gel Extraction* kit (QIAGEN), and eventually ligated with T4 DNA ligase (New England). A 20 µl reaction is prepared in a tube containing 100 ng of vector, a five-fold molar excess of insert fragment, the provided ligation buffer and 1 µl of enzyme. The ligation is incubated 2 hours at 20°C, after which 4 µl are used for the transformation of *E. coli* cells.

Colony-PCR screening

For a rapid screening of *E. coli* colonies obtained after transformation with the ligation reaction, a PCR is performed directly on bacterial cells.

Using a sterile tip, cells from a single colony are picked from the LB-agar plate and dissolved in 20 µl of PCR reaction mix with: 2. µl of 10x reaction buffer (Euroclone), dNTPs mix (0.2 mM of each dNTP, Invitrogen), forward and reverse primers (10 pmoles each), 1 unit of Taq DNA polymerase (Euroclone). If necessary, also 5% DMSO is included.

Cycling programme:

94°C	10	min	
95°C	1	min	↕ 30 cycles
55°C	1	min	
72°C	2	min	
72°C	10	min	
4°C		end	

The PCR reactions are analysed by agarose-gel electrophoresis and staining with ethidium bromide.

Plasmid DNA extraction from E. coli cultures

Single colonies picked from LB-agar plates (or from small LB cultures for colony-PCR screening) are inoculated in 5 mL for small-scale preparations (miniprep), or in 50 mL for medium-scale preparations (midiprep) of LB broth additioned with the appropriate antibiotics (100 µg/ml Amp); the culture is grown o.n. at 37 °C with shaking (250 rpm) and cells are recovered by centrifugation (5 minutes, 10000 rpm at RT in minifuge for

minipreps; 10 minutes, 4000 x*g* at RT for midipreps) and the bacterial pellet is processed using the *PureLink™ Qick Plasmid Miniprep kit* (Invitrogen) for minipreps and the *PureLink™ HiPure Plasmid Filter Purification kit* (Invitrogen) for midipreps, according to instructions provided by the manufacturers. Purity and concentration of plasmid DNA are assessed by agarose gel electrophoresis and ethidium bromide staining.

Production and purification of recombinant PSCA

Production of recombinant PSCA

A single colony of *E. coli* BL21 (λDE3) transformed with pET30a coding for the recombinant human PSCA (hPSCA) is inoculated in 10 ml of LB medium, 30 µg/ml kanamycin, 0.1% glucose and grown o.n. at 37 °C with shaking at 250 rpm. The o.n. culture is used to start a 1 l culture in the same medium, 30 µg/ml kanamycin which is grown at 37 °C with shaking to an OD_{600} between 0.7 and 0.8. Expression of the recombinant protein is induced by addition of 1 mM IPTG into the culture and incubation is continued for 3 hours at 30 °C with shaking. The bulk of the induced culture is centrifuged 15 minutes at 5000 rpm, at 4 °C and the bacterial pellet is stored at -20°C.

Isolation of inclusion bodies and purification by affinity chromatography

The pellet of the harvested induced culture is resuspended in 0.02 culture volume of Lysis Buffer (50 mM NaH_2PO_4, 1 M NaCl, pH 8.0) additioned with Protease Inhibitor (Roche), 0.1mg/ml Lysozyme (Sigma), DNase I (Roche) and 0.1 mM PMSF (Sigma). The suspension is incubated on ice for 30 minutes and then sonicated on ice until cells are lysed and the solution is no longer viscous. After centrifugation at 10000 x g, 10 minutes at 4°C the supernatant is collected (cytoplasm) and the pellet, that is inclusion bodies, are washed twice with Lysis Buffer. The inclusion bodies are resuspended in 0.015 culture medium of Denaturing Buffer (8 M urea, 100 mM sodium phosphate buffer, 10 mM tris HCl pH 8.0) and then incubated at room temperature (RT) for 30 minutes. After centrifugation at 10000 x g, 10 min at RT the supernatant is collected and added to NiNTA resin. The incubation is performed for 1h at RT on an orbital

shaker. After collecting the flow-through the resin is washed in three steps with Denaturing Buffer at decreasing pH (8.0, 6.3, 5.9). The PSCA protein is eluted with Denaturing Buffer at pH 4.5.

Refolding of PSCA from inclusion bodies

Refolding of urea-denatured proteins from inclusion bodies is attained by multi-step dialysis that gradually decreases the concentration of denaturant, therefore promoting protein refolding.

Tubular membranes with a 3 kDa cutoff (Carl Roth) are used.

The dialysis steps are performed at 4 °C according to the following scheme:

- Refolding Buffer with 6 M urea (24 h)
- Refolding Buffer with 4 M urea (24 h)
- Refolding Buffer with 2 M urea (24 h)
- Refolding Buffer with no urea (24 h)
- Tris 20 mM pH 8.0 (24 h)

Refolding Buffer:

TrisCl pH 8	20 mM
L-Arginine	400 mM
Urea	different concentrations
Oxidized glutathione	0.5 mM
Reduced glutathione	5 mM
PMSF	0.1 mM

PSCA purification by ionic exchange

The ionic exchange purification is performed with *Vivapure Mini Spin columns* (Vivascience) following the manufacturer's instructions. The PSCA is eluted with 20 mM Tris HCl pH 8.0 containing 500 mM NaCl and finally dialyzed in PBS buffer o.n. at 4°C.

Production and purification of anti PSCA scFv

Production of anti PSCA scFv

A single colony of *E. coli* HB2151 transformed with pHEN-2 coding for the anti PSCA scFv is inoculated in 10 ml of 2X YT medium, 100 µg/ml ampicillin, 0.1% glucose and grown o.n. at 37 °C with shaking at 250 rpm. The o.n. culture is used to start a 1 l culture in the same medium, 100 µg/ml ampicillin, 0.1% glucose which is grown at 37 °C with shaking to an OD_{600} between 0.7 and 0.8. Expression of the recombinant protein is induced by addition of 1 mM IPTG to the culture and incubation is continued for 20 hours at 26 °C with shaking. The bulk of the induced culture is centrifuged 15 minutes at 5000 rpm, at 4 °C and the bacterial pellet is immediately processed for periplasm extraction.

Extraction of periplasmic fraction

Proteins were extracted from the periplasm by osmotic treatment by resuspending the bacterial pellet in 0.05 culture volume of ice-cold extraction buffer (30 mM TrisCl pH 8.0, 20% sucrose, 1 mM EDTA). Cells were kept on ice for 1 hour and then centrifugated at 15000 x *g*, 10 minutes at 4 °C. The supernatant containing the soluble periplasmic fraction is separated from the cell pellet.

Purification of scFv by affinity chromatography

The periplasm containing scFv is diluted 1:2 with 2 M urea, 10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.2 mM DTT and then added to NiNTA resin so that the binding of scFv to NiNTA resin occurs in weak denaturing conditions. The incubation is performed o.n. at 4°C on an orbital shaker. After collecting the flow through the resin is washed in three steps with Binding Buffer with increasing concentrations of imidazole (5, 10 and 20 mM). The scFv is eluted by adding Binding Buffer containing 250 mM imidazole. Finally the scFv is dialyzed in PBS buffer o.n. at 4°C.

Binding Buffer:

Tris	10 mM
Urea	1 M
EDTA	0.1 mM
DTT	0.1 mM
Imidazole	5 mM, 10 mM, 20 mM or 250 mM

Adjust pH to 8.0

Production and purification of scFv-PE40 immunotoxin

Production of scFv-PE40 immunotoxin

A single colony of *E. coli* BL21 (λDE3) pLysS transformed with pET11d coding for the scFv-PE40 immunotoxin is inoculated in 10 ml of LB medium, 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 0.5% glucose, 0.05% $MgSO_4$ and grown o.n. at 37 °C with shaking at 250 rpm. The o.n. culture is used to start a 1 l culture in the same medium which is grown at 37 °C with shaking to an OD_{600} between 0.7 and 0.8. Expression of the recombinant protein is induced by addition of 1 mM IPTG into the culture and incubation is continued for 3 hours at 37 °C with shaking. The bulk of the induced culture is centrifuged 15 minutes at 5000 rpm, at 4 °C and the bacterial pellet is stored at -20°C.

Isolation, denaturation and refolding of proteins from inclusion bodies

Pellet of harvested induced culture is resuspended in 0.05 culture volume of IB Wash Buffer (20 mM Tris HCl pH 7.5, 10 mM EDTA, 1% Triton X-100) additioned with Protease Inhibitor (Roche), 0.1mg/ml Lysozyme (Sigma), DNase I (Roche) and 0.1 mM PMSF (Sigma). The suspension is incubated on ice for 30 minutes and then sonicated on ice until cells are lysed and the solution is no longer viscous. After centrifugation at 10000 x g, 10 minutes at 4°C the supernatant is collected (cytoplasm) and the pellet, that is inclusion bodies, are washed twice with IB Wash Buffer and then solubilised at 5 mg/ml in 50 mM CAPS pH 11, N-Lauroylsarcosine 1.2% and 1 mM DTT for 15 min at

RT. After centrifugation at 10000 x g, 10 min at RT the supernatant is collected and the refolding of denatured proteins is attained by multi-step dialysis.
The dialysis steps are performed at 4 °C according to the following scheme:

- Refolding Buffer 1 with 0.1 mM DTT	(20 h)
- Refolding Buffer 1 with 0.1 mM DTT	(8 h)
- Refolding Buffer 1 with no DTT	(20 h)
- Refolding Buffer 1 with no DTT	(8 h)
- Refolding Buffer 2	(24 h)
- Sodium phosphate buffer 5 mM pH 6.5	(24 h)

Refolding Buffer 1:

TrisCl pH 8.5	20 mM
PMSF	0.1 mM

Refolding Buffer 2:

TrisCl pH 8.5	20 mM
L-Arginine	400 mM
Oxidized glutathione	0.5 mM
Reduced glutathione	5 mM
PMSF	0.1 mM

Purification of scFv-PE40 immunotoxin

Purification was achieved by a first step of gel filtration on a TSK3000 SW with 20 mM Tris, 3.4 mM EDTA, pH 7.4. Protein identification was checked by Western Blot. Fractions containing scFv-PE40 were collected and then subjected to a second purification step by anion-exchange chromatography (QAE column) with a buffer containg 20 mM Tris, 3.4 mM EDTA, pH 7.4 and linear gradient of NaCl from 0 mM to 500 mM.

Western Blot

Proteins separated by SDS-PAGE are blotted on nitrocellulose membranes (*Trans-Blot transfer Medium*, BioRad) following the manufacturer's indications. The polyacrilamide gel and nitrocellulose membrane are assembled as a sandwich in a *Mini Trans-Blot Electrophoretic Transfer Cell* (BioRad) according to the manufacturer's instructions, and a tension of 100 V is applied for 1 hour.

Subsequently nitrocellulose membrane is incubated o.n. at 4 °C in blocking solution (3% w/v powder milk in deionized H_2O) under stirring. After blocking, the membrane is first incubated with a primary antibody recognizing a specific epitope of the protein analysed. This is followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody that interacts with the Fc portion of the primary. Both antibodies are diluted in blocking solution and the membrane is rinsed twice for 5 minutes in 0.05 % Tween-20, PBS and twice for 5 minutes in PBS after each incubation. Bands corresponding to the immunoselected polypeptides are finally detected by a chemiluminescent reaction using the *ECL Western Blotting Substrate* (Pierce), according to the manufacturer's instructions, and visualized by development of a photographic plate (*Hyperfilm MP High performance autoradiography film* - Amersham Biosciences).

Production of anti PSCA monoclonal antibodies

Monoclonal antibodies against PSCA were obtained by immunization of Balb/c mice with recombinant PSCA protein purified from bacteria. Polyclonal hybridomas were created following Khöler-Milstein protocol and PSCA-reactive polyclonal hybridomas were identified by ELISA and flow cytometry. Positive polyclonal hybridomas were adapted to growth in complete RPMI cell medium and then cloned by limiting dilution protocol. Monoclonal hybridomas were re-analyzed for the ability to recognize PSCA protein. mAbs were purified from hybridoma supernatants using the affinity chromatography on sepharose-protein G column.

Generation of anti PSCA mAb-RTA chemical immunotoxin

Anti PSCA mAb was derivatized with the heterobifunctional cross-linker SPDP which reacts with mAb amino groups introducing a dithiopyridylgroup. After derivatization the mAb contained 1.5 sulphidryl group/mAb molecule, on average. Anti PSCA mAb-SPDP molecule was conjugated via a reducible disulphide bond to the RTA toxin. The obtained anti PSCA mAb-RTA IT was separated from unconjugated RTA by gel filtration chromatography on TSK 3000 SW column with 5 mM sodium phosphate buffer, 50 mM NaCl pH 7.4. A further purification from unconjugated mAb was achieved by an affinity chromatography passage on Affi Blue gel column with 5 mM sodium phosphate buffer, 50 mM NaCl pH 7.4 and the elutions were performed with the same buffer containing 1 M NaCl. Fractions containing the IT, as identified by SDS-PAGE and Western Blot analyses, were concentrated with Centricon-10 and dialysed in PBS buffer. The IT concentration was determined evaluating absorbance at 280 nm and confirmed with BCA method.

Cells fixation by paraformaldehyde

Cells grown on a 96 wells plate were fixed with 2% paraformaldehyde, 2% sucrose in PBS for 5 min at RT. After five washings with 0.001% phenol red in PBS buffer the neutralization was performed by incubation with 0.1 M glycine in PBS phenol red buffer for 10 min at RT. After five washings with PBS phenol red fixed cells were stored with 1% BSA, 0.02% NaN3, 0.001% phenol red in PBS at 4°C.

ELISA

The ELISA assay was performed on fixed cells (see above) or on recombinant PSCA. The PSCA recombinant antigen was diluted at 2 µg/ml in PBS buffer and 50 µl/well were used to sensitize a 96-well plate (Maxisorp, Nunc). After incubation o.n. at 4°C, one wash was made with PBS and wells were quenched by addition of 200 µl/well blocking solution (3% w/v BSA in PBS) and incubation for 1 hour at 37°C. After a wash with PBS, the primary antibody was diluted at 5 µg/ml in blocking solution and 100 µl/well were dispensed. After 2 hours incubation at 4°C wells were rinsed twice with 0.05% Tween-20 in PBS and twice with PBS.

Secondary antibody-HRP diluted in blocking solution following manufacturer's instructions was dispensed at 100 µl/well and incubated for 1 hour at 4°C. After two washings with 0.05% Tween-20 in PBS and two washings with PBS the assay was developed by addition of 100 µl/well TMB substrate (Sigma) and the reaction stopped with 100 µl/well 1M HCl. The plate was analyzed using the *VERSAmax microplate reader* with a 450 nm beam and subtracting the background absorbance at 650 nm.

Surface Plasmon Resonance (SPR)

In recent years, surface plasmon resonance established itself as a powerful tool for the analysis of antigen-antibody interaction by the direct determination of the kinetic constants of association (K_{on}) and dissociation (K_{off}). SPR provides a real-time measure of the affinity through the monitoring of variations in the refraction index determined by the formation of molecular complexes on the surface of a solid substrate ("chip"), on which one of the two partners involved in the interaction (e.g. the PSCA antigen) is immobilized. A solution containing the second molecular partner (e.g. the anti-PSCA mAb or the derived scFv or Fab) is flowed over the chip by a microinjection system. At this stage (part 3 of curve in Fig. 1) the instrument (Biacore, GeHealthcare) detects an increase in the number of molecular complexes, signalled by a hike in RU (resonance units). From this portion of the sensorgram the K_{on} constant can be desumed. Next, a wash buffer is flowed through the instrument, thus initiating the dissociation stage (part 4 in Fig. 1), on which the K_{off} constant can be calculated. It is then possible to obtain the overall dissociation constant of the antigen-antibody interaction: $K_D = K_{off} / K_{on}$.

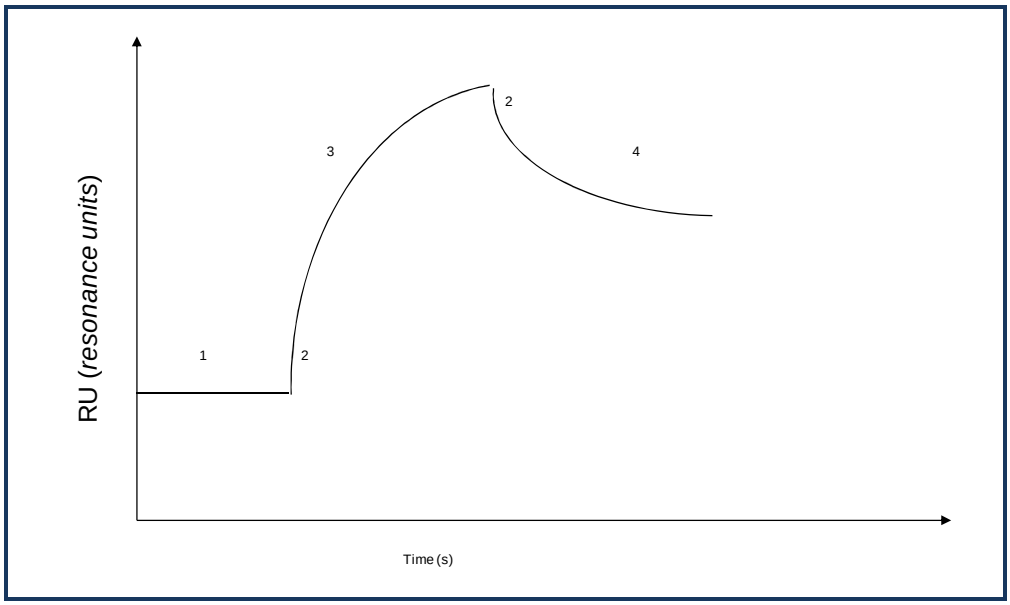

Figure 1. Sensorgram of an "antigen-antibody" interaction. 1 – Baseline (buffer flowed on the chip); 2 – Change in the refraction index; 3 – Association stage (K_{on}); 4 – Dissociation stage (K_{off}).

Flow cytometry

Cells from monolayer cultures were detached with Trypsin-EDTA 0.02%, harvested, washed two times with PBS-BSA 0.2% and re-suspended in cold PBS-BSA 0.2% containing different concentration of the mAbs under study or the control ones.

After 1 hr incubation on ice, cells were washed two times with ice-cold PBS-BSA 0.2% and incubated with saturating concentrations of a goat $F(ab')_2$ anti-mouse immunoglobulin (goat anti-mouse, GAM) fluorescein isothyocianate (FITC-labeled). Cell associated florescence was analyzed by a flow cytometer (FacsCanto, BD Biosciences). Because a myc tag is present at the C-terminal of the amino acid sequence of the scFv fragments, the cytometric analysis was also performed using mouse anti-myc tag antibody. The percent of positive cells and the mean fluorescence intensity (MFI) values were considered in evaluating antigen level of expression.

Competitive binding studies, comparing the binding properties of anti PSCA mAbs, were performed by analyzing the ability of anti PSCA mAbs to prevent binding of biotynilated clone 1 to SW780 cells; the binding of biotinylated clone 1 was detected by

a second labeling step carried out with FITC Avidin. Anti PSCA clone 1 was biotinylated with NHS-Biotin following the manifacturer's instructions.

Cytotoxicity assessment by thymidine incorporation

The effect on uptake of tritiated thymidine (^{3}H-TdR) by cells is taken as a measure of the growth inhibition caused by treatment with a toxin or immunotoxin. Cells are resuspended in standard medium (RPMI 1640, 10% FBS, 2 mM Gln) and 1.8 x 10^4 cells in a volume of 90 µl are seeded in each well of 96-well plates (Greiner Bio-one). The molecules to be tested are dialysed in PBS, filter-sterilized through Spin-X tubes (Costar) and diluted in sterile PBS containing 0.2% BSA. Ten µl of differently diluted antibody and (immuno) toxins are finally added in each well. The plate is incubated for 36 hours at 37 °C, 5% CO_2. Ten µl of ^{3}H-TdR from a 1-1.2x10^5 cpm/ml stock in plain RPMI are added 8 hours before the end time, after which the plate is kept at -20 °C and thawed at RT the day after. Using a cell-harvester (Wesbart), the content of each well is transferred to filter paper and radioactivity measured using a beta counter (Wallac 1409, Pharmacia).

Immunofluorescence

Permeabilized cells

Cells are grown on coverslips and after two washings with PBS are fixed with 4% paraformaldehyde for 1 hour at RT. Cells are washed twice with PBS and then incubated twice with 50 mM NH_4Cl for 10 min at RT to reduce the autofluorescence of cells. Permeabilization is then performed by incubation with Blocking Buffer (1% BSA, 0.5% saporin in PBS) for 30 min at RT. Cells are incubated at RT for 45 min with anti PSCA antibody diluted in Blocking Buffer. After 3 washings with Blocking Buffer, the anti mouse-Alexa 488 diluted in Blocking Buffer is added for 45 min at RT. The coverslips are washed twice in Blocking Buffer and twice in PBS and then mounted on objective glass with moviol. Samples are kept in the dark and observed by confocal microscopy.

Surface staining

Cells are grown on coverslips and after two washings with cold PBS are incubated at 4°C for 1 hour with anti PSCA antibody diluted in PBS. After 3 washings with cold PBS, the anti mouse-Alexa 488 diluted in PBS is added for 1 hour at 4°C. The coverslips are washed four times with cold PBS and then incubated at 37°C for 30 min to induce antigen internalization. Cells are then fixed with 4% paraformaldehyde for 1 hour at 4°C and after 4 washings with PBS the coverslips are mounted on objective glass with moviol. Samples are kept in the dark and observed by confocal microscopy.

RESULTS

Production of recombinant PSCA

To create new anti PSCA mAbs the project began with the generation of recombinant human PSCA (hPSCA) which was then used to immunize Balb/c mice.
Total RNA was extracted from DU145 cells with Trizol buffer and specific cDNA was retrotranscribed. PSCA specific cDNA was then cloned using Hind III restriction sites in the pET30a prokaryotic expression vector; this plasmid enabled the production of a tagged recombinant protein exploiting a 6-His Tag on C-terminal and N-terminal tails. Figure 1 shows the sequence of PSCA inserted in pET30a vector.

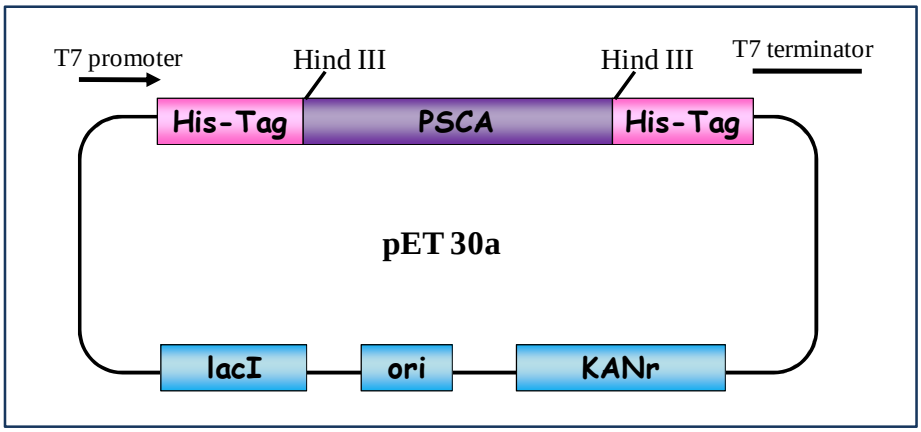

Figure 1. Schematic picture of the PSCA cDNA cloned in pET30a vector using Hind III restriction site.

The construct was expressed in the BL21 (λDE3) bacterial strain. Addition of IPTG to the culture medium triggers the overexpression of T7 RNA polymerase, which in turn superinduces the transcription of the PSCA gene being placed under the control of the T7 promoter in pET30a. After induction with IPTG inclusion bodies (IB) were isolated and then solubilized in urea containing buffer, as described in Materials and Methods. For protein purification solubilized IB were added to NiNTA resin. Protein purification and identification were checked by SDS-PAGE (Fig. 2). Fractions containing PSCA were collected and the protein subsequently renatured by multi-step dialysis in a "Refolding buffer" which allows the denaturing agent (urea) to be gradually removed. Because some contaminats were still present, a further ionic exchange purification using *Vivapure Mini Spin column* was necessary. The protein was eluted with a Tris buffer

containing 500 mM NaCl. An SDS-PAGE analysis shows a PSCA purity of about 90-95%; its identity was demonstrated by Western Blot (Fig. 3).

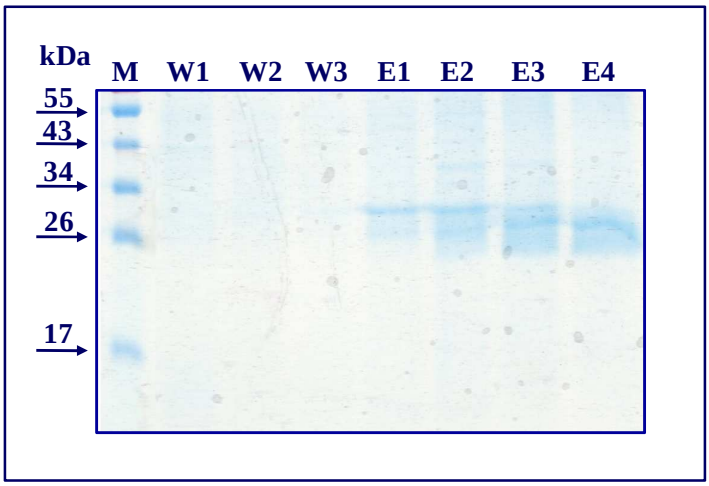

Figure 2. SDS-PAGE analysis of PSCA protein purified on NiNTA column. The PSCA protein shows an apparent molecular weight of 25-30 kDa and the smear is likely due to the presence of urea in the sample. Abbreviations: M, protein size standard; W1, wash with buffer at pH 8.0; W2, wash with buffer at pH 6.3; W3, wash with buffer at pH 5.9; E1, E2, E3 and E4, elution steps performed with buffer at pH 4.5.

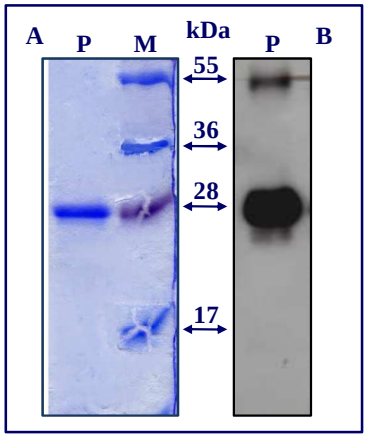

Figure 3. (A) SDS-PAGE and (B) Western Blot of purified PSCA. Coomassie staining shows the purity of PSCA; Western Blot performed with anti PSCA antibody (Abnova), recognizing the only recombinant PSCA, confirm the identity of the protein and shows the presence of a small amount of PSCA in dimeric form. Abbreviations: M, protein size standard; P, purified PSCA.

Generation and characterization of new anti PSCA mAbs

Balb/c mice were immunized with recombinant PSCA and their spleen used for hybridoma generation according to Khöler-Milstein protocol. After a step selection by ELISA with recombinant PSCA, positive hybridomas were cloned to obtain hybridoma cells secreting anti PSCA monoclonal antibodies (mAbs). Ten positive clones were obtained and mAbs secreted form hybridomas clone 1 and clone 2 were characterized.

The binding properties of our anti-PSCA mAbs clone 1 and clone 2 to the native cellular antigen were characterized by flow cytometry with SW780, PSCA positive, and PC-3 wild type (WT), PSCA negative, cell lines. As shown in Figure 4 and Table 1, both our mAbs clone 1 and clone 2 are able to recognize the native form of PSCA and they show a MFI (Mean Fluorescence Intensity) of 554 and 425 respectively on SW780 ($PSCA^+$). These values are similar to the positive control (a validated anti-PSCA antibody clone 7F5 [Morgenroth *et al.*, 2007]), showing a MFI of 587, and greater than the negative control (incubation with isotype control mAb, MFI=79). In the same experiment no staining was obtained on PC-3 ($PSCA^-$) cells demonstrating the specificity of our mAbs.

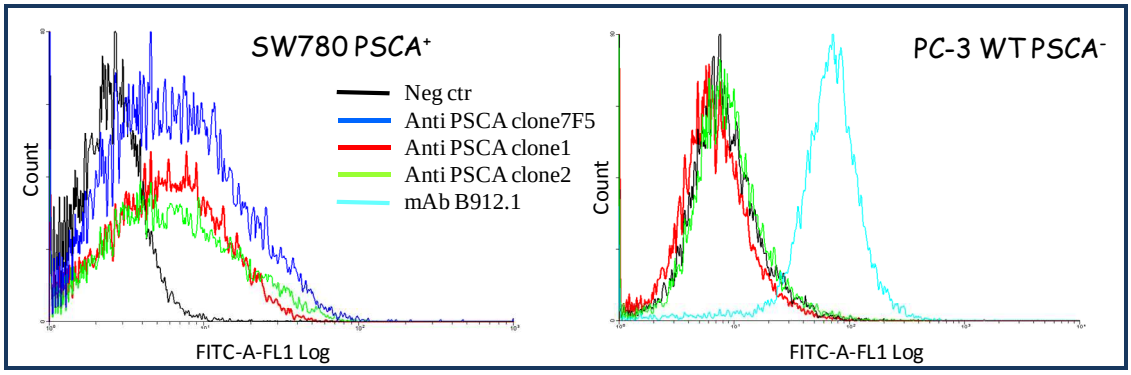

Figure 4. Flow cytometry with SW780 $PSCA^+$ and PC-3 WT $PSCA^-$ cell lines. Negative control is obtained with isotype control mAb; clone 7F5 is a validated anti PSCA mAb described in the literature [Morgenroth *et al.* 2007]; clone1 and clone2 are our anti PSCA mAbs; B912.1 is an anti MHC I and it is used as a positive control in the flow cytometry of PC-3 WT cells. FITC-A, *Fluorescein Isothiocyanate-Area*.

	SW780 MFI	PC-3 WT MFI
Isotype control	79	189
Anti PSCA mAb clone 7F5	587	n.d.
Anti PSCA mAb clone1	**554**	224
Anti PSCA mAb cone2	**425**	244
Positive control	n.d.	1926

Table 1. Binding specificity of anti PSCA mAbs analyzed by flow cytometry. Staining was measured considering two parameters: MFI (Mean Fluorescence Intensity) and the percentage of positive cells.

The IgG-isotype of our anti PSCA mAbs was determined by an ELISA assay; results summarized in Table 2 show that both our mAbs belong to the IgG2b subclass.

	Mouse serum	Neg ctr	mAb clone1	mAb clone2
IgM	2.41	0.23	0.27	0.3
IgG1	1.5	0.3	0.29	0.27
IgG2a	1.5	0.17	0.19	0.18
IgG2b	1.9	0.5	**1.9**	**2.0**
IgG3	1.51	0.22	0.21	0.22
IgA	0.73	0.21	0.19	0.18

Table 2. ELISA assay to determine the anti PSCA mAbs isotype. Mouse serum was used as a positive control and culture medium as a negative control. The values showed represent the adsorbance measured at 450nM.

We further characterized the binding properties of our mAbs in comparison with the reference anti-PSCA antibody clone 7F5 in order to define possible epitope shared during PSCA recognition. A competitive binding assay was set up using biotinylated mAb clone 1 and unmodified anti PSCA mAbs as competitors. Binding of biotinylated anti PSCA clone 1 was revealed by FITC-Avidin staining. As shown in Table 3, anti PSCA clone 1 and clone 2 are able to cross-inhibit the binding of each other, whereas the clone 7F5 is not able to inhibit their cell surface binding.

Therefore anti PSCA mAbs clone1 and clone2 recognize the same epitope or epitopes that are physically or functionally associated whereas clone 7F5 recognizes a different epitope.

Antibody	**MFI**
Isotype control	74
Anti PSCA mAb clone1 biotin	188
Anti PSCA mAb clone1 biotin + clone1	91
Anti PSCA mAb clone1 biotin + clone2	86
Anti PSCA mAb clone1 biotin + clone7F5	141

Table 3. Competition binding experiment performed with SW780 cells and analyzed by flow cytometry.

To better define the binding properties of our anti PSCA mAbs, their affinity was assessed through the SPR technology (Surface Plasmon Resonance). This study was performed in collaboration with Dr.ssa Mariangela Figini, INT Milan. Recombinant PSCA was chemically coupled to the surface of a "sensor chip" which was then analyzed by the instrument (BIACORE). The anti PSCA mAbs were tested at a concentration of 200 nM. As shown in Figure 5 the association/dissociation curves, also called sensorgrams, of the two anti PSCA mAbs are comparable. It can be observed that the binding properties of our mAbs are very similar. Moreover, the curves demonstrate a fast rate of association whereas the rate of dissociation is slow; these characteristics led to consider our mAbs as good tools for immunotherapy.

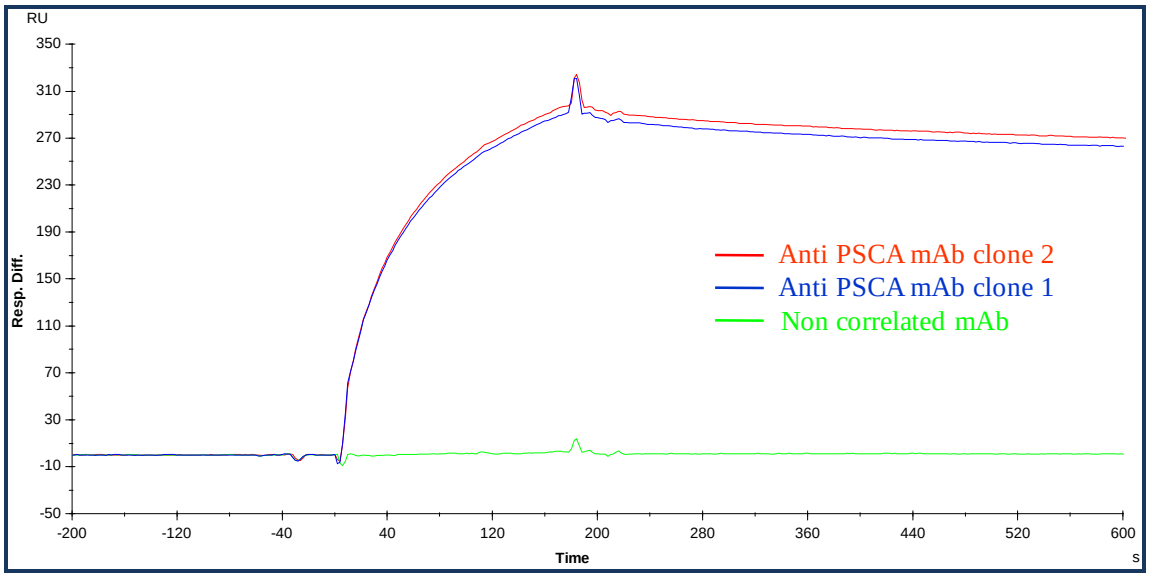

Figure 5. Biacore analysis of the binding properties of anti PSCA mAb clone 1 and clone 2. The sensorgram shows the association/dissociation curves obtained by flowing 200 nM of mAb over the chip on which recombinant PSCA was fixed.

Sequencing of V_H and V_κ regions of the two mAbs clone 1 and clone 2 showed that they differ only in the light chain. Because of that and because their binding characteristics are comparable from now on I will consider only the mAb from clone 1.

The characterization of the selected anti PSCA mAb continued by carrying out a flow cytometric analysis with a panel of positive and negative cell lines. At the present time SW780 cells are the only available cell line constitutively expressing PSCA but they present a variable antigen expression depending on as yet unknown factors.
For this reason PC-3, LNCaP, HEK 293 and MDCK cell lines were stably transfected with hPSCA-pcDNA3.1 and they showed a constant expression of PSCA. In Table 4 are summarized the MFI values obtained. It can be observed that the specificity of anti PSCA mAb is confirmed: the mAb recognizes the antigen PSCA in transfected cells but it does not so in mock transfected ones.

	PSCA positive cell lines (MFI values)					PSCA negative cell lines (MFI values)				
	SW780	PC-3* hPSCA	LNCaP* hPSCA	MDCK* hPSCA	HEK* 293 hPSCA	DU145	PC-3 WT	LNCaP WT	MDCK WT	HEK 293 WT
Isotype control	76	65	141	191	234	173	189	51	295	170
Anti PSCA mAb	**2168**	**571**	**3253**	**1057**	**3126**	166	224	58	248	164
Positive control	n.d.	n.d.	2833	n.d.	n.d.	1354	1926	2076	n.d.	760

Table 4. Binding specificity of anti PSCA mAb analyzed by flow cytometry with PSCA positive and negative cell lines. Staining was measured considering two parameters: MFI (Mean Fluorescence Intensity) and the percentage of positive cells. * = transfected cell lines.

To better define the binding properties of anti PSCA mAb, its affinity was then assessed by flow cytometry. A fixed amount of LNCaP hPSCA or PC-3 hPSCA cells were stained with increasing concentrations of anti PSCA mAb followed by incubation with a saturating concentration of secondary mAb, commonly a FITC-labeled goat anti mouse antibody. Figure 6 describes the results of the cytofluorimetric analysis; anti PSCA mAb reaches saturation of 50% of PSCA sites at the concentration of 23 nM and 16 nM respectively on LNCaP hPSCA and PC-3 hPSCA.

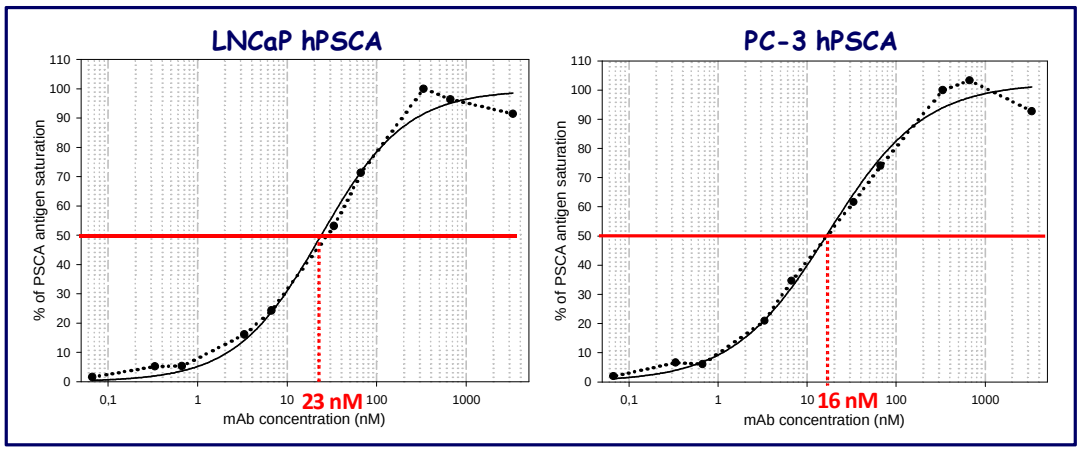

Figure 6. Binding of anti PSCA mAb on LNCaP hPSCA and PC-3 hPSCA. Cells were incubated with increasing concentrations of anti PSCA mAb and the signal was detected with FITC-labeled goat anti mouse IgG.

The WT cell line LNCaP is able to express the antigen PSMA, another tumor associated antigen. The PSMA expression is maintained in PSCA transfected LNCaP cells. In our laboratory a good anti PSMA mAb is available: it reaches saturation of 50% of PSMA sites at concentration of 8 nM, value consistent with the range of 10^{-7}-10^{-11} M for antibodies of biological interest. For this reason we compared the binding of our mAb to PSCA with the binding of anti PSMA mAb to PSMA. As shown in Fig. 7 the concentration of anti PSCA mAb necessary to saturate 50% of antigenic sites is only 3 fold higher than the anti PSMA mAb concentration. These data confirm the good binding properties of our anti PSCA mAb.

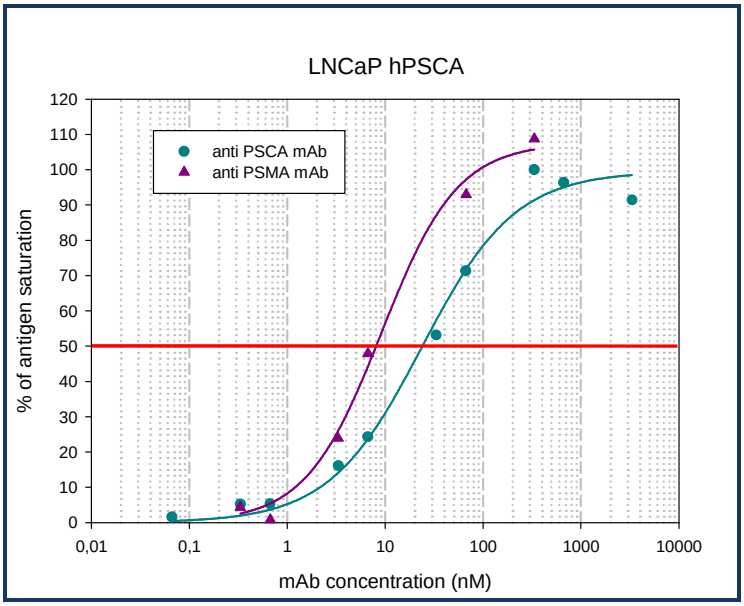

Figure 7. Binding of anti PSCA and anti PSMA mAbs to LNCaP hPSCA. Cells were incubated with increasing concentrations of anti PSCA (blue line) or anti PSMA (violet line) antibodies. Shown are the fitting curves obtained.

Anti PSCA mAb relevance for diagnosis

It is known that differences in the glycosylation pattern of antigens exist between healthy and malignant tissues [Pauli *et al.*, 2003; Jankovic and Milutinovic, 2008; Robbe-Messelot *et al.*, 2009]. To verify that our antibody is able to recognize possible different isoforms of PSCA a Western Blot assay was performed with cell lysate of

MDCK expressing PSCA and it was developed using our anti PSCA mAb as primary antibody. As shown in Figure 8 our mAb is able to recognize PSCA bounded to any sugars (lane F, obtained by treating the lysate with N-Glycosidase F), to hybrid sugars or complex glycans. In fact in the non treated total lysate (lane /) our mAb recognizes many bands with a molecular weight of about 25-40 kDa. When the total lysate is treated with endoglycosidase H (lane H) the mAb recognizes a band of about 40 kDa, corresponding to PSCA bounded to complex glycans, and about 22 kDa, deriving by the cleavage of mannose rich glycans from PSCA bounded to hybrid sugars. When the total lysate is treated with N-Glycosidase F the mAb recognizes a band of about 15 kDa, corresponding to PSCA bounded to any sugar.

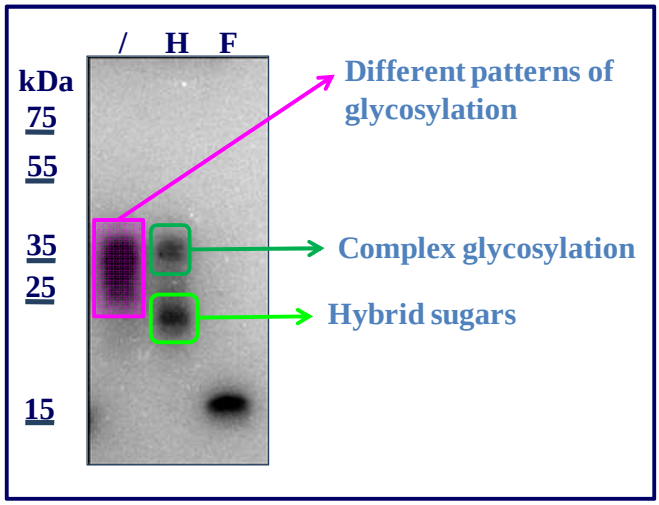

Figure 8. Western Blot of MDCK hPSCA cell lysates. The cell lysate is loaded without treatment (/), after treatment with endoglycosidase H (**H**), after treatment with N-Glycosidase F (**F**). Endoglycosidase H cleaves mannose rich glycans, N-Glycosidase F cleaves complex glycans. The Western Blot was performed using our anti PSCA antibody as primary antibody and anti mouse-HRP as secondary antibody.

Moreover to verify the ability of our antibody to recognize the antigen in tumor tissues our mAb was used as primary antibody in Western Blot performed with tissue lysates from patients. As shown in Figure 9 the anti PSCA mAb is able to detect the presence of denatured PSCA in 3 out of 3 prostate neoplastic tissue lysates and in 2 out of 3 pancreatic neoplastic tissue lysates, whereas no signal is detected in normal tissues (stomach, uterus and spleen) used as negative controls. These data demonstrate the

specificity of the signal due to the binding of anti PSCA mAb to the antigen expressed in neoplastic tissues.

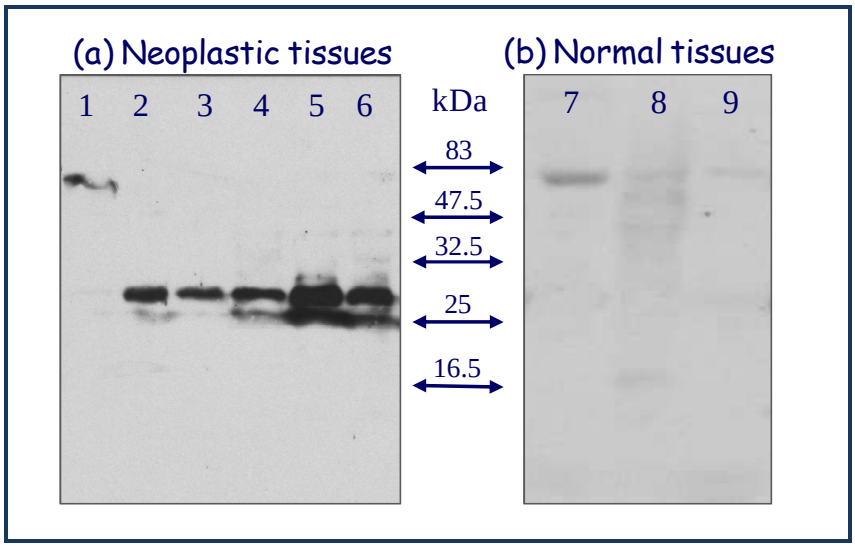

Figure 9. Recognition of PSCA in neoplastic tissues by Western Blot. (**a**) lanes 1, 2, 3: pancreatic tumor tissue lysates; 4, 5, 6 prostate tumor tissue lysates; (**b**) lanes 7, 8, 9: normal tissue lysates: stomach, spleen and uterus respectively. The Western Blot was performed using our anti PSCA mAb as primary antibody and anti mouse-HRP as secondary antibody.

To further characterize the diagnostic potential of our anti PSCA mAb an ELISA assay was performed with cells fixed with 2% paraformaldehyde. Anti PSCA mAb is able to stain SW780 cell surface whereas no signal is detected on DU145 cell surface, a PSCA negative cell line (Fig. 10). The ability to identify PSCA on tumor tissues of patients and on fixed cells leads us to consider our mAbs a possible tool in both diagnostic (for instance immunohistochemistry, IHC) and therapeutic approaches.

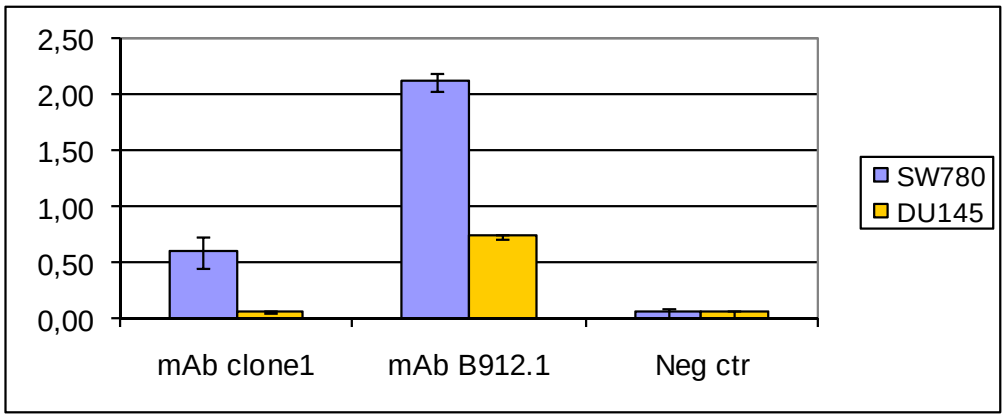

Figure 10. Whole mAb recognizes native PSCA by ELISA with whole fixed SW780 cells. Our anti-PSCA mAb clone 1 recognizes native hPSCA on SW780 cells fixed with 2% paraformaldehyde. The ability of the mAb to recognize fixed cells could allow its use in immunohistochemistry (IHC). The antibody B912.1 was used as positive control because it binds to MHC I.

Anti PSCA mAb relevance for immunotherapy

Gu *et al.* [2005] described the ability of an anti PSCA mAb to induce cell death *in vitro* through a direct, Fc-independent mechanism. Shown in Figure 11 are results from the work by Gu *et al.* [2005]. For this reason we first evaluated if our anti PSCA antibody had cytotoxic effects by itself on a PSCA positive cell line. When PC-3 hPSCA cells are incubated for 36 h with anti PSCA mAb we observed no cytotoxic effect (Fig. 12). Therefore, our anti PSCA mAb is not able to induce cell death through a direct mechanism probably because our mAb binds to a different epitope from the epitope recognized by the anti PSCA Ab described in literature. It is therefore interesting that the apoptosis induction mechanism activated throught PSCA could be dissected using mAb to different epitopes.

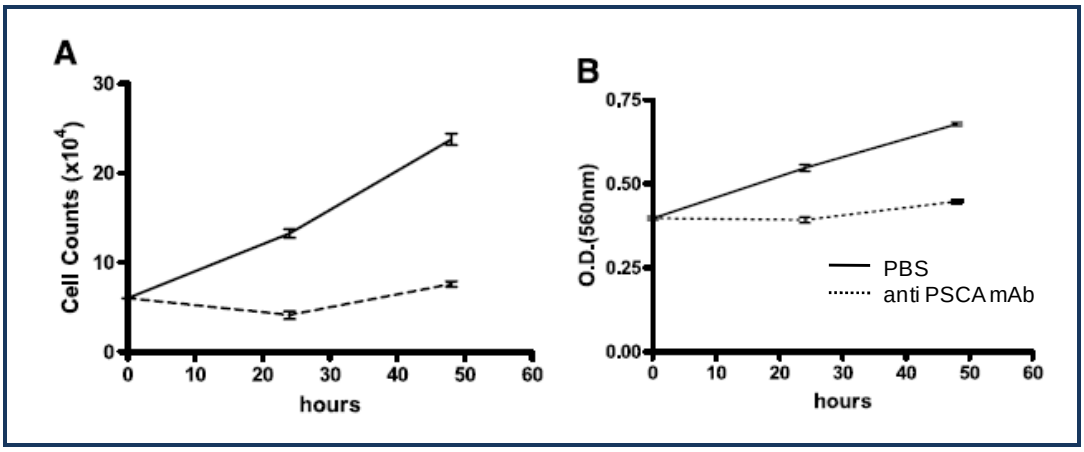

Figure 11. Anti PSCA mAb, described by Gu, leads to cell growth inhibition through a direct effect. LNCaP-PSCA cells were incubated with PBS or anti PSCA mAb for the indicated time. Cell proliferation was measured by (**A**) counting viable cells after Trypan Blue staining or (**B**) via MTT assay. (Modified from Gu *et al.*, 2005).

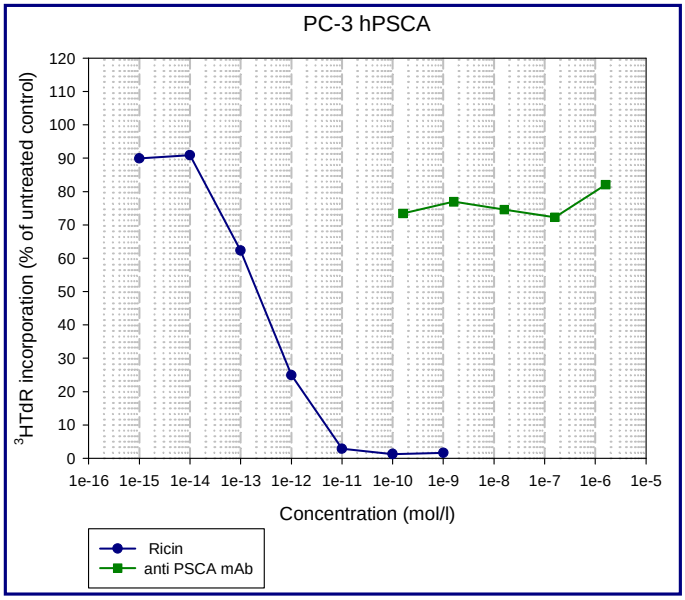

Figure 12. ***In vitro*** **cytotoxicity using the PC-3 hPSCA cell line.** The cytotoxic effect of our anti PSCA mAb is compared with a potent toxin, Ricin. Shown is the percentage of ^{3}HTdR incorporation as a function of concentration.

Because in our case the direct approach is not sufficient to kill tumor cells, my research was focused on the generation of immunotoxins (ITs).

To make sure that anti PSCA mAb represents a suitable targeting molecule for the design of an immunotoxin, the internalization of the antibody following binding to PSCA on target cells was evaluated by flow cytometry. This assay was performed on SW780 cells and we used Trypan Blue which is able to quench about 50-60% the amount of surface fluorescence. All MFI values were obtained by pre-incubating cells at 4°C with a FITC labelled antibody. "MFI sample" (see Table 5), obtained incubating the cells with our anti PSCA mAb, was normalized using a negative control obtained incubating the cells with irrelevant antibody (MFI sample/MFI negative control). In this experiment (Tab. 5) it can be observed that the ratio MFI sample/MFI negative control is 2.2 when cells are analysed immediately, it decreases to 1.07 after Trypan Blue treatment, but it increases again to 2.07 when cells are incubated at 37°C (to induce internalization) and then treated with Trypan Blue. In the last sample Trypan Blue is not able to quench the Ag-Ab complex fluorescence because the complex has translocated inside the cell. To confirm these results the same experiment was performed with LNCaP WT cells ($PSMA^+$) incubated with anti PSMA-FITC Ab because the PSMA-Ab internalization is known [Liu *et al.*, 1998]. Comparing the two experiments we can demonstrate that the anti PSCA mAb is 87% internalized after incubation at 37 °C. For this reason the work continued by generating chemical immunotoxins.

MFI sample/ MFI neg ctrl	**+ 4°C**	**+ 4°C + TB**	**+4°C + 37°C + TB**	**% internalized Ab**
SW780	2.2	1.07	2.07	87%
LNCaP WT	7.3	3.6	5.3	45%

Table 5. Internalization assay. MFI sample/MFI negative control ratio value on SW780 or LNCaP WT cells incubated with mAb at +4°C and analyzed immediately (+4°C), after treatment with Trypan Blue (+4°C + TB), after incubation at +37°C and treatment with Trypan Blue (+4°C + TB). Results obtained with SW780 cells are compared with results obtained with an anti PSMA mAb and LNCaP WT cells ($PSMA^+$), known to be able to internalize PSMA-Ab complexes.

Construction of chemical immunotoxin

The anti PSCA mAb (whole molecule) was chemically linked to ricin A chain toxin (RTA) to create an anti PSCA mAb-RTA immunotoxin (IT).

Chemically linked immunotoxin was synthesized by conjugating RTA toxin to the anti PSCA mAb via a disulfide bond, using the bifunctional cross-linker SPDP. After reduction of cysteines on RTA protein by DTT and derivatization of the mAb with SPDP, the cross-linking reaction of these two reagents took place for about 2 days at 4°C; the immunotoxin obtained was then separated from unconjugated reagents by a first step of gel filtration chromatography. The chromatogram of Figure 13 displays a purification run obtained using a Bio-Rad FPLC apparatus. A second step of chromatographic purification on an Affi-Blue gel column allowed the removal of unconjugated mAbs from the selected fractions. It is mandatory to eliminate these free mAbs, because they could be able to compete with immunotoxin for the binding to the target Ag; this competition could mask the real killing efficacy of the immunotoxin in the cells assay.

Coomassie-staining of a SDS-PAGE and a Western Blot (Fig. 14) confirmed effective binding of RTA to the mAb and the absence of free mAb and RTA in the purified immunotoxin.

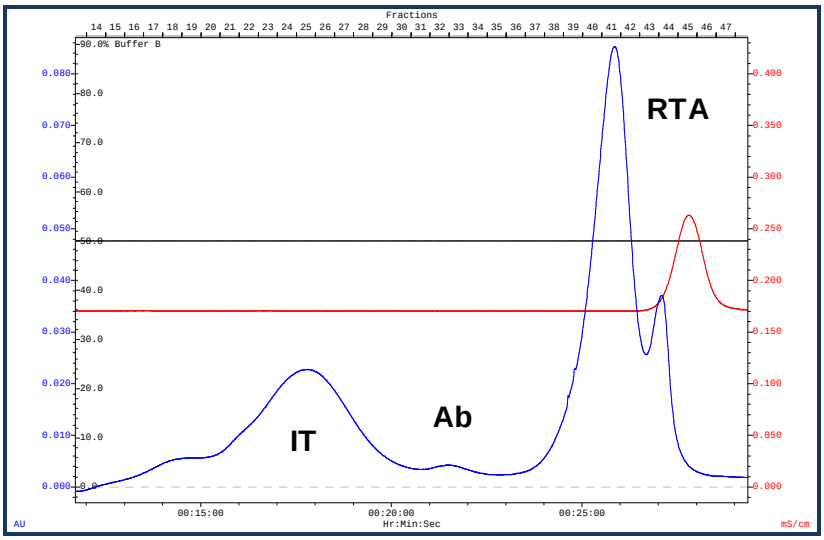

Figure 13. Chromatogram of a purification run of the immunotoxin on a gel filtration column. Abbreviations: Ab, anti PSCA mAb; IT, anti PSCA-RTA immunotoxin

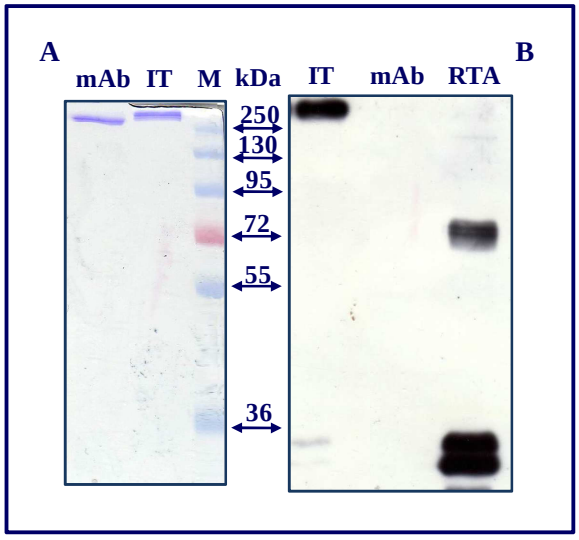

Figure 14. (A) SDS-PAGE and (B) Western Blot performed using anti RTA antibody. Abbreviations: mAb, anti PSCA mAb; IT, anti PSCA-RTA immunotoxin; M, protein size standard.

Cytotoxicity of the immunotoxin

The cytotoxic potential of the chemical immunotoxin was evaluated by checking the effect of increasing concentrations of the immunotoxin on the incorporation of tritiated thymidine (^{3}H-TdR) by target and non-target cells. For comparison, also the anti PSCA

mAb and RTA alone were tested. Cytotoxic effects were compared in terms of IC_{50}, which defines the reagent concentration able to inhibit cell proliferation of about 50% with respect to an untreated control. The plots in Figure 15 clearly show that the immunotoxin can inhibit the proliferation of cells expressing the PSCA antigen, while the mAb has no detectable toxic activity within the range of the concentrations used. The immunotoxin has a more potent cytotoxic action as compared with RTA toxin alone: the IC_{50} value of the anti PSCA mAb-RTA is 100 times lower than the IC_{50} of the RTA toxin (Tab. 6). This difference is not detectable in a PSCA negative cell line: the IC_{50} value is $1x10^{-7}$ (mol/l) and $5x10^{-7}$ (mol/l) respectively, for the anti PSCA mAb-RTA and RTA. Comparing the IC_{50} value of our immunotoxin after targeting PC-3 hPSCA cells with another irrelevant anti CD5 immunotoxin, it can be observed that the cytotoxic effect of anti PSCA mAb-RTA is 100 fold higher than that of anti CD5 mAb-RTA. These results signify that the greater toxicity of our immunotoxin is due to the specific binding of anti PSCA mAb to the PSCA antigen.

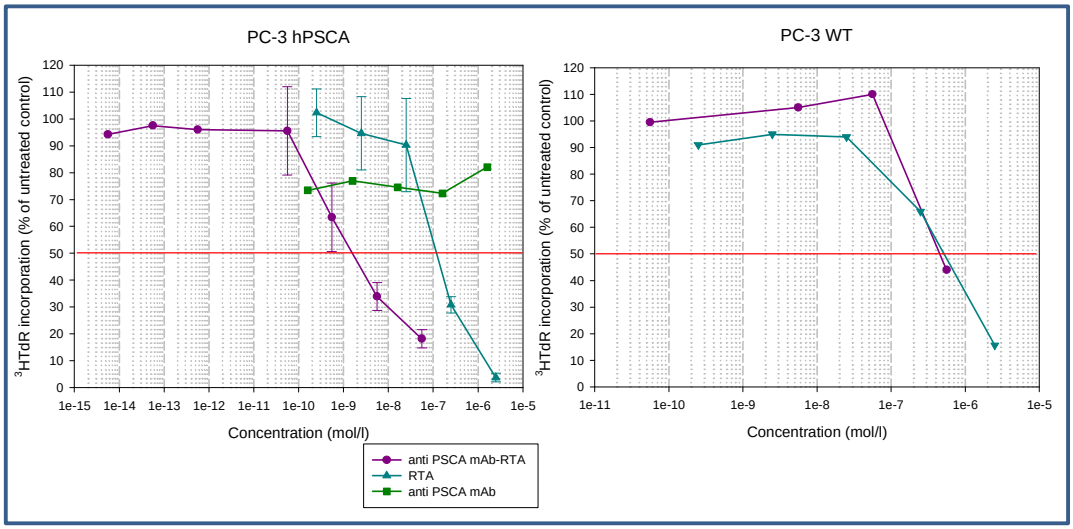

Figure 15. Dose-response curves obtained by treating PC-3 hPSCA and PC-3 WT cell lines with increasing concentrations of the molecules described in the picture. Shown is the percentage of ^{3}HTdr incorporation as a function of concentration.

Monensin	**-**	**+**	**-**	**+**
Toxin/Immunotoxin	**PC-3 hPSCA IC_{50} (mol/l)**		**PC-3 WT IC_{50} (mol/l)**	
anti PSCA mAb-nRTA	**$1{,}3x10^{-9}$**	**$1{,}2x10^{-10}$**	$1x10^{-7}$	$1{,}5x10^{-7}$
nRTA	$1{,}2x10^{-7}$	$2x10^{-8}$	$5x10^{-7}$	$1{,}3x10^{-8}$
Ricin	$2x10^{-13}$	$4x10^{-14}$		
mAb OKT9-nRTA	$2x10^{-11}$	$3x10^{-12}$		
anti CD5 mAb-nRTA	$1{,}2x10^{-7}$	$1{,}3x10^{-8}$		
anti PSCA mAb	$>10^{-6}$	$>10^{-6}$		

Table 6. Values of concentrations inhibiting 50% of the maximal proliferation (IC_{50}) for different molecules on positive cell line (PC-3 hPSCA) and negative cell line (PC-3 WT). Monensin is added (+) or not (-) to cells during the experiment at the concentration of 50 nM.

The carboxylic ionophore monensin is known to increase the cytotoxic activity of immunoconjugates [Candiani *et al*., 1991]. The potentiating effect is mediated by the enhancement of lysosomal pH and the inhibition of IT degradation [Ippoliti *et al*., 1998]. For this reason we assessed the cytotoxic effects of toxins also in the presence of 50 mM monensin. As shown in Table 6, monensin is able to enhance the cytotoxic effect by 10 times with all tested toxins and immunotoxins. Also in this case the cytotoxic effect of anti PSCA mAb-RTA is 100 fold higher than that of RTA.

Construction of an anti PSCA scFv antibody

Because our home-made anti PSCA antibody shows a good affinity and specificity we decided to generate anti PSCA scFv by cloning the variable domains of heavy (V_H) and light (V_L) chains.

The cDNA obtained from cells of our hybridoma clone was used as a template for the PCR screening of an array of primers designed to amplify DNA fragments coding for the V_H and V_L. Since mouse Ig use preferentially κ light chains (1:10 ratio λ/κ), we employed degenerate primers for V_κ and J_κ domains only. Primers for this first

amplification were designed to match the leader regions of the V_H and V_κ chains and both constant regions of heavy and light chains. Twenty-five PCR reactions were carried out for each V chain combining 25 different V_H or V_κ murine domain forward primers with a mix of four J_H or J_κ corresponding reverse primers, as showed in Figure 16. The products of V_H or V_κ amplifications can be visualized on a 2% agarose gel and are approximatly ~350-390 bp long; in the picture it can be observed that due to the degeneration of primer sequences we obtain amplification products from several V primers. Among them, according to band intensity and low amplification background, we have chosen one V_H and one V_κ forward primers.

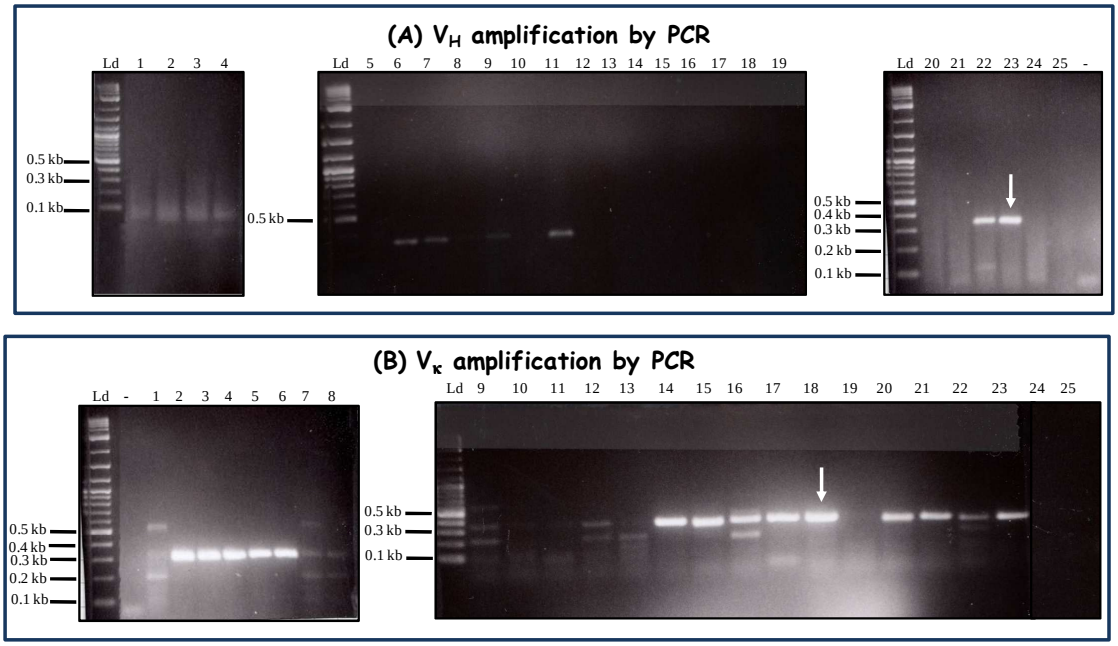

Figure 16. Screening of forward primers for the amplification of DNA sequences coding for the variable domains of the anti PSCA mAb. PCR amplificates were analyzed by agarose gel electrophoresis. V_H (A) and V_κ (B) amplifications were tested with different forward primers (lanes 1-25). The negative control (-) was a PCR reaction with no template. Arrows indicate amplificates from primers selected for further characterization. Ld = DNA Ladder.

At this point it was important to test the "joining" (J) primers separately in order to identify J_H and J_κ ones leading to the best matching pair for heavy and light chain amplification. Figure 17 shows that two J_H primers and two J_κ primers, associated with

the variable respective V_H and V_κ, are able to amplify the V domains. Also in this case we have chosen the reverse primer corresponding to the single and most abundant amplificate.

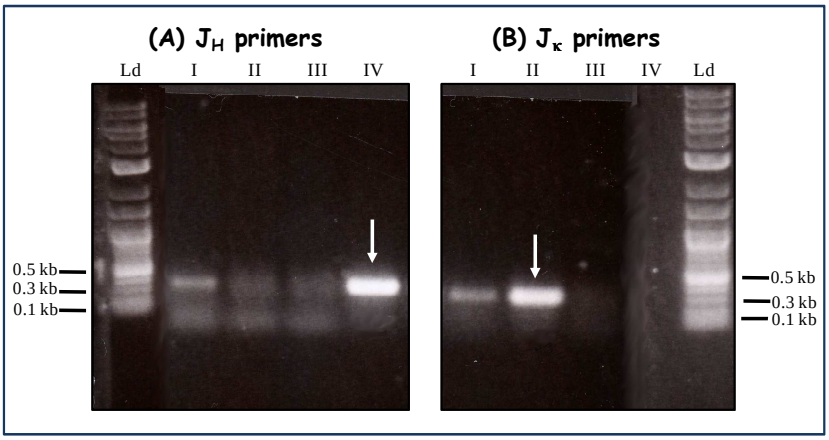

Figure 17. Screening of reverse primers for the amplification of DNA sequences coding for the variable domains of the anti PSCA mAb. PCR products obtained amplifying V_H region using the best forward primer with each of four reverse J_H primers (**A**) and V_κ region using the best forward primer with each of four reverse J_κ primers (**B**). Arrows indicate amplificates from primers selected. Ld = DNA Ladder.

V genes obtained by PCR were sequenced using the same forward and reverse primers as for PCR reactions; to confirm that these sequences matched V domain of mouse Ig protein we compared by computer analysis our V amplified DNA using Blast NCI Software.

A second amplification was carried out to insert desired restriction sites (Sfi I/Xho I and Apa LI/Not I for V_H and V_κ respectively) to facilitate directional cloning of the V_H and V_κ domains in pHEN-2 plasmid. Figure 18 represents a schematic picture of the synthesized plasmid pHEN2-anti PSCA scFv.

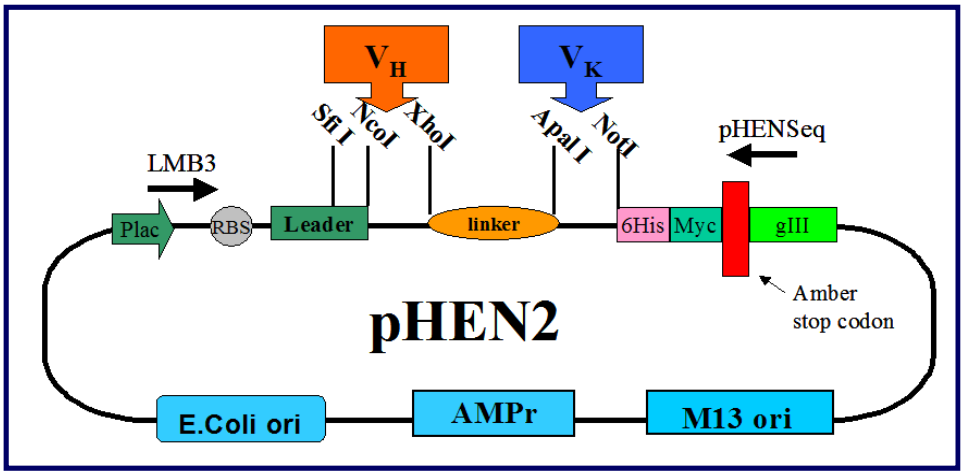

Figure 18. Schematic picture of the plasmid pHEN2-anti PSCA scFv.

Production and purification of anti PSCA scFv antibody

The scFv protein was produced in HB2151 *E. Coli* bacterial strain using the pHEN-2 vector which contains a pelB leader sequence up-stream and c-myc and His-tag sequences down-stream to the scFv insert; this allows an easier detection and purification of the protein.

The level of scFv synthesis in HB2151 was first assessed in small-scale cultures. The intracellular localization and solubility of the single-chain antibody expressed in HB2151 *E. coli* was investigated with the intention of maximizing the share of induced protein being stored in the periplasmic compartment, which normally allows the recovery of more appropriately folded and soluble polypeptides. Different conditions for protein induction were tested (Table 7). In each case the cell extracts, corresponding to the soluble cytoplasmic and periplasmic fractions and to the insoluble protein fraction, were prepared and analyzed by Western Blot using an anti His Tag antibody (Fig. 19). The band of size about 30 kDa represented scFv protein. Finally we decided to grow bacteria in 2X YT medium added with glucose 0.1% and ampicillin 100µg/ml and induced with 1 mM IPTG for 20h at 26°C.

Induction Temperature	Induction duration
37°C	3 h
	20 h
30°C	3 h
	20 h
26°C	3 h
	20 h

Table 7. Table summarizing the induction conditions tested with the anti PSCA scFv.

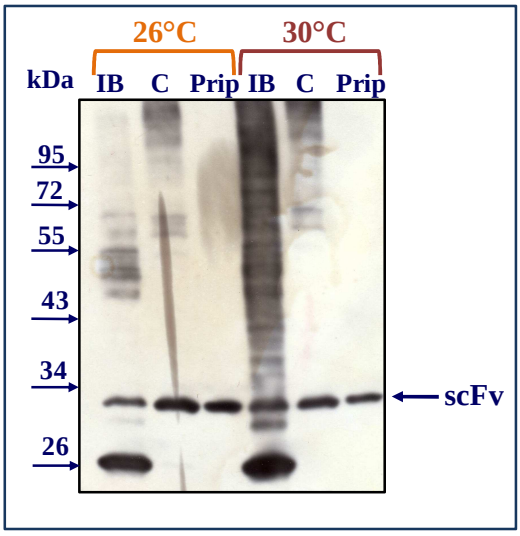

Figure 19. Western Blot of bacterial fractions. Each fraction was obtained after induction with 1 mM IPTG for 20 h at 26°C or 30°C. The band at about 30 kDa represents our scFv protein. The Western Blot was performed with anti His Tag antibody. Abbreviations: IB, inclusion bodies; C, cytoplasmic soluble fraction; Perip, periplasmic soluble fraction.

To purify the scFv I have tried different methodologies (ion exchange and affinity chromatography, gel filtration) using cytoplasmic, periplasmic or insoluble protein fractions (data not shown). The method supplying the highest yield and level of purification was the purification from the periplasmic fraction by affinity chromatography. The binding of scFv to NiNTA resin, which is able to bind 6-His tagged proteins, occurred in weak denaturing conditions (see Materials and Methods).

The periplasmic fraction was added to NiNTA resin and then incubated o.n. at 4°C on an orbital shaker. After sequential washings with Tris 20mM pH=8.0 additioned with increasing concentrations of imidazole (from 0 to 20 mM), the scFv protein was eluted from the resin with a Tris buffer containing 250 mM imidazole. Protein purification and identification were checked by SDS-PAGE and by Western Blot (Fig. 20). The band of size between 28 and 36 kDa represented scFv protein.

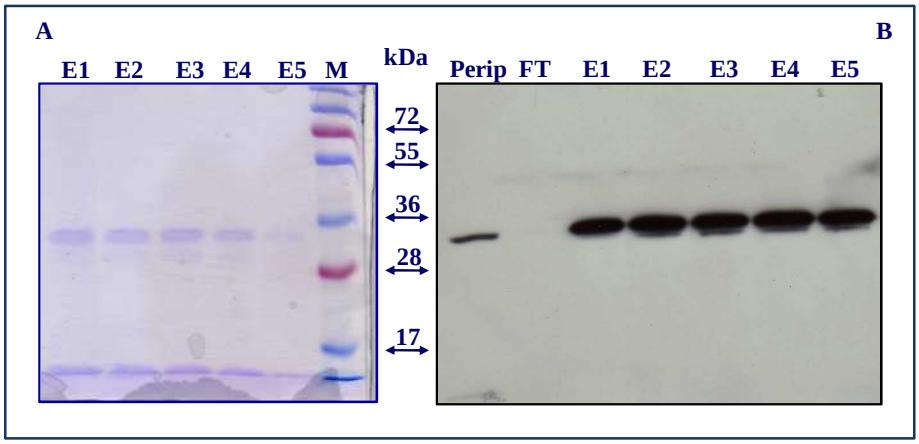

Figure 20. (A) SDS-PAGE analysis and (B) Western Blot of anti PSCA scFv protein purified on NiNTA column. Coomassie staining shows the purity of anti PSCA scFv; Western Blot performed with anti His Tag antibody confirms the identity of the protein. Abbreviations: M, protein size standard; Perip, periplasmic fraction of induced bacterial culture; FT, flow through of the NiNTA resin; E1, E2, E3, E4 and E5, elution steps performed with Tris buffer containing 250 mM Imidazole.

Anti PSCA scFv antibody characterization

Functional and binding properties of anti PSCA scFv were investigated by flow cytometry and by ELISA using anti PSCA whole mAb as control.

The immunoenzymatic assay performed on recombinant PSCA confirmed that the antibody fragment produced in *E. Coli* retains the binding specificity of the parental anti PSCA mAb, with nearly no background and no reactivity on an unrelated protein (BSA) (Fig. 21). The signal obtained with scFv is lower than that obtained with the whole mAb. This difference can be due to the monovalent binding property of the scFv in comparison with the divalent binding of the whole mAb. In order to create a scFv in divalent form scFv was preincubated with anti myc antibody for 1h at room

temperature. In this way two scFv monomers are cross linked via an antibody-mediated myc-Tag interaction. Staining of cells with scFv in divalent form is similar to that obtained with the whole mAb.

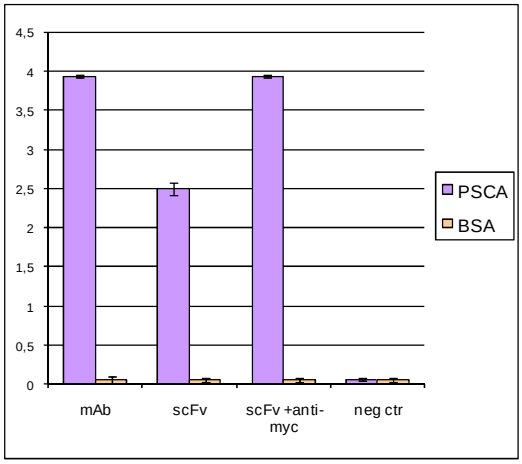

Figure 21. ELISA of recombinant PSCA. Anti PSCA scFv shows a specific binding to recombinant PSCA. The scFv staining is lower than that of the mAb. Staining of scFv preicubated with anti myc antibody is similar to that obtained with the whole mAb.

The flow cytometry analysis (Fig. 22, Tab. 8), performed with positive cell lines (SW780, LNCaP hPSCA) and negative cell lines (PC-3 WT, LNCaP WT), confirmed the interaction of our scFv with the PSCA antigen expressed on the surface of target cells.

When assayed on LNCaP hPSCA cells the scFv shows a MFI value of 834 whereas the MFI of the negative control is 369; the mAb (whole molecule) specifically detects $PSCA^+$ cells with a MFI of 4401. Also in this case when the scFv is preincubated with an anti myc antibody the staining increases (MFI value of 4010) and it reaches a value close to that obtained with whole mAb.

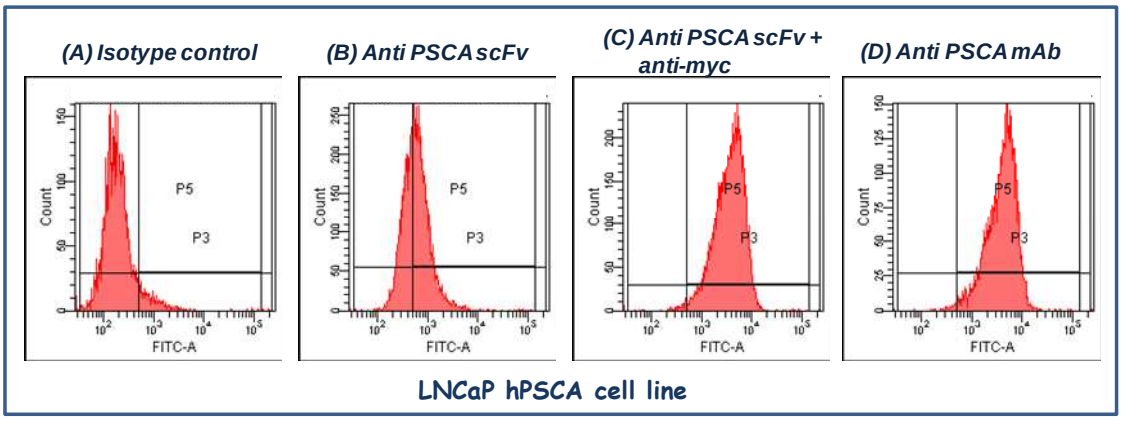

Figure 22. Flow cytometry analysis of LNCaP hPSCA cells. (A) Negative control. (**B**) Binding of anti PSCA scFv(**C**) Binding of anti PSCA scFv in divalent format obtained by preincubating it with anty myc antibody. (**D**) Binding of mAb anti PSCA (whole antibody).

	PSCA positive cell lines MFI values		PSCA negative cell lines MFI values	
	SW780	**LNCaP hPSCA**	**PC-3 WT**	**LNCaP WT**
Isotype control	64	369	111	184
Anti PSCA scFv	**382**	**834**	142	167
Anti PSCA scFv + anti-myc	**931**	**4010**	124	n.d.
Anti PSCA mAb	2662	4401	133	58
Positive control	n.d.	n.d.	525	2076

Table 8. Binding specificity of anti PSCA scFv analyzed by flow cytometry with PSCA positive and negative cell lines.

To better define the binding properties of the single chain fragment its kinetic parameters were determined by Biacore assay. Thanks to a collaboration with Dr.ssa Mariangela Figini, INT Milan, the dissociation constant (K_D) of the interaction between our scFv and recombinant PSCA was determinated by the SPR technology (Surface Plasmon Resonance). Association/dissociation curves were obtained by flowing different diluitions of the scFv on chips coated with a fixed amount of recombinant PSCA (Fig. 23). From this curves the following kinetic constants were deduced: k_{on} = 1.16 x 10^5 (1/Ms), k_{off} = 2.65 x 10^{-3} (1/s). The resulting K_D (k_{off} / k_{on}) is 2.29 x 10^{-8} M. This value is consistent with a K_D of 1.41 x 10^{-8} M determined for the Fab derived from

our anti PSCA mAb. This means that the recombinant fragment scFv produced in *E. Coli* and purified from the periplasm maintains the same binding properties as the parental Fab.

Moreover we compared the association/dissociation curves obtained by injecting 200 nM of anti PSCA scFv, Fab or whole mAb on the same PSCA coated chip (Fig. 24). As expected the scFv and Fab curves are comparable whereas the mAb shows a greater affinity.

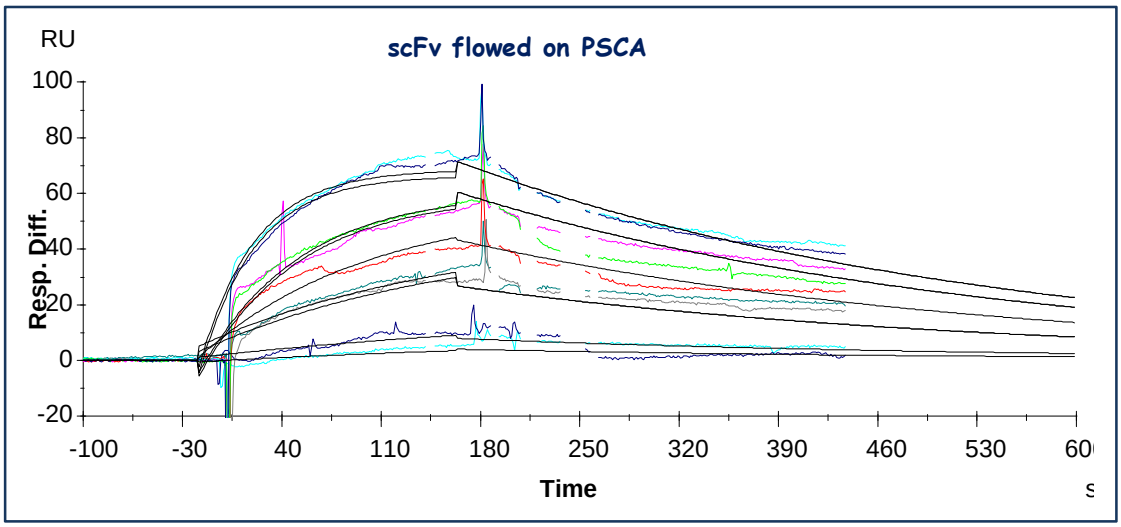

Figure 23. Biacore analysis to determine scFv kinetic parameters. The association/dissociation curves were obtained by flowing different dilutions of the scFv over a PSCA coated chip.

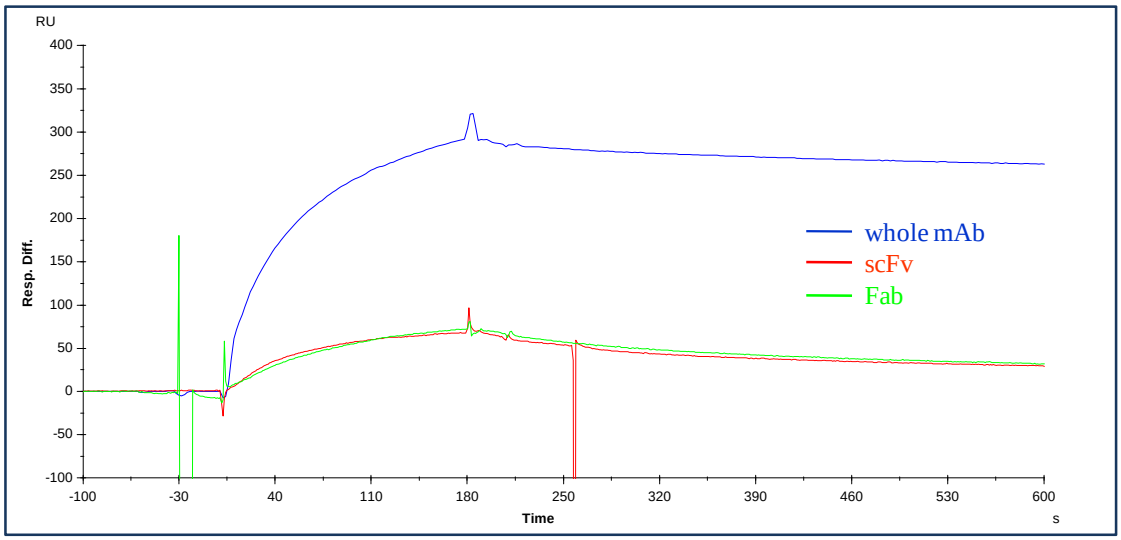

Figure 24. Biacore analysis of the binding properties of anti PSCA whole antibody and its fragments.

Generation of a recombinant immunotoxin

PE40 toxin is known to follow a translocation route similar to RTA [Falnes and Sandvig, 2000]. Moreover PE40 toxin bears in its sequence a cleavable site (5' to the enzymatic domain), which, once recognized by furin or furin like-proteases, allows the intracellular separation of the vehicle from the toxic moiety of the IT. If the toxin moiety is not released from the carrier molecule, the toxin is not able to translocate in the cytosol and it cannot intoxicate the cells. To create the fusion IT anti PSCA scFv-PE40 we used a vector available in the laboratory: a pET 11d plasmid carrying the PE40 toxin sequence. The scFv sequence was modified by PCR to add a Hind III site at 3'. As shown in Figure 25 the PCR product is a band of about 800-900 bp; this DNA was cloned in pET 11d plasmid with Nco I/Hind III restriction sites; a scheme of the plasmid is displayed in Figure 26.

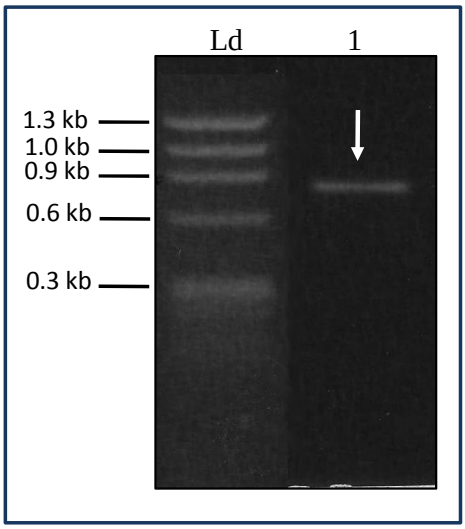

Figure 25. PCR product obtained by amplifying the anti PSCA scFv sequence. The arrow indicates the amplificate of about 800-900 bp (lane 1). Ld = DNA Ladder.

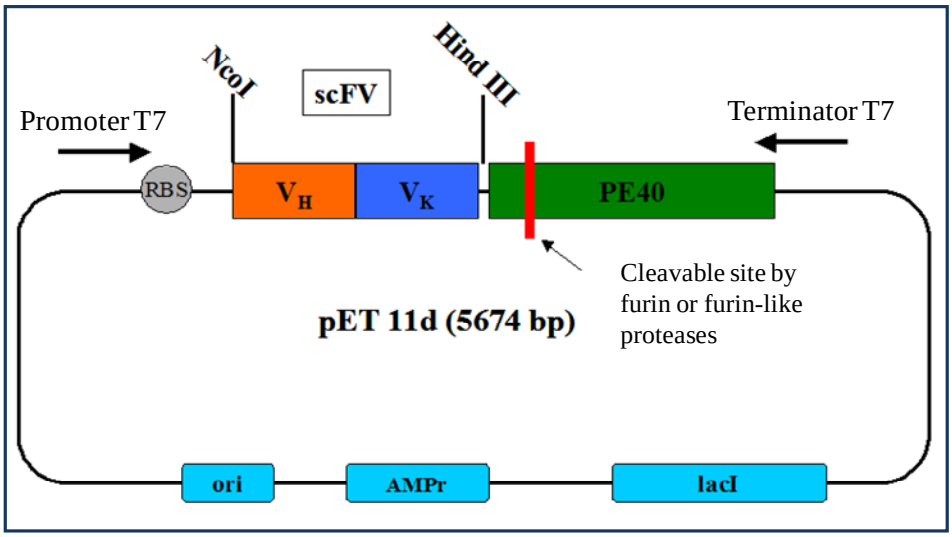

Figure 26. Schematic picture of the plasmid pET11d-anti PSCA scFv-PE40.

A small-scale expression of the IT in BL21 (λDE3) pLysS *E. Coli* yielded an induced protein of approximately 70kDa, as visualized by Western Blot (Fig. 27), consistent with the expected size for a fusion between the scFv (30 kDa) and PE40 (40 kDa).

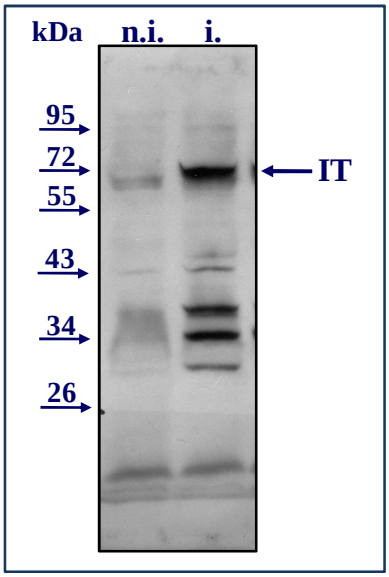

Figure 27. Western Blot of total bacterial lysate. The Western Blot was performed using an anti PE antibody. The same amount of total protein was loaded form non induced (n.i.) and induced (i.) bacterial cultures. The band at about 70 kDa represents the immunotoxin (IT).

To maximize the yield of the immunotoxin the bacteria were induced with 1 mM IPTG for different times (3 h, 5 h, 20 h) at different temperatures (30-37°C). The amount of produced protein and its site of subcellular storage (inclusion bodies, cytoplasm) were analyzed by Western Blot using an anti PE antibody; with an incubation time of 3h at 37°C the highest amount of protein was detected in the inclusion bodies fraction (Fig. 28). Moreover it was observed that the amount of produced immunotoxin increases when bacteria are harvested just before induction and then induced in LB medium lacking glucose (Fig. 29). Also in this case the immunotoxin is detected in the inclusion bodies fraction.

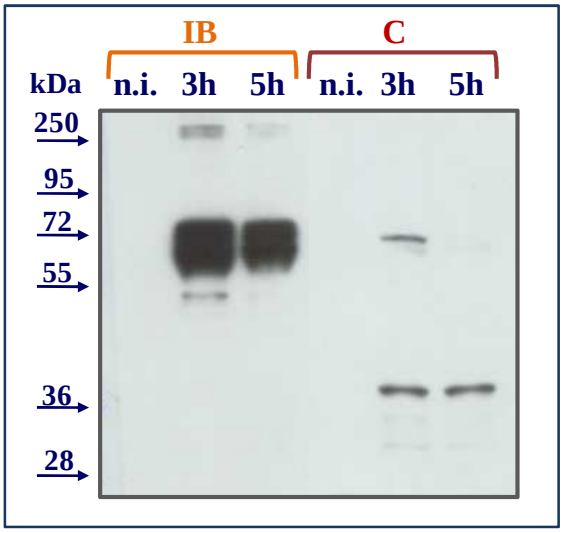

Figure 28. Western Blot of bacterial fractions using anti PE antibody. Each fraction is obtained after induction with 1mM IPTG at 37°C for 0h (n.i.), 3h or 5h. The band at about 70 kDa represents the anti PSCA scFv-PE40. Abbreviations: IB, inclusion bodies; C, cytoplasmic soluble fraction.

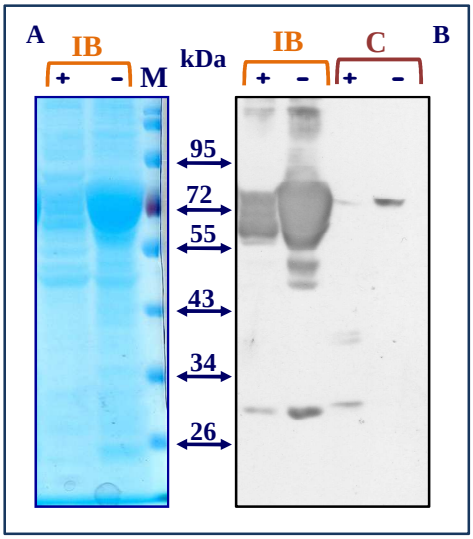

Figure 29. (A) SDS-PAGE and (B) Western Blot of bacterial fractions. A bacterial culture was induced with IPTG 1 mM at 37°C for 3 h in LB medium added (+) or lacking (-) of glucose. The Western Blot was performed with an anti PE antibody. Abbreviations: IB, inclusion bodies; C, cytoplasmic soluble fraction; M, protein size standard.

Before beginning protein purifications, proteins located in inclusion bodies were solubilized and refolded as described in Materials and Methods. As shown in Figure 30, during the refolding steps the immunotoxin aggregates generating multimers with a

molecular weight greater than 170 kDa. The refolding procedure is currently being optimized.

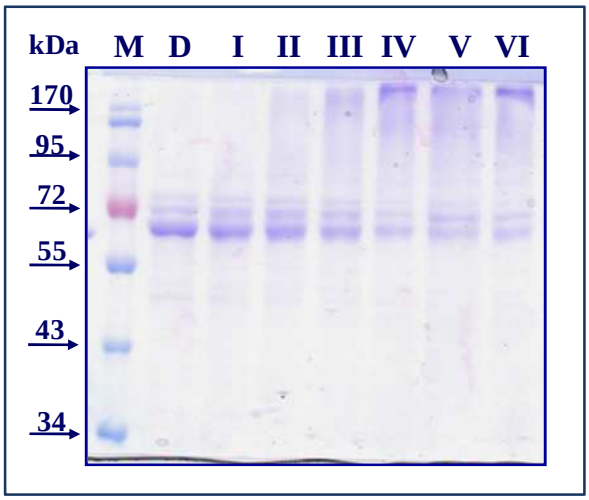

Figure 30. SDS-PAGE of samples taken at different steps of the refolding procedure. Abbreviations: M, protein size standard; D, denatured inclusion bodies; I-VI, inclusion bodies during refolding dialysis.

An ELISA assay with recombinant PSCA provided a qualitative indication of binding selectivity for the refolded inclusion bodies containing scFv-PE40 (Fig. 31).

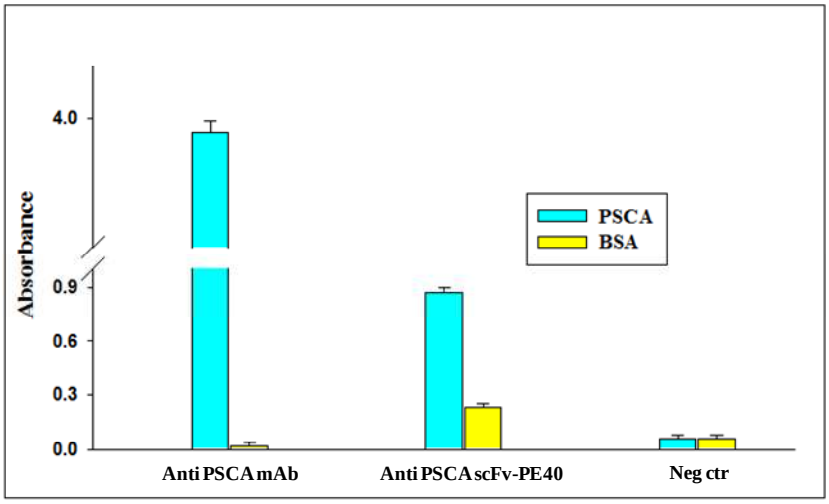

Figure 31. ELISA assay performed with recombinant PSCA. A whole refolded IB extract containing scFv-PE40 shows a specific binding on recombinant PSCA.

The whole refolded IB were then assessed by flow cytometry on positive (LNCaP hPSCA) and negative (LNCaP WT) cell lines (Fig. 32). When assayed on LNCaP hPSCA cells the refolded IB showed a MFI value of 1100, 5 fold higher than that of Isotype control (MFI = 222). Whereas when assayed on LNCaP WT cells the refolded IB showed a MFI value of 441, 1.6 fold higher than that Isotype control (MFI = 269). This low staining on negative cells is due to non specific binding of contaminants present in the exctract. This assay confirmed that the interaction of scFv with the PSCA antigen expressed on the surface of target cells is preserved after fusion to PE40 toxin.

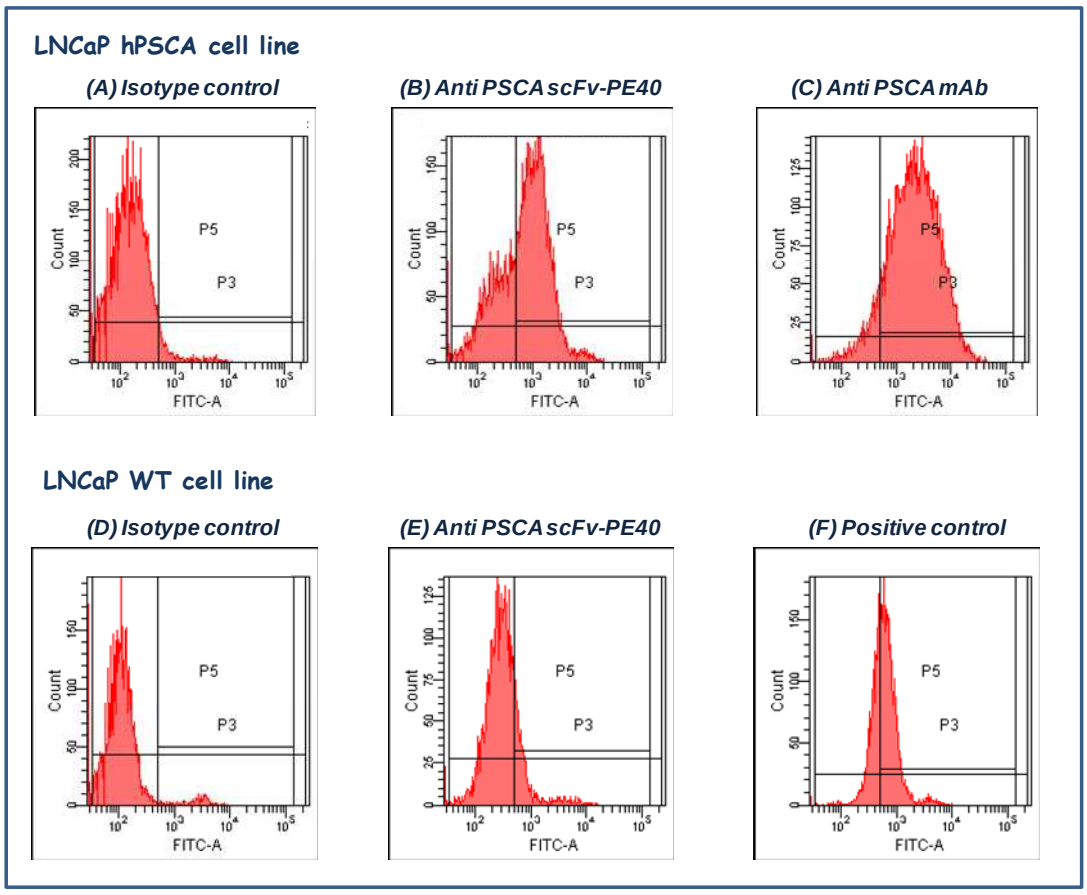

Figure 32. Flow cytometry on LNCaP hPSCA cells (A-B-C) and LNCaP WT (D-E-F). (A-D) Isotype control is used as negative control. (**B-E**) Binding of whole refolded IB extract containing scFv-PE40 immunotoxin. (**C**) Binding of anti PSCA whole antibody and (**F**) anti MHC I antibody were used as positive control.

Different purification methods were tested to obtain the highest amount and level of purity of immunotoxin. In my hands the best purification protocol consists in the purification from refolded inclusion bodies by a first step of gel filtration. Protein purification and identification were checked by Western Blot (Fig. 33). Fractions containing scFv-PE40 were collected and, because of a low protein pI (5.3), were then subjected to a second purification step through adsorption on an anion-exchange resin (QAE).

This protocol allows to obtain scFv-PE40 with no contaminants but it does not separate immunotoxin monomers from multimeric forms (Fig. 34). To overcome this problem and also to increase the yield of purified protein the purification protocol is still being optimized.

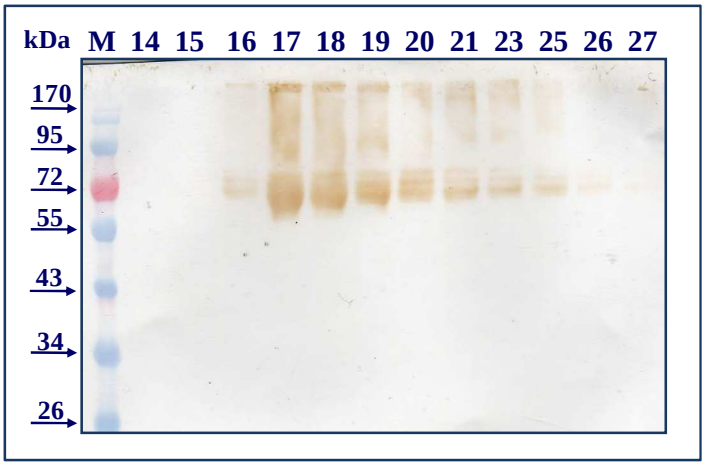

Figure 33. Western Blot of fractions obtained from the first chromatographic separation of scFv-PE40 on gel filtration. The Western Blot was performed with anti PE antibody. Abbreviations: M, protein size standard; 14-27, fractions collected from gel filtration.

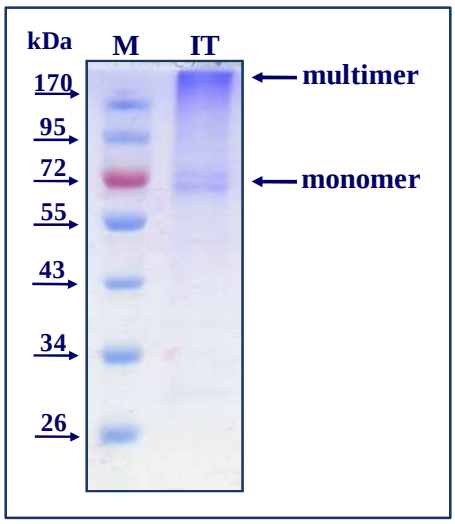

Figure 34. SDS-PAGE of purified immunotoxin. The scFv-PE40 (IT) is present as monomer (molecular weight of about 70 kDa) and multimer (molecular weight more than 170 kDa). Abbreviations: M, protein size standard.

Generation of a cellular model of polarized epithelial tissue

The PSCA distribution and trafficking processes post-internalization could be different in polarized cells both normal and tumoral. To study the efficacy of anti PSCA immunotoxins in polarized cells a cellular model of polarized epithelial tissue expressing PSCA was generated. Madin-Darby canine kidney (MDCK) cells are a well differentiated cell line with tight junctions that form polarized monolayers in culture that closely resemble epithelial tissue. For this purpose MDCK cells were transfected with hPSCA-pcDNA3.1 plasmid and immunofluorescences were permormed using our anti PSCA mAb.

Immunofluorescence on permeabilized cells (Fig. 35A) showed the distribution of PSCA in the whole molecule: it localizes on the cell surface, both apical and basolateral membrane, and in its closeness. When the immunofluorescence is performed on non permeabilized cells the anti PSCA antibody can reach only the apical membrane because the tight junctions do not permit the mAb to reach the basolateral membrane (Fig. 35B). In this case we can appreciate that PSCA is distributed on all apical membrane. A preliminary study on PSCA routing is shown in Fig. 35C where cells,

after anti PSCA mAb binding, are incubated at 37°C for 1 h and then subjected to the same conditions as previous sample. It is possible to observe that PSCA from the apical membrane can reach the basolateral membrane through small vescicules represented as small green dots in the middle of the cell. This cell model could be used to study the PSCA routing in order to find the best toxic portion to create potent immunotoxin and also to study the cellular intoxication adding immunotoxins to apical or basolater or both membranes.

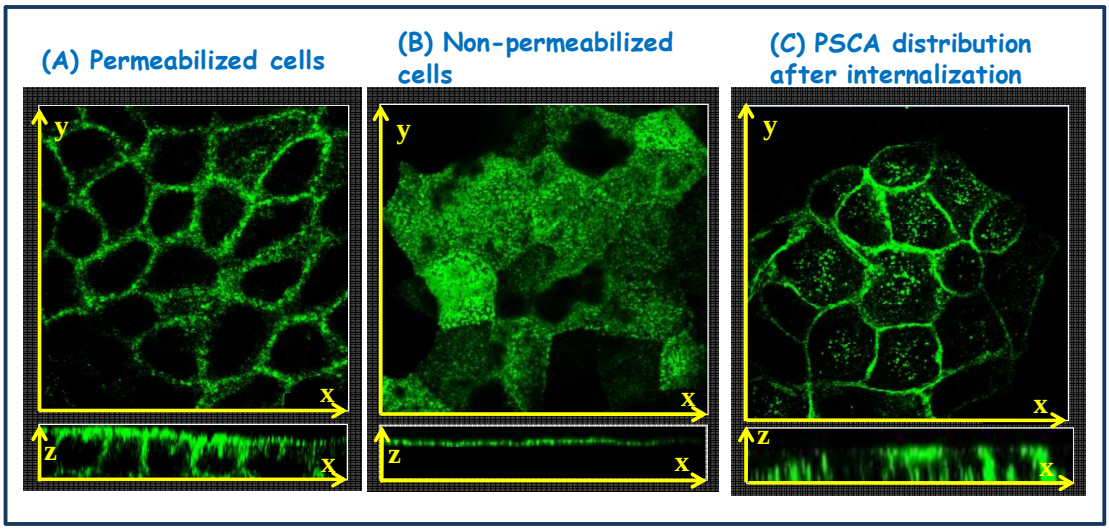

Figure 35. Immunofluorescence on MDCK hPSCA cells stained with anti PSCA mAb. (**A**) To evidence PSCA distribution in the whole cells, after fixation and permeabilization MDCK were incubated with anti PSCA antibody at 4°C. (**B**) To investigate the distribution of PSCA on the sole apical membrane, cells were incubated with anti PSCA mAb at 4°C and then fixed. (**C**) The internalization of PSCA from the apical membrane is obtained by staining cells with anti PSCA mAb at 4°C and, after washing, incubating them at 37°C for 30 min. PSCA from the apical membrane can reach the basolateral membrane by vescicules.

DISCUSSION

Cancer is the second leading cause of human death next to coronary disease. Worldwide, millions of people die from cancer every year and deaths generally are on the rise. Conventional therapies, like surgery, radiotherapy and chemotherapy, are not always sufficient to eliminate the tumor and the possible metastases; moreover these therapies are responsible of many side effects. For this reason there is an urgent need for new treatments strategies, the most promising one is probably immunotherapy.

In this context prostate stem cell antigen (PSCA) represents an ideal choice for immunotherapy because it is highly expressed by a large proportion of human prostate, pancreatic and bladder tumors, including metastatic and hormone-refractory prostate cancers, but has limited expression in normal tissues [Gu *et al*., 2000; Wente *et al.*, 2005; Elsamman *et al*., 2006]. This restricted expression profile of PSCA also makes it a potential diagnostic and prognostic biomarker for PSCA expressing cancers.

This project started with the generation of anti PSCA monoclonal antibodies (mAbs) obtained by mice immunization with recombinant PSCA and subsequent hybridoma generation according to Köhler-Milstein protocol. Ten clones were able to recognize the recombinant PSCA in the ELISA assay and among them the two having highest affinity for the antigen were chosen: clone 1 and clone 2. As the mice were immunized with the recombinant PSCA, it was important to evaluate if the two mAbs were able to recognize the native PSCA expressed on the cell surface. For this reason the mAbs were analyzed by flow cytometry performed with SW780 ($PSCA^+$) and PC-3 WT ($PSCA^-$) cells. The MFI values obtained with our mAbs were similar and comparable to the clone 7F5, a validated anti PSCA antibody described in literature [Morgenroth *et al*., 2007]. These results demonstrate that our mAbs are able to recognize the native PSCA with a good specificity.

The research continued with the characterization of the two mAbs.

It is interesting to observe that the two mAbs have similar characteristics. The ELISA assay determinated that clone 1 and clone 2 belong to the same subclass: IgG2b. Moreover in a binding competition experiment, performed with SW780 and analyzed by flow cytometry, the two mAbs were able to cross inhibit the binding of each other. From this experiment it could be supposed that the two mAbs recognize the same or at least partially overlapping epitopes on the antigen PSCA. Thanks to the Surface Plasmon Resonance technology, which studies the affinity of the mAbs for the PSCA, it

was possible to observe that the binding properties of clone 1 and clone 2 are similar. In fact the association/dissociation curves are comparable and they show a fast rate of association and a slow rate of dissociation demonstrating that the mAbs assessed have a good affinity for PSCA. Subsequently the variable region of the light (V_L) and heavy (V_H) chain of the two mAbs were cloned. The comparison of the nucleotidic and deduced aminoacidic sequences showed that the two mAbs have the same V_H but different V_L. Kabat and Wu [1991] examined a large database of amino acid sequences of antibodies of various specificities and they found that for many antibodies the specificity is due to the V_H region and in particular to the CDRH3 (Complementary-Determining Region 3 of the heavy chain). Indeed, they found that many antibodies of distinct specificities assemble identical V_L domains with different V_H domains. In contrast they found that rarely antibodies with different specificities have the same V_H domain and very different V_L. In the case of our anti PSCA mAbs we can suppose that the binding specificity and affinity of the antibodies is mainly due to the V_H domain because it is the same in clone 1 and clone 2 and the two mAbs demonstrate comparable binding properties, whereas the different V_L domains could be involved in a different role than the binding properties, like antibody stability. Since the two mAbs have no different binding properties clone 1 was selected for further characterization.

The selected anti PSCA mAb was assessed by flow cytometry with different PSCA positive and negative cell lines. As SW780 is the only available cell line constitutively expressing PSCA with a variable expression depending on as yet unknown factors, PC-3, LNCaP, HEK 293 and MDCK cell lines were stably transfected with hPSCA-pcDNA3.1. The flow cytometric analysis confirmed the antibody clone 1 binding properties to the antigen and its specificity. To assess the possible use of our antibody for diagnostic/therapeutic purposes, the concentration of antibody necessary to saturate the 50% of antigenic sites was determined by flow cytometry. The binding curves obtained incubating cells with increasing concentrations of mAb were determined for two different cell lines: PC-3 hPSCA and LNCaP hPSCA. The antibody reaches saturation of 50% of PSCA sites at the concentration of 16 nM and 23 nM respectively on PC-3 hPSCA and LNCaP hPSCA. To verify the reliability of the values obtained, the binding curve of anti Prostate Specific Membrane Antigen (PSMA) antibody was determined for LNCaP hPSCA cells which are able to express another tumor associated

antigen, namely PSMA. The anti PSMA mAb reaches saturation of 50 % of PSMA sites at the concentration of 8 nM. The values obtained for anti PSCA mAb are consistent with that obtained for the anti PSMA mAb and with the rage of 10^{-7}-10^{-11} M for antibodies of biological interest. For this reason the anti PSCA mAb clone 1 was assessed for its diagnostic potential. First of all it has to be considered that the antigen PSCA has multiple N-glycosylation sites [Reiter *et al*.,1998], and that differences in the glycosylation pattern of antigens exist between healthy and malignant tissues [Pauli et al., 2003; Robbe-Messelot et al., 2008; Jankovic and Milutinovic, 2008] and could exist also between different patients. As our anti PSCA mAb was obtained by mice immunization with PSCA purified from bacteria, which is non glycosylated, the mAb was assessed by Western Blot with MDCK hPSCA cell lysate and subsequently with tumor tissue lysates from different patients to verify its ability to recognize the possible glycosylated isoforms of the antigen. MDCK cells were chosen because they are a well differentiated cell line with tight junctions that form polarized monolayers in culture closely reproducing the epithelial tissue organization *in vivo*. A Western Blot assay performed with this transfected cell line demonstrated that our mAb is able to recognize different isoforms of the PSCA. In fact in the total lysate are present many bands with a molecular weight between 25 kDa and 40 kDa. The PSCA pattern of glycosylation was verified by treating the total lysate with two endoglycosidases: Endoglycosidase H and N-Glycosidase F. The band at 40 kDa corresponds to the antigen bound to complex sugars, in fact it shifts to 15 kDa only after treatment with N-Glycosidase F which cleaves mannose rich, hybrid and complex glycans. The bands at 25-35 kDa correspond to the antigen bound to hybrid sugars, in fact they partially shift to 22 kDa after treatment with Endoglycosidase H, which cleaves only mannose rich glycans, and completely to 15 kDa after treatment with N-Glycosidase F. Because our antibody is able to recognize different PSCA isoforms it was assessed also in Western Blot with prostate and pancreatic tumor tissue lysates from patients. It is interesting to observe that also in this case our anti PSCA mAb is able to recognize the antigen, that the PSCA has a unique pattern of glycosylation in each tissue lysate and that the PSCA molecular weight is the same in the tumor tissue analyzed. The antigen could be supposed to be glycosylated because it has a molecular weight of about 28 kDa. In the literature there is no information available as to the molecular weight of PSCA expressed in tumor tissues

because the PSCA expression analysis was performed by immunohistochemistry (IHC) or by flow cytometry. In future experiments it could be interesting to investigate if there is a correlation between the tumor stage and the pattern of glycosylation of PSCA which could be involved in the PSCA function which at the present time is still unknown.

The Western Blot assay performed with tumor tissue lysates showed also that our antibody can recognize the antigen in three out of three prostate neoplastic tissue lysates and in two out of three pancreatic neoplastic tissue lysates. These results are consistent with the evidence that in IHC 94% of prostate tumors [Gu *et al*., 2000] and 60% of pancreatic adenocarcinomas [Argani *et al*., 2001] are positive for PSCA expression. Moreover our antibody could recognize a linear and non glycosylated epitope because it binds to the denatured and both glycosylated and non glycosylated antigen as assessed by Western Blot.

The diagnostic potential of the anti-PSCA antibody was assessed also by ELISA with cell fixed with 2% paraformaldehyde. Paraformaldehyde, used in IHC to fix tissues, could alter the antigen structure and prevent antibody binding to the antigen. This experiment demonstrated that the antibody can recognize the antigen also on fixed cells. All the binding properties examinated for our anti PSCA mAb clone 1 led us to consider it as a potential tool for diagnostic purposes. It will be important to assess the antibody performance also by the IHC technique. Moreover the antibody could be conjugated to fluorescent tracers or radionuclides and then be assessed for *in vivo* immuno-imaging approaches; this technique could improve the diagnosis of micrometastases.

Preliminary studies were also performed to analyze the therapeutic potential of our anti PSCA mAb clone 1. Many antibodies are approved by FDA or are in clinical trials to evaluate their therapeutic use in cancer therapy. First of all the proliferation assay performed with PC-3 hPSCA cells demonstrated that the antibody alone is not able to induce cell death through a direct effect, but it can kill cells after linkage to the A chain of Ricin (RTA). It is interesting to observe that Gu *et al.,* 2005 demonstrated that their anti PSCA mAb, 1G8, induces cell death *in vitro* through a direct, Fc-independent mechanism. Probably the difference in cell killing between the two anti PSCA antibodies, clone 1 and 1G8, could be due to the recognition of different epitopes. It is interesting to observe that the binding to different PSCA epitopes could induce or not cell apoptosis.

The anti PSCA-RTA immunotoxin obtained by chemical linkage of anti PSCA clone 1 mAb to RTA was assessed on PC-3 hPSCA and PC-3 WT cell lines. A proliferation assay demonstrated that the chemical immunotoxin has a 100 fold higher cytotoxic activity in PC-3 hPSCA cells compared with the RTA toxin alone, that is the active enzymatic domain. Moreover the anti PSCA-RTA immunotoxin has a 100 fold lower toxicity on PC-3 WT cells than on PC-3 hPSCA cells and the same toxicity as RTA on PC-3 WT cells. These results demonstrate that the greater toxicity of the immunotoxin is due to the specific binding of anti PSCA mAb to the PSCA antigen. The anti PSCA clone 1 antibody could be considered as a good vehicle for the delivery of toxic molecules to tumor cells overexpressing the antigen PSCA.

The monoclonal antibody described in this thesis is murine and because of this it cannot be administered to patients who could develop human–antimouse antibodies (HAMAs). Moreover the large size of the antibodies limits tumor penetration, and their long serum half-life is not suitable for applications such as radioimmunotherapy or imaging. To overcome these problems the recombinant DNA technology allows to generate fragments that retain the binding activity of the full-length molecule and that, thanks to their reduced size, demonstrate high tumor penetration, very rapid plasma clearance and lower immunogenicity. For this reason the variable heavy (V_H) and light (V_L) chain of our anti PSCA antibody were cloned to obtain the variable fragment scFv.

The anti PSCA scFv was produced in a bacterial strain and then purified from the periplasmic fraction. The scFv binding properties were assessed by ELISA, using recombinant PSCA, by flow cytometry, using positive and negative cell lines, and by the SPR technology. The scFv demonstrated to be able to recognize the antigen PSCA with the same specificity but lower affinity than the parental whole mAb. This is consistent with the monovalent binding moiety of the scFv and the divalent binding moiety of the whole mAb; in fact the ability to bind to two antigen sites greatly increases the functional affinity [Holliger and Hudson, 2005]. The Biacore analysis moreover determined the kinetic parameters of scFv and Fab, derived from the whole mAb. The K_D of scFv and Fab are 2.29×10^{-8} M and 1.41×10^{-8} M respectively. This means that the recombinant fragment scFv maintains the same binding properties as the parental Fab. It is interesting to observe that the scFv partially recovered its affinity when it was made artificially divalent by cross-linking scFv monomers via an antibody-

mediated myc-tag interaction. This was possible because the scFv was cloned in an expression vector which appended a myc-tag to the C-terminus of the scFv. These data suggest that it could be possible to generate PSCA specific fragments with higher affinity by generating a divalent minibody or diabody. For the generation of antibody fragments for cancer therapy/diagnosis, it is important to consider another factor: "the binding site barrier effect". Antibodies with high affinity bind tightly to their antigen upon the first encounter, that is, at the periphery of the tumor. So they do not penetrate deeper inside the tumor until all antigen molecules are saturated in the periphery. By contrast moderate binders are released from these first encountered antigens and penetrate deeper into the tumor, ultimately leading to uniform intratumoral distribution and higher tumor uptake. For this reason an scFv with low affinity could be better suited for tumor targeting. The recombinant DNA technology could be also exploited for the humanization and the engineering of the optimal affinity of the anti PSCA scFv characterized in this thesis. As regards the affinity optimization by protein engineeering techniques it would be important to balance the binding properties of the antibody leading to an efficient tumor targeting with the tumor retention effects that influence the diffusion of therapeutic molecules within the tumor.

A fusion immunotoxin (scFv-PE40) was also created to obtain a more stable immunotoxin with reproducible chemical properties due to the obligatory 1:1 antibody:toxin ratio. The immunotoxin was produced in a bacteria strain and it was accumulated in the inclusion bodies. Preliminary results obtained by flow cytometry with PSCA positive cell lines showed that the interaction of scFv with the PSCA is preserved after fusion to PE40. The efficacy of purified scFv-PE40 will be analysed *in vitro* using positive and negative cell lines. Moreover it will be important to study the efficacy of anti PSCA immunotoxins in MDCK cells, a polarized cell line which closely resemble epithelial tissue. Subsequently *in vivo* models will also be useful to study the *in vivo* effects of this new drug.

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