ABSTRACT

This study describes the genetic construction and expression of a fusion protein consisting of a human monoclonal antibody in a single chain fragment variable (scFv) format (E8) specific for a well defined determinant of the CEA cell surface antigen family and cytosine deaminase from yeast (yCD). DNA sequence encoding for the scFvE8 human monoclonal antibody recognizing an epitope shared by CEACAM1 (CD66) and CEACAM5 isoforms was assembled with a monomer of yCD. The scFvE8:yCD fusion protein possessed the binding specificity of the immuncompetent part of protein which include the recognition of melanoma (Mel P5) and colon carcinoma (LoVo) cell lines. The scFv8:yCD system showed the ability to make tumour cells naturally resistant to chemotherapy, susceptible to the non toxic substrate 5-fluorocytosine (5-FC) by its enzymatic conversion into 5-fluorouracil (5-FU). In vitro pre-treatment of Mel P5 and LoVo cell lines with scFvE8:yCD followed by cell washing and incubation with 5-FC, resulted in a significant cell killing supporting the utility of this fusion protein as an agent for selective tumor therapy by in loco prodrug activation.

A convincing demonstration that such system can be developed for clinical use requires evidence that each of the components of the antibody complex functions by the mechanisms proposed This can be provided by well defined measurements including the concentration levels of the antibody-enzyme conjugate , in plasma, tumour and healthy tissues. To this aim we select from ETH-2 synthetic fagic library, a human monoclonal antibody in single chain fragment (scFv) format against a recombinant CD from yeast (yCD). This antibody proved to be functionally active in NMR and in *in*

vitro studies to convert the antifungal drug 5-FC into the anticancer compound 5-FU. The specificity of the human scFv was confirmed by Western blot and ELISA analyses. With this antibody, yCD expression can now be monitored without interfering with its enzymatic function in ADEPT.

INTRODUCTION

1 ANTIBODIES

1.1 ANTIBODIES AS NEW THERAPEUTIC PROTEINS

Antibodies have proven to be an excellent paradigm for the design of high-affinity protein -based binding reagents. Innovative recombinant DNA technologies, including chimerization and humanization, have enhanced the clinical efficacy of murine monoclonal antibodies by reducing their potential adverse effects due to immunogenicity of the xenogenic rodent protein. Then several recombinant monoclonal antibodies and have been approved by regulatory agencies for in vivo diagnosis and treatments of cancer, infectious and inflammatory diseases. With 21 monoclonal antibodies (mAb), (www.fda.gov/) currently on the market and more than 100 in clinical trials, it is clear that engineered monoclonal antibodies have come of age as biopharmaceuticals (table1).

Table 1

Brand name	Common name	Target	Therapy area	approval date
MURINE (3)				
Orthoclone OKT3	Muromonab-CD3	Tcell CD3 receptor	Transplant rejection	1986
Zevalin (conjugated)	Ibritumomab tiuxetan	CD20	Non-Hodgkin lymphoma	2002
Bexxar (conjugated)	Tositumomab-I131	CD20	Non-Hodgkin lymphoma	2003
CHIMERIC (5)				
ReoPro	Abciximab	glycoprotein IIb/IIIa	Cardiovascular desease	1994
Rituxan	Rituximab	CD20	Non-Hodgkin lymphoma	1997
Simulect	Basiliximab	IL2 repeptor a	Transplant rejection	1998
Remicade	Infliximab	TNF	Inflammatory deseases	1998
Erbitux	Cetuximab	EGFR	Colorectal cancer	2004
HUMANIZED (11)				
Zenapax	Daclizumab	CD25	Transplant rejection	1997
Synagis	Palivizumab	F protein of RSV	Viral infection (RSV)	1998
Herceptin	Trastuzumab	ErbB2	Breast cancer	1998
Campath	Alemtuzumab	CD52	Chronic lymphocytic leukemia	2001
Raptiva	Efalizumab CD11a		Inflammatory deseases	2003
Xolair	Omalizumab	IgE	Asthma	2003
Avastin	Bevacizumab	VEGF	Colorectal cancer	2004
Mylotarg (conjugated)	Gemtuzumab/ozogamicin	CD33	Acute myelogenous leukemia	2000
Soliris	Eculizumab	C5 protein (complement)	Inflammatory desease	2007
Tysabri	Natalizumab	Tcell VLA4 receptor	Inflammatory deseases	2006
Lucentis	Ranibizumab	VEGF	Macular degeneration	2006
HUMAN (2)				
Humira	Adalimumab	TNF	Inflammatory deseases	2002
Vectibix	Panitumumab	EGFR	Colorectal cancer	2006

1.2 ANTIBODIES STRUCTURE AND FUNCTION

Antibodies (eg, IgG, which is the most commonly used immunoglobulin form), (Figure 1) are highly specific targeting reagents and they are unique proteins with dual functionality. All naturally occurring antibodies are multivalent, are a Y-shaped, with IgG having two binding 'arms.' The ability to bind to two antigen greatly increased their functional affinity and confers high retention time (also called avidity) on many cell surface receptors and polyvalent antigens. Antigen-binding specificity is encoded by three *complementarity-determining regions* (CDRs), while the *Fc-region* is responsible for binding to serum proteins (eg, complement) or cells. An antibody is not usually capable for killing target cells, but after binding and in co-operation with other

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components of the body's immune system including cells with effector function may exert cytolytic mechanisms. Furthermore the antibody binding on cell determinant may initiate a cascade of events leading to the cell's self-destruction (Figure2) or apoptosis. The above mentioned mechanisms include antibody-dependent *complement mediated cytotoxicity* (CMC) and *antibody dependent cellular cytotoxicity* (ADCC). ADCC involves the recognition of the antibody by immune cells that engage the antibody-marked cells and either through their direct action, or through the recruitment of other cell types, lead to the tagged-cell's death. CMC is a process where a cascade of different complement proteins become activated, usually when several IgGs are in close proximity to each other, either with one direct outcome being cell lysis, or one indirect outcome being attracting other immune cells to this location for effector cell function. Antibodies, when bound to key substances found on the cell surface, also can induce cells to undergo *programmed cell death*, or *apoptosis* (Figure 2).
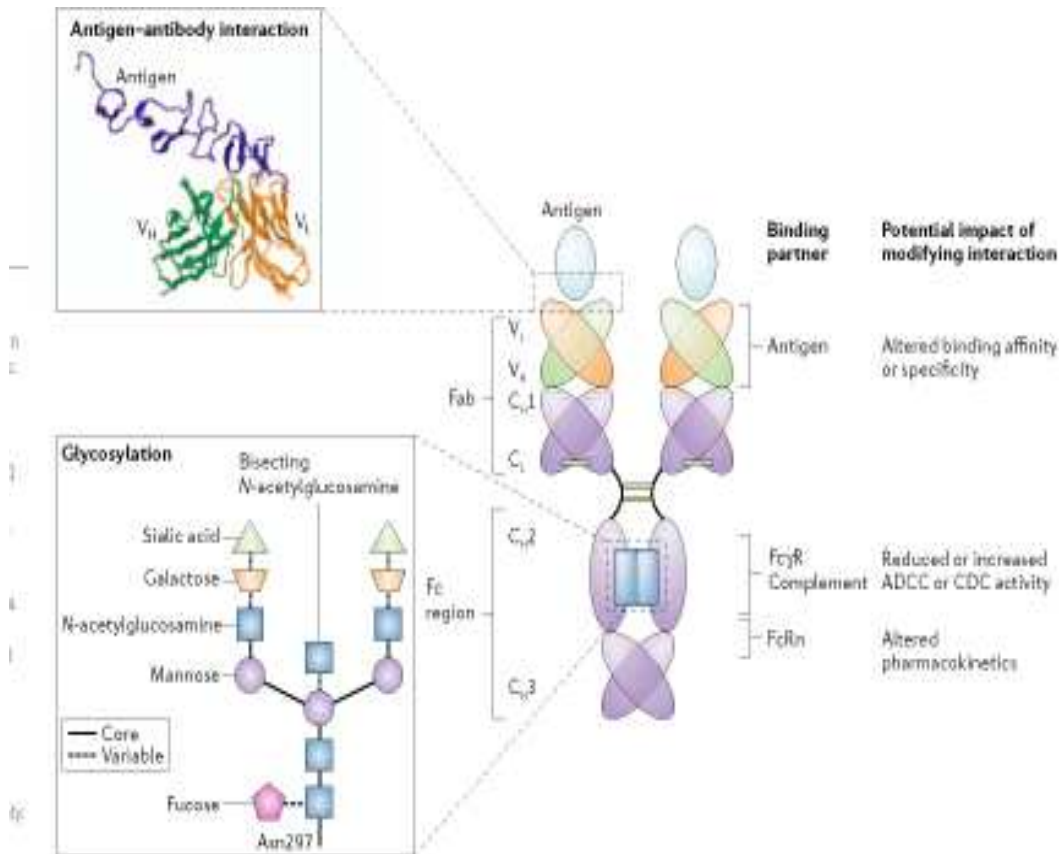
Figura 1 The basic structure of an antibody: two identical light chains and two identical heavy chains which are linked by disulphide bonds. Each of the heavy and the light chains contains a variable sequence (VH and VL respectively) in the amino-terminal 110 residues, and constant sequences (CH and CL respectively) in the remaining portion of the chain . The antibody variability, which accounts for their different binding specificity, is located in the VL and VH, clustered in several hypervariable regions: the Complementary Determining Regions (CDRs). There are three CDRs in VH sequence (named HCDRs) and three in VL sequence (named LCDRs). These regions form the antigen binding site of the antibody molecule and determine its specificity.
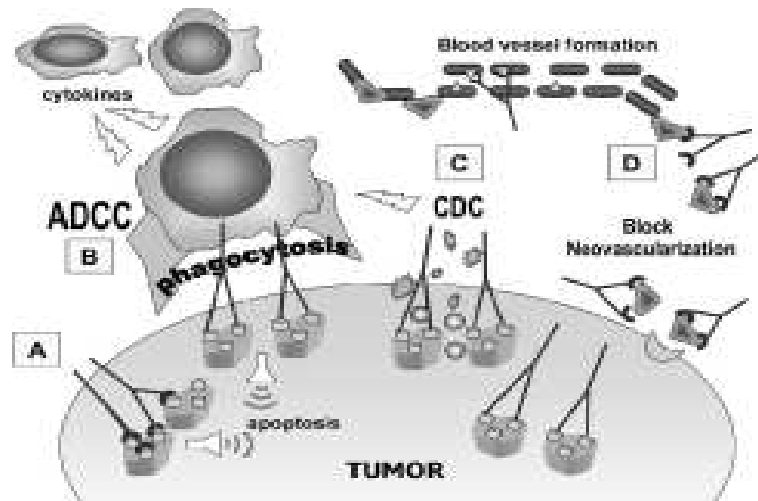
FIGURE 2 Mechanisms of action associated with antibodies. In this example, the antigen is shown to befloating in lipid rafts within the tumor cell membrane. (A) Antibodies can activate apoptotic signals by cross-linking antigen,particularly across different lipid rafts. Additional cross-linking of antibody by immune cells can also enhance cellular signaling. (B) Immune cells themselves can attack the antibody-coated cell (eg, phagocytosis), and/or they can liberate additional factors, such as cytokines that attract other cytotoxic cells. (C) If antibodies are positioned closely together, they can initiate the complement cascade that can disrupt the membrane, but some of the complement components also are chemo-attractants for immune effector cells and stimulate blood flow. (D) Tumors also can produce angiogenic factors that initiate neovascularization. Antibodies can neutralize these substances by binding to them, or they can bind directly to unique antigens presented in the new blood vessels, where they could exert similar activities.

1.3 CONVENTIONAL AND RECOMBINANT MONOCLONAL ANTIBODIES

The antibody response to a typical antigen is highly heterogeneous, so conventional antiserums contain mixtures of antibodies. Theoretically, a single lymphocytes cell (the precursor of plasma-cells) would be a source of large amounts of identical antibody: unfortunately antibody secreting cells can not be maintained in a culture medium. However there are malignant tumors of the immune system called myelomas, whose rapidly proliferating cells can be cultured indefinitely. In 1975 Cesar Milstein and

Georges Kohler were able to fuse mouse myeloma cells with lymphocites from the spleen of mice immunized with a particular antigen (Kohler and Milstein, 1975). The resulting hybrid-myeloma or "hybridoma" cells express both the lymphocyte's property of specific antibody production and the immortal character of the myeloma cells. Individual hybrid cells can be cloned and each clone produces large amount of identical antibody to a single antigenic determinant. Highly specific monoclonal antibodies produced by this general method become at the same time a fundamental tool for both functional and molecular biological studies. The first monoclonal antybody approved for human treatement (1986), is a murine Mab directed to human CD3 for renal allograft rejection. This and other early murine Mabs were hampered by toxicity and limited efficacy resulting from a high incidence of adverse immune-mediated events (Kuus-Reichel K et al., 1994), poor effector activity and rapid intrinsic clearance due to weak interaction with human FcRs (Ober RJ et al., 2006), and difficulties associated with large-scale production of grade material This immune-mediated events are a consequence of a potent human anti-mouse antibody response (HAMA) to foreign protein constituents of murine Mabs. (Reynolds et al., 1989).The techniques of monoclonal antibody production and recombinant DNA technology tried to resolve these problems. The primary strategy for overcoming these limitations was the develop antibodies with a higher continent of human protein sequence. The first successful approach to engineering was chimerization, whereby the V regions of non-human Mab are grafted onto human C regions replacing the native human V segments (Figure 3). Nonetheless human-anti-chimeric antibody-response (HACA) to these chimeras is observed in clinical use, (Mascelli M.A., et al 2007), although the impact of the response on the pharmacology and safety of the agent it not always clear and is debated

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(Clark M., 2000). As a further step, the antigen binding sites, which are formed by three "Complementary Determining Regions" (CDRs) of the heavy chain and by three CDRs of the light chain are excised from cells secreting rodent MAb and "grafted" into the DNA coding for the framework (FW) of the human antibody (Jones et al.1986; Vaswani et al., 1998). The resulting humanized antibodies are less immunogenic than the first generation chimeric antibodies. However during the immunization process the antibody affinity is frequently reduced. This reduction in affinity might be minimized by reintroduction of important murine FW residues back into the engineered antibody (Riechmann, 1988). After repeated administration of therapeutic mAbs elicited a human-anti-human antibody response (HAHA). (Szolar OHJ. Et al., 2007). Therefore accurate methods for the timely detection of HAHA response are mandatory for clinical trials to ensure the patients safety. Although the humanization of antibody might result sometime in an efficacious strategy, it involves some practical limitations, such as the cost and the laboriousness of required methods, as well as the necessity to do in any case the monoclonal antibody with desired specificity. (Vaughan et al., 1998).
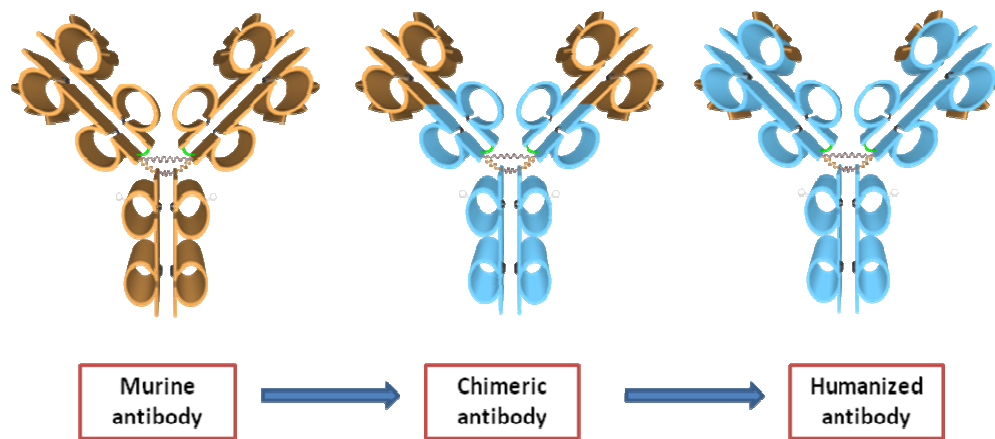
FIGURE 3 Engineered antibody development. In red and in blu are represented murine and human portions respectively

An alternative strategy for producing fully human mAbs is offered by transgenic mice (xenomice) (Figure 4) (Vaughan TJ et al., 1998). This technology, which ultimately remains dependent on immunization, involves two prior in vivo genetic manipulation: inactivation of the endogenous mouse Ig genes and the subsequent introduction of unrearranged human Ig gene segments. (Bruggemann M et al., 1997). Because the human Ig loci are extremely large and complex structures comprising hundreds of genes spanning several Mb of DNA, large fragments of these loci must be introduced into the mouse germ line if functional human Mabs are to be produced. Essentially, this has been achieved using either minigene constructs where up to 80 Kb of a limited number of cloned human Ig genes are artificially juxtaposed or through yeast artificial chromosomes (YACs) that enable the cloning of very large contiguous Ig gene fragments that can be over 1Mb in size. To achieve maximal expression of the human antibody transgenes, it also essential to silence the endogenous Ig expression prior to

introduction of the human loci. (Bruggemann M et al., 1996). This has been achieved by knockout of critical mouse V-genes in embryonic stem cells, thereby blocking B-cell development and endogenous antibody production. Consequently the levels of human Ig in the serum of transgenic mice are typically around 100 µg/ ml, but levels up to 800 µg/ ml have also been reported (Mendez Mj et al., 1997).
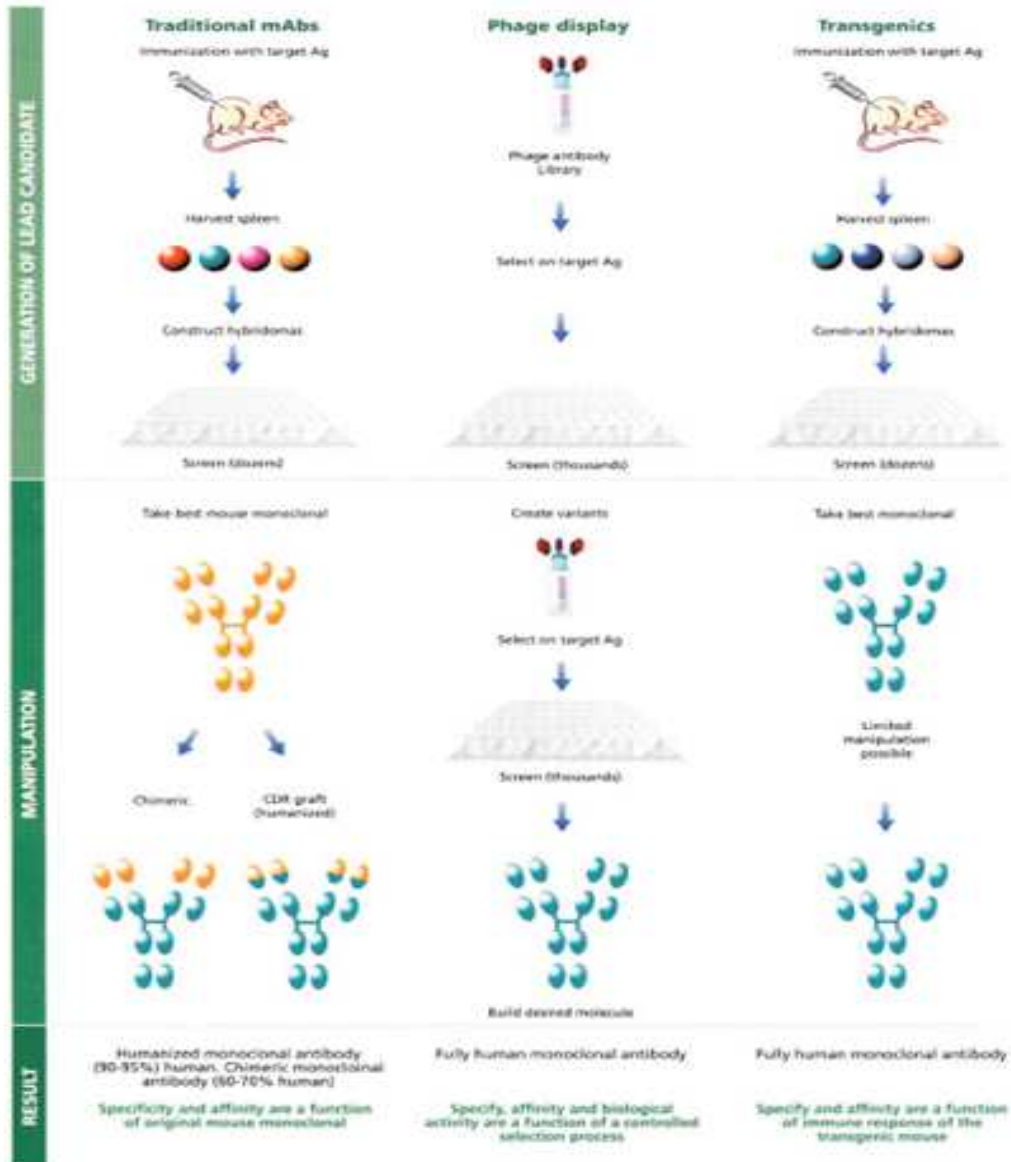
FIGURE 4 comparison of the different approaches for producing monoclonal antibodies. With traditional or transgenic technology, the mAB is a whole IgG. In phage display, the panel of lead Mabs are either in scFv or Fab format. This facilitates further engineerin for desired characteristics and this may take from zero (none necessary) to several months.

1.4 ANTIBODY PHAGE DISPLAY TECHNOLOGY

Display of antibody fragments as F(ab)s or scFvs on filamentous phage was first described in 1990, the first human antibodies were generated soon after. Apart from the completely human nature of the antibodies, at the time the maior advantage of phage display was viewed as the speed to Mab isolation, with antibody selection and ELISA screening taking just 1-2 weeks.

1.4.1 Phage display technology

Phage display is a powerful technology which allows to select a particular phenotype (for example a protein which specifically bind to an antigen) from repertoires of proteins displayed on phages. The technology was originally described in 1985 by Smith (Smith, 1985; Smith, 1991) who presented the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power of such methodology was further enhanced by groups of Winter (McCaferty et al., 1990) and Wells (Lowman et al., 1991), who demonstrated the display of functional folded proteins on the phage surface. The technology is based on the fact that a polypeptide (capable of performing a function, typically the specific binding to a target of interest) can be displayed on the phage surface by inserting the gene coding for the polypeptide in the phage genome. It is possible to create repertoires of phages, called phage display libraries, in which the proteins displayed on each phage are slightly different from each other. If one is able to purify from this large repertoire a phage particle by virtue of the phenotype (for instance the binding specificity) displayed on its surface, then he also isolates the genetic information coding for the binding protein, and he can amplify the corresponding phage by bacterial infection. (figure 4). As an

example, let us consider the selection of a binding specificity from repertoires of binders. The library on phages is biopanned against the antigen of interest; unbound phages are discarded, while specifically binding phages are collected and amplified in bacteria. Several rounds of selections can be performed (Figure 5).
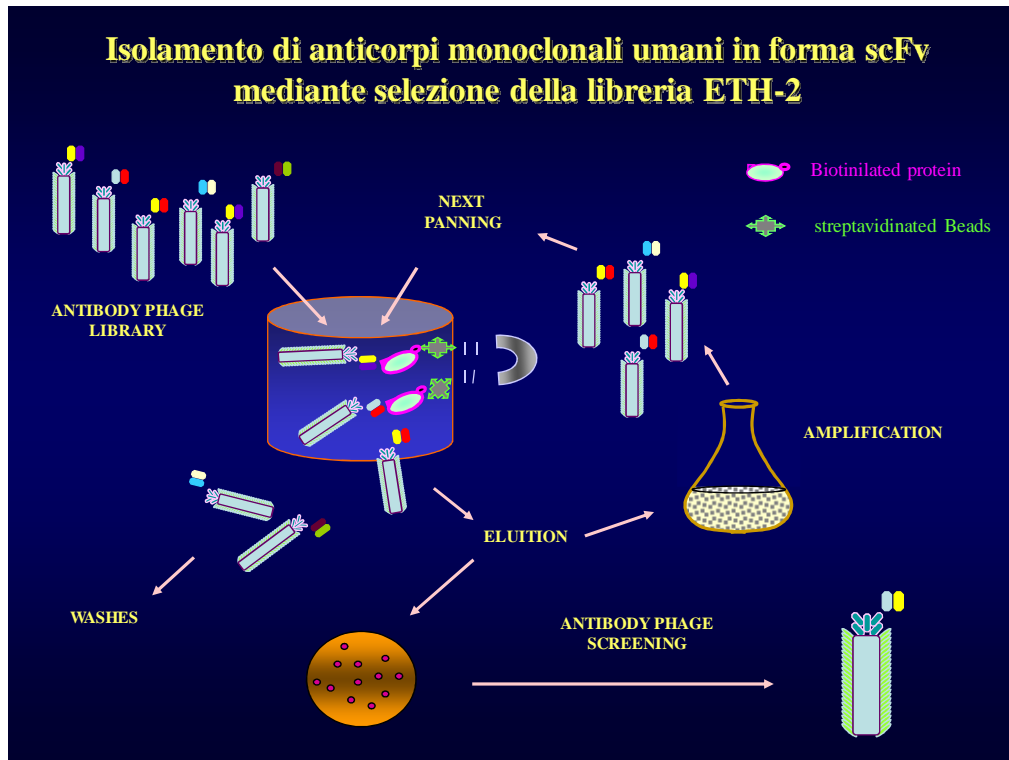

FIGURE 5 Selection of a binding specificity from a phage display library

As a consequence, even very rare phenotypes present in large repertoires can be selected and amplified from background of phages carrying undesired phenotypes. Phage remains infective when treated with acids, bases, denaturants and even proteases. These properties allow a variety of selective elution protocols and have been used for applications other than selection for binding, such as the selection of proteins with

altered thermal stability (Bothmann and Plückthun, 1998) or the selection of catalytically active enzymes (Demartis et al., 1999). Filamentous phage infects strains of E. coli that harbours the F conjugative episome (for a phage biology review see Webster et al., 1996). Filamentous phage particles are covered by approximately 3000 copies of a small major coat protein (pVIII). The minor coat protein pIII, the product of gene III, is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus. Peptides and/or proteins have been displayed on phage as fusions with the coat proteins pIII (Smith, 1985) or pVIII (Greenwood et al., 1991). The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pVIII or pIII fusions and an antibiotic resistance gene). Phagemids, a more efficient and popular vector for display, are plasmid vectors that carry gene III with appropriate cloning sites and a phage packaging signal (Hoogenboom et al., 1991; Hust and Dubel, 2005). Phagemids encoding the polypeptide-pIII fusion are preferentially packaged into phage particles using a helper phage that contains a slightly defective origin of replication, such as M13K07 or VCS-M13, which supplies all the structural proteins. The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion, encoded by phagemid. Depending on the type of phagemid, growth conditions used and the nature of the polypeptide fused to pIII, ratios of (polypeptide-pIII) : pIII ranging between 1: 9 and 1: 1000 have been reported (Bothman and Plückthun, 1998; Demartis et al., 1999).

1.4.2 Phage display of antibody fragments

Phage antibody technology refers to the display and use of repertoires of antibody fragments on the surface of bacteriophages. Antibody fragments, containing at least the VH and VL of a full immunoglobulin, can retain the binding specificity of the parental molecule. Different formats of recombinant antibody fragments can be expressed on phage surface (Figure 6). In Fab fragments the association of the variable domains is stabilized by the first constant domain of the heavy chain and the constant domain of the light chain. More often antibodies are displayed on phage as single-chain Fv fragments (Barbas, 1995). scFvs consist of a single polypeptide chain, including an antibody heavy chain variable domain (VH) linked by a flexible polypeptide linker to a light chain variable domain (VL). The most frequently used format, VH-(Gly4Ser)3-VL has been used extensively for the construction of phage libraries (Clackson et al., 1991; Marks et al., 1991). For practical applications, scFv are generally preferred to Fv fragments, since the polypeptide linker prevents the VH and VL from falling apart. scFv and Fv fragments are the smallest antibody fragments that conserve the same binding affinity (although not the avidity) of the parental immunoglobulin (Neri et al., 1995). They have a molecular mass of approximately 30 kDa and are not glycosylated.
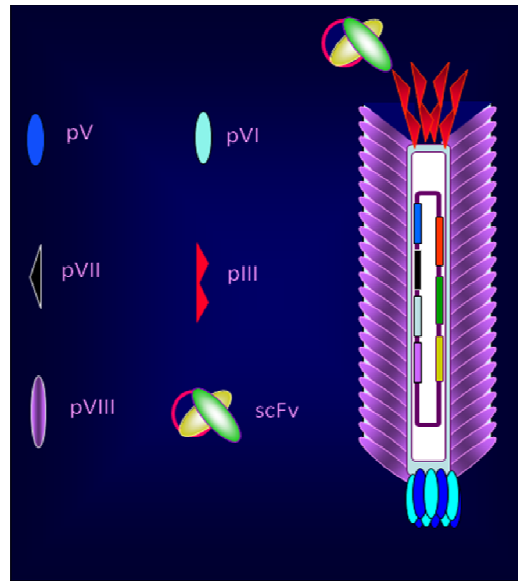
FIGURE 6 scFv displayed on phage M13 surface

1.4.3 Antibody repertoires

In 1990, the display of a scFv on the surface of the filamentous phage was demonstrated, together with the possibility of selecting specific antibodies from a mixture of phages with irrelevant binding specificity (McCafferty et al., 1990). Phage display libraries of recombinant antibodies are artificial immune systems that reliably yield specific monoclonal antibody fragments in 1-2 weeks of experimental work, provided that a small amount of pure antigen is available (Winter et al., 1994). From repertoires of billions of different binding specificities on phages is therefore possible to obtain specific recombinant antibodies without immunisation and against both foreign and self-antigen. The fragments are secreted into the bacterial periplasm and culture medium and can be produced on a large scale (Carter et al.,1992). Like in immune systems, the recombinant antibodies of a phage display library have a common scaffold

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and diversity is inserted in the positions which determine the specificity of binding. Therefore, CDR3s are the positions in which diversity is usually concentrated. There are different ways to create diversity when building an antibody phage display library; however they all rely on the possibility to harvest VH and VL genes by PCR performed with primers matching the genes. Thanks to the extensive characterisation of the V-genes and their flanking regions, several sets of "universal" PCR primers have been described for the cloning of human (Marks et al., 1991; Tomlinson et al., 1992), murine (also usable for rat) (Clackson et al., 1991; Kettleborough et al., 1993), rabbit (Lang et al., 1996) or chicken (Davies et al., 1995) V-genes repertoires. On the basis of the strategy followed to obtain diversity, the antibody phage display libraries can be classified in:

Immune repertoires   (antigen-biased)

Single-pot libraries   (antigen-unbiased)

Immune antibody phage display libraries

Immune antibody phage display libraries take advantage of the diversity created in vivo by the immune system: in this case the source of variable immunoglobulin genes are B-cells from an animal immunized with antigen of interest or an immune patient (Clackson et al., 1991; Chester et al. 1994).The resulting libraries are enriched in antigen-specific immunoglobulin domains, some of which have already been matured by the immune system, and may therefore yield high-affinity antibodies even when the library size is not spectacular (e.g., 10^7 clones). Plenty of examples (referenced in de

Haard et al., 1998) of monoclonal antibodies isolated from immune phage display libraries have been reported so far. Immune libraries were used to retrieve antibodies to 'difficult' antigens, including native T-cell receptor (TCR)-Vα and specific major histo-compatibility complex (MHC)/peptide. Recently, even the derivation of repertoires from rabbit (Lang et al., 1996) and chicken (Yamada et al., 1996) were reported, both describing the isolation of high affinity antibody fragments. There are some disadvantages in isolating antibodies from immune repertoires. When the source of V genes is an immunized animal, the resulting antibodies are not human and therefore potentially immunogenic. Animal immunization and library construction are necessary for each individual antigen, making the whole procedure long and somewhat labour intensive. However, the isolation of human anti-tumour antibodies from phage repertoires of antibodies derived from cancer patients immunized with autologous tumour cells (Cai et al., 1995), or from their tumour-draining lymph nodes is a powerful strategy for the isolation of novel tumour-associated binding specificities (Rothe et al., 2004).

Single-pot libraries

Single-pot libraries contain virtually all possible binding specificities and are not biased for a particular antigen. They are cloned once, with the aim to reach a complexity $>10^8$ clones and, if possible, $>10^9$-10^{10} clones. The corresponding phages are stored frozen in aliquots and can directly be used in panning experiments against a variety of different antigens. In general, both library design and library size contribute to the performance of the library, and to the quality of the antibodies isolated. Larger libraries have higher probability of containing high affinity antibodies (Griffiths et al., 1994; Vaughan et al.,

1996). It is technically possible to make phage-display libraries of complexity $>10^9$ using brute force electroporation, and $>10^{11}$ using combinatorial infection and cre-lox mediated recombination (Griffiths et al., 1994). However, the combinatorial diversity that can in practice be explored in panning experiments is limited by several factors, including the solubility of phage particles (typically 10^{13} transforming units/ml), the efficiency of antibody display on phage, and the phage recovery yields in biopanning experiments (de Haard et al., 1998). Single-pot libraries can be classified as naive or synthetic repertoires.

☞ Naive repertoires

In this case V-genes are isolated from unimmunized animals or human donors, and are combinatorially assembled to create large arrays of antibodies (Rojas et al., 2005). The murine naive repertoire has been estimated to contain $<5 \times 10^8$ different B-lymphocytes, while the human repertoire may be a hundred to a thousand times bigger (Winter et al., 1994). This array of antibodies may be cloned as a "naive" repertoire of rearranged genes, by harvesting the V genes from the IgM mRNA of B-cells isolated from Peripheral Blood Lymphocytes (PBLs), bone marrow or spleen cells. Several naive human antibody phage libraries have been cloned so far. The first library of Marks et al. (Marks et al., 1991) was made from the PBLs of two healthy human volunteers and has yielded several antibodies with different specificities, including scFv fragments directed against cerbB2, a tumour-associated antigen over-expressed in approximately 30-40% of breast cancers, as well as in other solid tumours (Schier et al., 1995). Recently others large naive libraries have been reported to yield high-affinity antibodies against protein antigens (Vaughan et al., 1996; Sheets et al., 1998). While it is by now clear that high-

affinity antibodies can easily be isolated from large naive libraries if the corresponding pure antigen is available, the main disadvantage is that the content of the library is largely unknown and uncontrolled.

☞ Synthetic repertoires

In synthetic repertoires, antibodies are entirely created outside their natural host. To construct a synthetic antibody library, V-genes are typically assembled by introducing randomized CDRs into germline V-gene segments (Hoogenboom et al., 1997). Since the HCDR3 is the most diverse loop, in composition, length and structure, this is the region which is usually chosen for partial or complete randomization. The choice of the germline V-genes into which one can insert combinatorial diversity can greatly vary. The variable regions of human antibodies are assembled from 51 different VH germline genes (Chothia et al., 1992) and 70 different functional VL segments (40 Vκand 30 Vλ; Tomlinson et al. 1995). One can choose to use only one type of scaffold, based on qualities of the scaffold (Pini et al., 1998), or keep one of the heavy or light chains constant and use the different scaffolds of the other one as much as possible (Nissim et al., 1994), or take full advantage of the diversity of the scaffolds and combine the different heavy and light chains as much as possible (Griffiths et al.,1994).Since not all of the different chain variants are equally well represented in the functional repertoire, there might be a disadvantage using such a great variation of scaffolds. Indeed there is evidence that only a few germline V-genes dominate the functional repertoire (Tomlinson et al. 1995). By using scaffolds that are not often represented amongst the binders, library diversity would be wasted. Several synthetic antibody repertoires have been reported so far, and are described in de Haard et al. (1998). The strategies range

from using all the different heavy and light antibody scaffolds available, to keep the light chain constant and use all the heavy chain variants, or to use just one specific heavy chain and one specific light chain (Silacci et al., 2005). The library that we use, called ETH-2 library and described in detail in materials and methods, makes use of only one heavy chain variant (DP-47) and two light chain variants (DPK-22 and DPL-16) based on the fact that these chains dominate the natural functional antibody repertoire (Kirkham et al. 1992) and that they are commonly found in antibody fragments selected from naive libraries.One of the main advantage of synthetic antibody phage display libraries, is that the content of the library (antibody structure, codon usage, knowledge of the antibody portions that are randomised and of those that are kept constant) is defined a priori. Moreover, since antibody genes have not undergone any immunological selection, the library is not biased against self antigens. Indeed, synthetic libraries have already yielded good-quality antibodies against conserved antigens such as calmodulin (Griffiths et al., 1994), the ED-A (Borsi et al., 1998) and ED-B domain of fibronectin (Carnemolla et al., 1996) or against "difficult" antigens such as BiP (Nissim et al., 1994).

1.5 ENGINEERED ANTIBODY FRAGMENTS

IgGs have been dissected into constituent domains, initially through proteolysis, with such enzyme as papain and pepsin, and fragments obtained later are genetically engineered into either monovalent or bivalent fragments. The nature and size of the immunoglobulin or its smaller constructs, will determine how quickly it reaches the target antigen and clears from the blood, and the extent, penetration, and duration of its

binding to the tumor *vs.* normal tissues. IgG, clears very slowly from the blood, requiring several days before a sufficient amount leaves the circulation to achieve a specific concentration in the tumor *vs*. blood and adjacent tissue. Its slow clearance is in part due to its large size, ~150 kD, which impedes its extravasation, resulting in a slow tumor accretion (maximum tumor uptake achieved within 1–3 days). As the molecular size of an antibody is reduced from a divalent F(ab')$_2$ fragment (~100 kDa) to the monovalent binding Fab fragment (~50 kDa), there is a progressively faster clearance from the blood, and the maximum tumor uptake is achieved more quickly and often with better tumor penetration, but this at the cost of having proportionally less of the injected product reaching the tumor and with a commensurately shorter residence time (Buchsbaum DJ 1995; Sharkey RM et al 1990). Molecular engineering has provided even smaller antibody structures, such as scFv (~25 kDa), which are cleared more rapidly from the blood, and have an even lower uptake and shorter retention in tumors. However, the rapid clearance of these molecules from the blood and adjacent, antigen-negative tissues, can result in early, high tumor to-background ratios, achieving relatively strong signals compared to background. (Batra SK, et al., 2002; Wittel UA et al., 2005). Among different monovalent fragments there are (figure 7):

- **Fab**: a 50 KDa fragment, constituted by CH1,CL constant domains and VH, VL variable domans, showing improved pharmacokinetics for tissues penetration (Holliger P and Hudson P, 2005)

- .**scFv:** (single chain fragment variable) consist of a single polypeptide chain of 30 KDa, including an antibody heavy chain variable domain (VH) linked by a flexible polypeptide linker to a light chain variable domain (VL). The most

frequently used format, VH-$(Gly_4Ser)_3$-VL has been used extensively for the construction of phage libraries (Clackson et al., 1991; Marks et al., 1991).
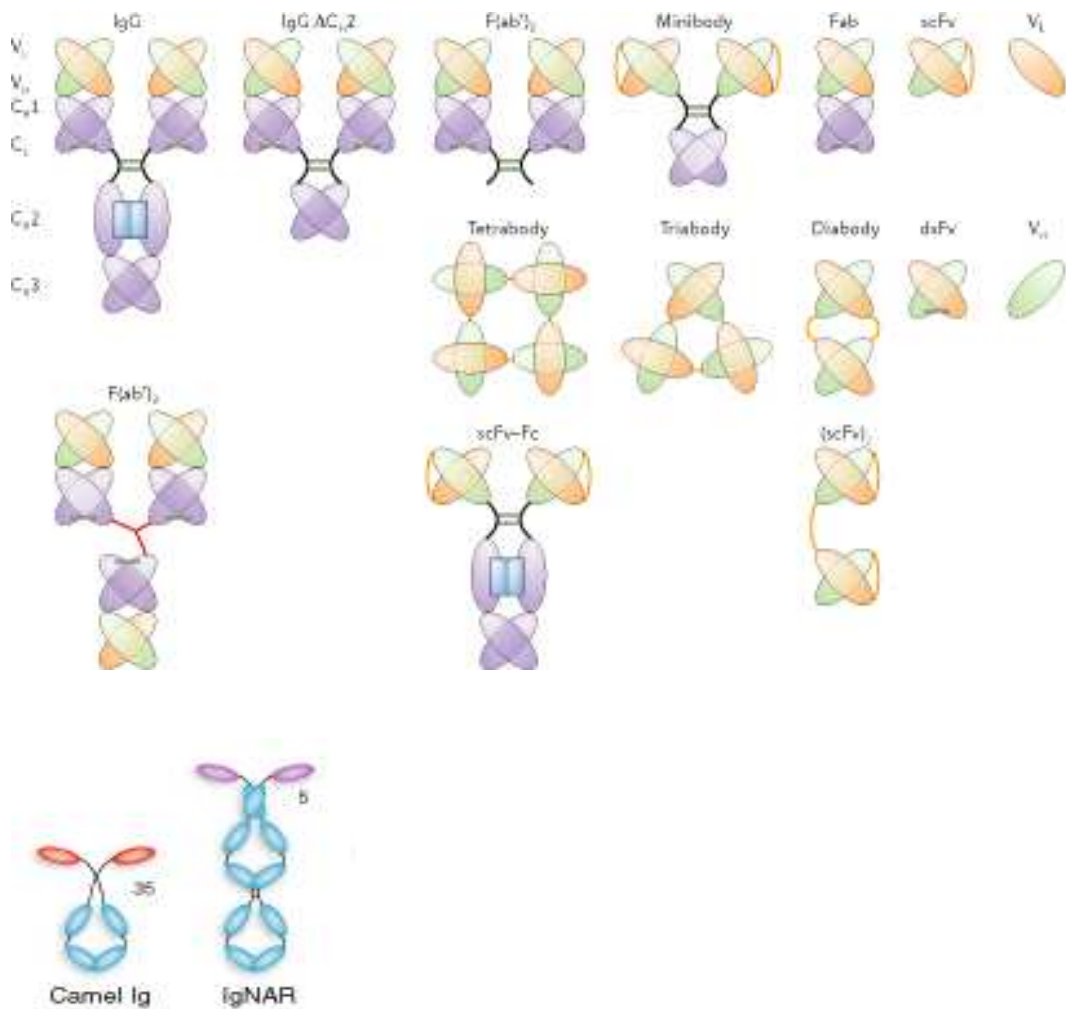

FIGURE 7 Different engineered antibody fragments. The modular domain architecture of immunoglobulins has been exploited to create a growing range of alternative antibody formats that spans a molecular-weight range of at least 12–150 kDa and a valency (n) range from monomeric (n = 1), dimeric (n = 2) and trimeric (n = 3) to tetrameric (n = 4) and possibly higher12,16. For simplicity, all antibody formats are shown as being monospecific: that is, having one or more copies of identical antigen-binding sites. However, formats with a valency of two or more have also been used to create antibodies that have two or more (up to the valency of the format) distinct antigen-binding sites, which bind different antigens or different epitopes on the same antigen.

- **Single V-type domains** : it was discovered that at least two types of organisms, the camelids (Bactrian camels, dromedaries and llamas), (Hamers-Casterman et al., 1993) and cartilaginous fish (wobbegong and nurse sharks)(Greenberg et al., 1995) have evolved high affinity single V-like domains (called VhH in camelids and V-NAR in sharks) mounted on an Fc equivalent constant domain framework as an integral and crucial component of their immune system. This antibodies, called also "*nanobodies*", (Cortez-Retamozo V et al., 2004) devoid of light chain, and their heavy chain lack the CH1 domain, (figure 7) each display long surface loops, often larger than for conventional murine and human antibodies, and are able to penetrate cavities in target antigens, such as enzyme active sites (for example lysozyme) and canyons in viral and infectious disease biomarkers (including malaria apical membrane antigen-1),(Holliger et al., 2005). Methods to isolate antigen-specific VHHs from immune, non-immune, or semisynthetic libraries (Harmsen et al., 2007) using phage,yeast or ribosome display are now well estabilished (Muyldermans 2001, Dufner et al., 2006)

Bivalent fragment bind to two different epitopes usually on distinct antigens, as the intact immunoglobulin. F(ab')₂, diabodies, minibodies, are some examples of bivalent formats (figure 7) Moreover these frangments can be ulteriorly engineered to generate recombinant bi-specific fragments (BsAb). They bind to two different epitopes usually on distinct antigens. Many of these products are now in clinical and preclinical trials. (table 2)

Table 2 Fragment type/ format	Brand name	Target antigen	Stage	Indication
Fab/ chimeric	ReoPro (abciximab)	GpIIb/gpIIa	FDA approved	Cardiovasclar desease
Fab /ovine	CroFab	Snake venom	FDA approved	Rattlesnake bits (antidote)
Fab /ovine	DigiFab	Digoxin	FDA approved	Digoxine overdose
Fab /ovine	Digibind	Digoxin	FDA approved	Digoxine overdose
Fab /mouse	CEA-scan (arcitumomab)	CEA	FDA approved	Colorectal cancer imaging
Fab/humanized	Lucentis	VEGF	Phase 3	Acular degeneration
Fab/humanized	THROMBOVIEW	d-dimer	Phase 1	Deep vein thrombosis imaging
Fab/PEGhumanized	CDP791	VEGF	Phase 1	Cancer (antiangiotensin)
Fab/PEGhumanized	CDP870	TNF alfa	Phase 3	Crohn desease
Fab/bispecific humanized	MDX-H210	Her2/Neu	Phase 2	Breast cancer
Scfv humanized	Pexelizumab	Complement C5	Phase 2\3	Coronary artery bypass
Scfv fused to b-lactmase h	SGN-17	P97 antigen	preclinical	Melanoma ADEPT
Scfv fused to PEG human	F5 scfv PEG immunoliposmr	Her 2	Preclinical	Breast cancer as drug targeting
Diabody human	C6.5K-A	Her 2\Neu	preclinical	Ovarian and Breast cancer
Diabody human	L19 L19-gamma IFN	EDB domain fibronectin	Preclinical	Antiangiogenesis and atherosclerotic Plaque imaging
Diabody human	T84.66	CEA	Preclinical	Colorectal cancer imaging
minibody human	T84.66	CEA	Preclinical	Colorectal cancer pretherapy
Minibody murine-human chimera	10H8	Her 2	Preclinical	Ovarian breast cancer
Scfv dimer Fc	T84.66	CEA	Preclinical	Colorectal cancer
Bispecific scfv	r28M	CD28 and MAP	Preclinical	Melanoma (MAP antigen)
Bispecific scfv	BiTE MT103	CD19 and CD3	Phase 1	B cell tumor
Bispecific scfv	biTE	Ep-CAM and CD3	Preclinical	Colorectal cancers
Bispecific tandem diabody	Tandab	CD19 and CD3	Preclinical	B cell tumor
VhH- b-lactmase fusion camelid	Nanobody	CEA	Preclinical	Cancer imaging
Dab\ human	Anti TNF alfa dAB	TNF alfa	Preclinical	Rheumatoid arthritis and Crohn desease
VhH\ camelid	Nanobody	TNF alfa	Preclinical	Rheumatoid arthritis and Crohn desease
VhH\ camelid	Nanobody	Von Willebrand factor	Preclinical	antithromotic

2 IMMUNOTHERAPY

The goal of cancer therapy remains as the long-term eradication of tumor cells without adverse effects on normal tissue. Conventional approaches utilizing chemotherapy and radiotherapy are limited by both their toxicity and lack of specificity. Antibodies are finally realizing their potential as anticancer. Several strategies are being explored to increase the efficacy of such antibodies, including enhancement of effector functions, direct and indirect arming, and pre-targeting of prodrugs or radionuclides (Figure 8). In addition, potent antitumour activity might be achieved with antibodies that prevent soluble growth factors from binding to their cognate receptors, such as the epidermal-growth-factor receptor (EGFR), (Yang, XD. et al., 1999)10 and ERBB$_2$, (Agus, D. B. et al 2000; Fitzpatrick VDet al., 1998). Promising and potentially complementary alternative strategies to direct tumour targeting include targeting tumour vasculature, angiogenic growth factors and their receptors (Halin, C. & Neri, D, 2001).
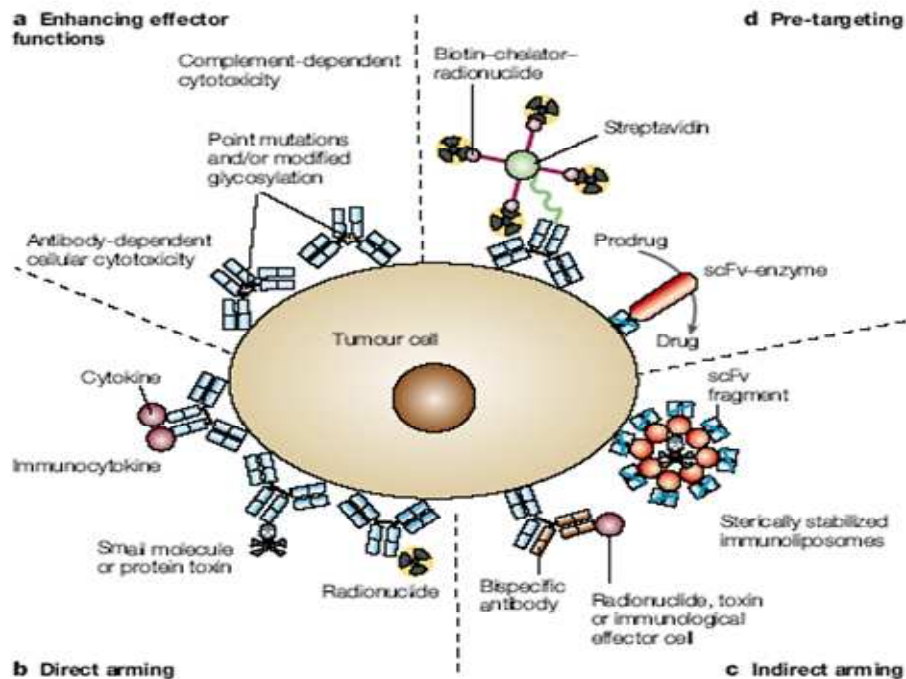
Figure 9 Strategies for enhancing the potency of antitumour antibodies. Numerous strategies for improving the efficacy of antitumour antibodies are now being tested, including the representative examples shown here . a | Enhancing effector functions involve improving antibody-dependent cellular cytotoxicity 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.

2.1 ENHANCING EFFECTOR FUNCTIONS

Human antibodies of the IgG1 and IgG3 isotypes can potentially support the effector functions of antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) as described before in the text. The first demonstration that the Fc–Fcγreceptor interaction is important for the antitumour activity of an antibody *in vivo* came with the development of mice that lack FcγRI and FcγRIII (Clynes R et al., 1998). An anti-melanoma antibody had potent antitumour activity in a mouse model of metastasis, but this benefit was lost in mice that lack FcγRI and FcγRIII receptors. ADCC is likely to be the mechanism underlying the antitumour effects of the Fc–Fcγreceptor interaction. Alternatively antitumour activity of an antibody by manipulating the Fc region to increase its affinity for the activation receptor(s) and/or by abrogating its ability to bind to the inhibitory receptor. Indeed, point mutations in the Fc region,which result in improved binding to FcγRIII, yielded up to a twofold enhancement in ADCC *in vitro* (Shields RL et al. 2001). The *in vivo* and clinical significance of this *in vitro* improvement is unknown. Moreover glycosylation plays an important role in a number of therapeutic proteins including monoclonal antibodies. For example Glycosylation of IgG molecules at Asn 297 helps to maintain the tertiary structure of their CH2 domains36, and is necessary for effector functions (Wright A and Morrison SL, 1997). The cells producing the antibody (the 'production host') and, to a lesser extent, culture conditions, can significantly affect the resulting antibody glycoforms which, in turn, can influence the ability of the antibody to participate in ADCC (Lifely MR, 1995).

2.2 DIRECT ARMING

The most widely explored strategy for enhancing the efficacy of antitumour antibodies is direct arming by covalent linkage to toxins or radionuclides (*Sharkey et al 2008*). Armed antibodies typically show more potent antitumour activity in preclinical tumour xenograft studies than their 'naked' parents. Radionuclides more commonly used conjugated to antibodies for cancer treatment are listed in table 3.

Table 3

Radionuclide	Emission	Half-life	Range	Approximate # Cell Diameters*
^{131}Iodine	B	8.0 d	0.08–2.3 mm	10 to 230
^{90}Yttrium	B	64.1 h	4.0–11.3 mm	400 to 1100
^{177}Lutetium	B	6.7 d	0.04–1.8 mm	4 to 180
^{188}Rhenium	B	17.0 h	1.9–10.4 mm	200 to 1000
^{67}Copper	B	61.9 d	0.05–2.1 mm	5 to 210
^{211}Astatine	A	7.2 h	60 µm	6
^{213}Bismuth	A	46 min	84 µm	8
^{125}Iodine	Auger	60.5 d	<100 nm	(1)
^{111}Indium	Auger	3.0 d	<100 nm	(1)

*Assuming a tumor cell is 10 µm in diameter.

Because the radioactivity can be detected easily by external scintigraphy, it is also noteworthy to mention the additional application of radiolabeled antibodies for imaging. ^{99m}Tc- and ^{111}In-radioconjugates have been commonly used for this application, but with the advent of positron-emission tomography (PET), investigators are now beginning to take advantage of this technologically superior imaging system by radiolabeling tumor-associated antibodies with positron-emitters. (McBride WJ et al., 2006; Goldenberg DM, 1997). The primary concern for using radionuclide labeled IgG is that it remains in the blood for an extended period of time, which continually exposes the highly sensitive red marrow to radiation, resulting in dose-limiting

myelosuppression. Smaller forms of the antibodies, such as a F(ab') or F(ab')$_2$, and more recently, molecularly engineered antibody subfragments with more favorable pharmacokinetic properties, are removed more rapidly from the blood, thereby improving tumor/blood ratios. (Kenanova V et al., 2005). There have been reports of improved therapeutic responses using smaller-sized antibodies, but these smaller entities frequently are cleared from the blood by renal filtration, and as a result, many radionuclides (eg, radiometals) become trapped in a higher concentration in the kidneys than in the tumor (Behr TM et al., 1998). As a consequence of their more rapid blood clearance, the fraction of the injected activity delivered to the tumor is lower with an antibody fragment than with an IgG. When it comes to choices of radionuclides for therapy, tumor size is the primary consideration. Medium-energy beta-emitters, such as ^{131}I (0.5 MeV) and ^{177}Lu (0.8 MeV), can traverse 1.0 mm, while high-energy beta-emitters, such as ^{90}Y or ^{188}Re (2.1 MeV), can penetrate up to 11 mm, making it possible for beta-emitters to kill across several hundred cells, referred to earlier as a *bystander* or *crossfire* effect. (Kassis AI and Adelstein SJ, 2005). Although higher energy beta-emitters have the potential of killing cells across a longer path-length, the absorbed fraction is higher for the lower energy beta-emitters (ie, probability of hitting the nuclear DNA), making them efficient killers. Alpha emitters, such as ^{213}Bi and ^{211}At, traverse only a few cell diameters, but an alpha particle is also a far more efficient (energetic) killer than even a low-energy beta particle, requiring fewer "hits" to damage cellular processes. (Kassis AI and Adelstein SJ, 2005). Low-energy electrons, such as are produced by Auger emitters (^{125}I, ^{67}Ga, or ^{111}In, for example) have to be in close contact, preferably inside a cell or in the nucleus, to exert a cytotoxic effect. Most immunotoxins comprise either a plant toxin, such as ricin A chain, or a bacterial toxin,

for example, Pseudomonas exotoxin, conjugated or genetically fused to an antibody or antibody fragment (Farah RA, et al., 1998; Pastan, I, 1997). Immunotoxins have occasionally been associated with antitumour responses in patients (Kreitman RJ et al., 2000; Pai LH, et al., 1996). Unfortunately, clinical development of immunotoxins has also been plagued with toxicity problems, such as vascular leak syndrome, and by immunogenicity that often precludes multiple dosing. Site-specific pegylation of one recombinant immunotoxin improved its antitumour activity in animal models, and also decreased its immunogenicity and toxicity (Tsutsumi Y. et al., 2000).. Gemtuzumab ozogamicin (Mylotarg) is an example of an antibody conjugated with small toxin, that are 100–1000-fold more potent than conventional chemotherapeutics. Mylotarg is a humanized anti-CD33 IgG linked to calicheamicin, a potent antitumor agent isolated from a bacterium. Conjugation of these small-molecule toxins to antibodies converts them to inactive prodrugs that can selectively target tumors. Activation of these prodrugs involves release of the drug from the antibody and occurs primarily in the tumor following receptor binding to antigen-positive cells and antibody internalization. In the case of calicheamicin, release from the antibody is followed by a chemical rearrangement to create diradicals that can cause double- stranded DNA breaks and compromise cell viability. Calicheamicin contains a sugar component that contributes to its potency by binding to the minor groove of DNA. The humanized anti-CD33 antibody–calicheamicin conjugate Mylotarg has been approved for treatment of CD33-positive acute myeloid leukaemia in first-relapse patients of ≥60 years old and who are not candidates for cytotoxic chemotherapy (Carter P, 2001).

2.3 INDIRECT ARMING

Engineered antibody fragments can be attached to the surface of stealth liposomes for selective tumour targeting of large payloads of drugs (Park, J. W. *et al., 1995; .* Park, J. W. *et al., 1997)*, toxins or even DNA for gene therapy. Such large payloads offer an important potential advantage over direct antibody arming, in which only one or a few molar equivalents of the payload are attached per antibody to avoid compromising antigen binding, conjugate solubility or promoting aggregation. Delivery of chemotherapeutics using immunoliposomes offers substantial benefits over the use of free drugs. For example, anti-ERBB2 immunoliposomes loaded with doxorubicin show greater antitumour activity than free drug or drug loaded in non-targeted liposomes in several tumor xenograft models (Park JW., Hong K, 1997). Moreover, the systemic toxicity of the immunoliposome- targeted doxorubicin was much less than that of free doxorubicin. Despite such encouraging progress with immunoliposomes, several underlying difficulties remain, including their inherent complexity and extravasation due to their large size (commonly ~100 nm in diameter), (Bendas G, 2001). Using immunoliposomes to target tumor vasculature, rather than the tumor *per se*, obviates the need for extravasation. BsAb have potential clinical utility in targeting tumor cells or tumor vasculature with cytotoxic machinery including, immune effector cells, radionuclides, drugs and toxins (figure 8). In clinical oncology, BsAbs have been used most widely for delivering immune effector cells and, to a lesser extent, for delivery of radionuclides, drugs and toxins to tumors (van Spriel, A. B et al., 2000) Abs that bind to a tumor-associated antigen and a so-called trigger antigen on an immune effector cell can recruit the effector cell to kill a tumour cell that it would otherwise disregard. Encouraging local antitumour responses have been seen for BsAb targeting of T cells to

ovarian cancer cells in small-scale clinical trials (Lamers CH, 1997). Unfortunately, treatment failure occurred because of metastasis outside the peritoneal cavity, which was refractory to systemic BsAb therapy. Another common clinical problem associated with BsAb is that the systemic activation of effector cells causes widespread cytokine release, which leads to serious side effects. So, more effective strategies are needed for the targeting of effector cells and selective activation in the context of tumour cells.

2.4 PRETARGETING PRODRUG

Advances in molecular engineering have greatly enhanced the ability to provide uniform and highly novel pretargeting agents (Rossi EA et al 2006; . Lin Y et al.,2006). Other pretargeting approaches have been studied, each showing improved tumor/blood ratios, as well as improving therapy when compared with directly-radiolabeled antibodies (Sharkey RM et al., 2005) Dosimetry data from a pilot clinical study with ^{90}Y-biotin pretargeted by a new recombinant streptavidin-anti-TAG-72 antibody are promising, and in other indications, such as medullary thyroid cancer and glioma, encouraging therapeutic results using pretargeting methods have been reported.(Goldenberg DM   et al., 2006; Chatal J-F et al., 2006). Two anti-CD20 IgG-radioconjugates are currently FDA-approved for the treatment of indolent and transformed forms of NHL, ^{90}Y -ibritumomab tiuxetan (Zevalin, biogenidec) and ^{131}I-tositumomab (Glaxo SmithKline). (Sharkey RM, et al., 2005). Both of these treatments improve the objective response rate compared with the unlabeled anti-CD20 antibody used to deliver the radionuclide (Witzig TE et al., 2002; Davis TA et al., 2004). Initially, there was some concern that while objective response rates were significantly improved, the pivotal trial performed with 90Y-ibritumomab tiuxetan did not show a

statistical improvement in the duration of the response compared with its unlabeled antibody (ie, rituximab). However, continued follow up has shown the complete responses have been more durable (Gordon LI et al 2004; Wiseman GA et al., 2005). Pretargeting approaches also have been applied to drugs. Most often referred to as ADEPT (antibody-directed enzyme prodrug therapy), (Bagshawe KD, 2006)

3 ADEPT (Antibody directed enzyme prodrug therapy)

Antibody-direct enzyme prodrug theraphy was proposed in the mid -1980s as a means of restricting the action of cytotoxic drugs to tumor sites, thereby increasing their efficacy and reducing their normal tissue toxicity. This proposed method of selective drug delivery is two step approach. In ADEPT, selectivity for the target is achieved by an antibody (Ab) in an   Ab-enzyme conjugate that binds antigen preferentially expressed on the surface of tumour cells, or in the tumour interstitium. In the first step, the Ab- complex is administered and accumulates at the tumour site (figure 10).
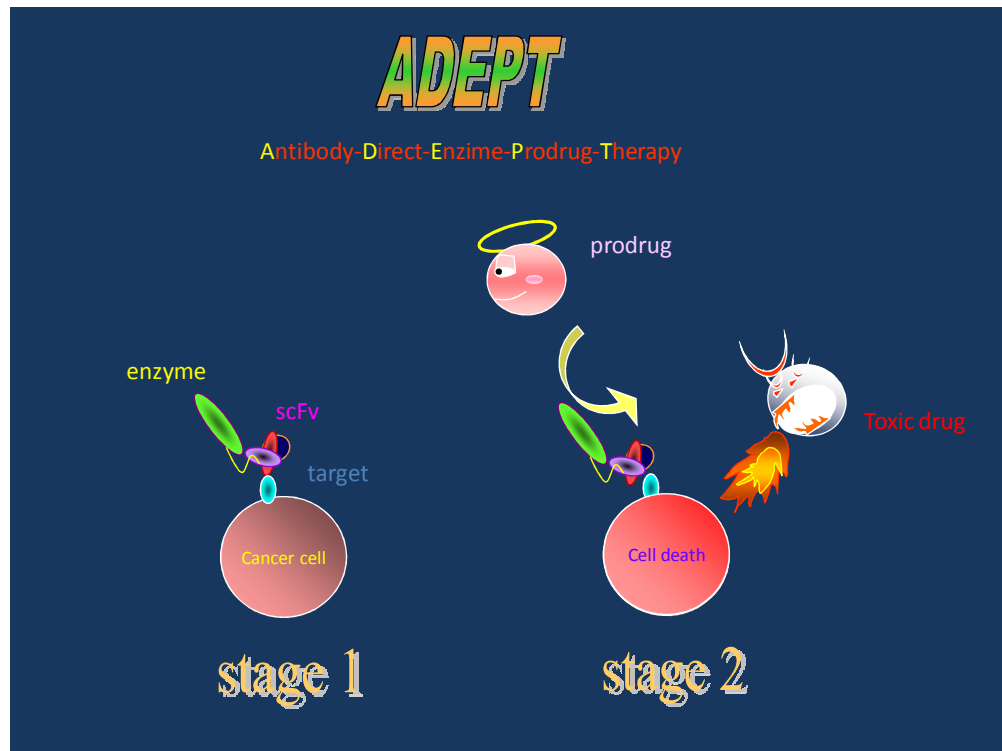
FIGURE 10 ADEPT strategy. A prodrug activated by the enzyme is given when the enzyme has cleared from the circulation but is retained in the tumor, so that active drug is generated selectively in the cancer

Time is allowed for clearance of the conjugate from blood and normal tissues. In the second step, a non-toxic prodrug is injected, which is converted into a cytotoxic drug by the enzyme in the at the tumor (Senter PD and Springer CJ, 2001). An amplification feature of this system means that one molecule of enzyme catalyses the conversion of many molecules of prodrug into the cytotoxic drug. This is an inherent feature of ADEPT, which potentially enables higher drug concentrations at the tumor compared to direct injection of drug alone Another is the bystander effect which effects killing of surrounding tumour cells that do not express tumor. The main drawback of ADEPT is the immunogenicity of the Ab-enzyme conjugates which may preclude the administration of repeated doses of the conjugate. However, up to three courses of

treatment have been administered to patients by the co-injection of the immunosuppressant drug cyclosporine (Springer CJ, Niculescu-Duvaz I I, 1997).

3.1 ENZYME IN ADEPT

There are specific requirements made of the enzymes used for ADEPT. They must be able to catalyse a scission reaction of the prodrug. They should have catalytic properties different from any circulating endogenous enzyme. They should be and stable under physiological conditions. Ideally they should effect high catalytic turnover. It is also of benefit if they are able to activate a panel of anticancer prodrugs. (Springer CJ, Niculescu-Duvaz I I, 1997). The enzymes used for ADEPT can be characterised into three categories: (i) Enzymes of non-mammalian origin with no mammalian homologue, e.g., carboxypeptidase G2 (CPG2); cytosine deaminase (CD); b-lactamase (b-L); penicillin G amidase (PGA); penicillin V amidase (PVA). This avoids activation of the prodrug by endogenous enzymes in blood and normal tissues. These enzymes are readily available on a large scale due to their lack of post translational modification. Also, many have good kinetic parameters. Their main disadvantage is their (ii) Enzymes of non-mammalian origin with a mammalian homologue, e.g., βglucuronidase (β-G). The advantage is that only low levels of this class of enzymes are present in the blood. However the β-G human enzyme is less efficient as a catalyst than the bacterial form: the mammalian form potential to elicit an immune response in humans The human enzyme also has a lower turnover rate. (iii) Enzymes of mammalian origin, e.g., alkaline phosphatase (AP); α-galactosidase (α-g). Their main advantage resides in the reduction of their potential to elicit an immune response. Unfortunately their presence in humans

is likely to preclude specific activation of the prodrugs only in the tumor (Senter PD et al., 1993).

3.2 ANTIBODIES IN ADEPT

The Abs that bind tumor-associated antigens are a key component in ADEPT since they ensure the selectivity of the localisation of prodrug activation The main requirement of Ab-conjugates used in ADEPT is that they must localize on the tumor ideally with high affinity. They should also have minimum binding to normal sites. In addition, the covalent binding of the enzyme must not destroy the ability of the Ab to bind to its associated antigen, nor should it alter the enzyme activity. Ideally, there should be a rapid clearance of the conjugate from body fluids. There are two opposing factors in the penetration of tumors by Ab-enzyme conjugates. Firstly, the blood vessels and interstitium of tumors are more 'leaky' than those of normal tissues which provides advantages for the localization of macromolecules (Jain RK et al., 1988). Secondly, there is inadequate distribution which leads to poor uptake of macromolecules Attempts were made to overcome this limitation by using Ab fragments, e.g., F(ab') , $F(ab')_2$ and scFv, to increase the interstitial rate of transport. These fragments provide better penetration properties than intact Ab and also show more rapid clearance as demonstrated in animals and in patients.

3.3 PRODRUGS IN ADEPT

The poor vascularisation of tumors is a major problem for cancer therapy in general. The delivery and penetration of molecules across the physiological barriers of the tumor

are extremely important for efficacy. Two factors govern the uptake of a compound into the tumor :extraction coefficient by the tumor and the blood flow. The fraction of the prodrug extracted from the blood flow by the tumor depends on its chemical structure (e.g., its lipophilicity) and the properties of the physiological barrier. The prodrugs designed for ADEPT must be less cytotoxic than their corresponding active drugs. The prodrug must also be chemically stable under physiological conditions and have good pharmacological and pharmacokinetic properties. The prodrugs must are be suitable substrates for the activating enzyme under physiological conditions. Most ADEPT prodrugs are derived from well-known anticancer agents or their close counterparts as model molecules. An additional advantage of this choice is that the pharmacokinetic parameters of the drugs are known. One exemple of enzyme-prodrug utilized in ADEPT is the cytosine deaminase (CD)/5FC. This system was designed to take advantage of the conversion of the antifungal agent 5-fluorocytosine (5-FC) into the well known anticancer agent, 5-fluorouracil (5-FU) (De Angelis PM et al., 2006). Another reason for developing this system was for use in human colon cancer which is refractory to many other chemotherapeutic approaches.

3.4 CYTOSINE DEAMINASE

Cytosine deaminase catalyzes the deamination of cytosine to uracil and 5-methylcytosine to thymine. The enzyme has been found in bacteria and fungi, where it plays an important role in pyrimidine salvage. however it is not present in mammalian cells, which utilize cytidine deaminase (CDA) instead (Nishiyama T et al., 1985). The bacterial and fungal CDs are distinct from each other and have evolved separately. The

yeast protein structure is composed of a central five-stranded β -sheet (β1- β5) sandwiched by six α-helices (αA - αF) (figure 11 A) Interestingly, the 426-residue hexameric *E. coli* CD belongs to superfamily, whereas the 158-residue dimeric yeast counterpart is grouped into the CDA superfamily (Ireton gc). The active site of yeast CD contains one tightly bound zinc ion, which is tetrahedrally coordinated by His62, Cys91 , Cys94 , and a bound inhibitor. (figure 11B)The complex structure reveals that yeast CD converts the inhibitor 2-hydroxypyrimidine into 4-(R)hydroxyl-3,4-dihydropyrimidine, which is enantiomeric to the configuration observed in *E. coli* CD. Therefore, the crystal structures of bacterial and fungal CDs provide an excellent example of convergent evolution, in that they have evolved from unrelated ancestral proteins but have achieved the same deamination reaction.
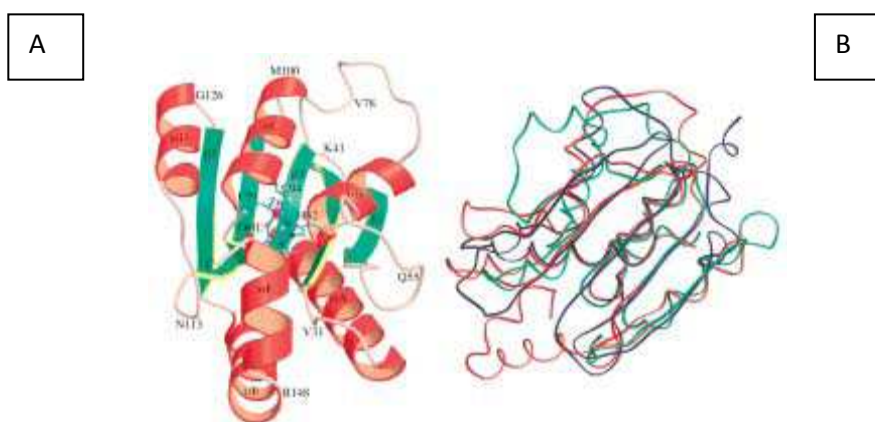

FIGURE 11 (a) The monomeric structure of yeast CD with the zinc ion shown as a magenta sphere with its ligands and the inhibitor (DHU) as ball-and-stick representations. **(b)** Structural superposition of yeast CD (red), *B. subtilis* CDA (blue), and the subdomain 2 of AICAR transformylase (green)

3.5 5-FLUOROURACIL

5-fluorouracil is a chemotherapeutic drug used worldwide in the treatment of metastatic colorectal cancer, either alone or in combination with irinotecan, a topoisomerase I inhibitor. 5-FU is considered to be purely an S phaseactive chemotherapeutic agent, with no activity when cells are in G0 or G1 (Shah MA, Schwartz GK , 2001). It is well-established that treatment of cells with 5-FU causes DNA damage, specifically double-strand (and single-strand) breaks, during S phase due to the misincorporation of FdUTP into DNA (Curtin NJ et al., 1991; Peters GJ et al., 2000). However, damage to DNA can occur in all cell cycle phases in proliferating cells, and the repair mechanisms involved vary in the different phases of the cell cycle. DNA damage checkpoint pathways in G1, S, and G2 couple DNA damage detection to inhibition of cell cycle progression, activation of DNA repair, maintenance of genomic stability, and when damage is beyond repair, to initiation of cellular senescence. The position of tumor cells in the cell cycle and the ability to undergo apoptosis in response to drug treatment together play an important role in the sensitivity of tumor cells to chemotherapy. 5-FU has a complicated mechanism of action with several enzymes involved in its metabolic activation. It inhibits thymidylate synthase as its main mechanism of action, leading to depletion of dTTP. Overexpression of thymidylate synthase has been shown to be associated with 5-FU resistance in colorectal cancer, but it is also likely that other alterations, for example, to crucial genes on cell cycle and apoptotic regulatory pathways, underlie the development of resistance (De Angelis PM et al., 2006).

4 CARCINOEMBRYONIC ANTIGEN (CEA)

The carcinoembryonic antigen (CEA) was first described by Gold and Freedman in 1965 (Gold and Freedmann, 1965). It was hypothesised that CEA was an antigen expressed in colonic tumours and in foetal colon, but not in healthy adult colon. With the development of more sensitive immunoassays, raised circulating CEA was also found in cases of breast, lung, ovarian tumors and it has also proven to be expressed in a few of normal epithelial tissues (Nap et al., 1988). Despite this, CEA continues to play an important role in diagnostic pathology and to be studied as a "tumor associated antigen" involved in metastasis and carcinogenesis.

4.1 CEA STRUCTURE

In mature form CEA is a glycoprotein with a molecular weight (MW) of about 180 kDa containing approximately 50% carbohydrate comprising mannose, galactose, N-acetylglucosamine, fucose and sialic acid (Thompson, 1995) (Figure 12).
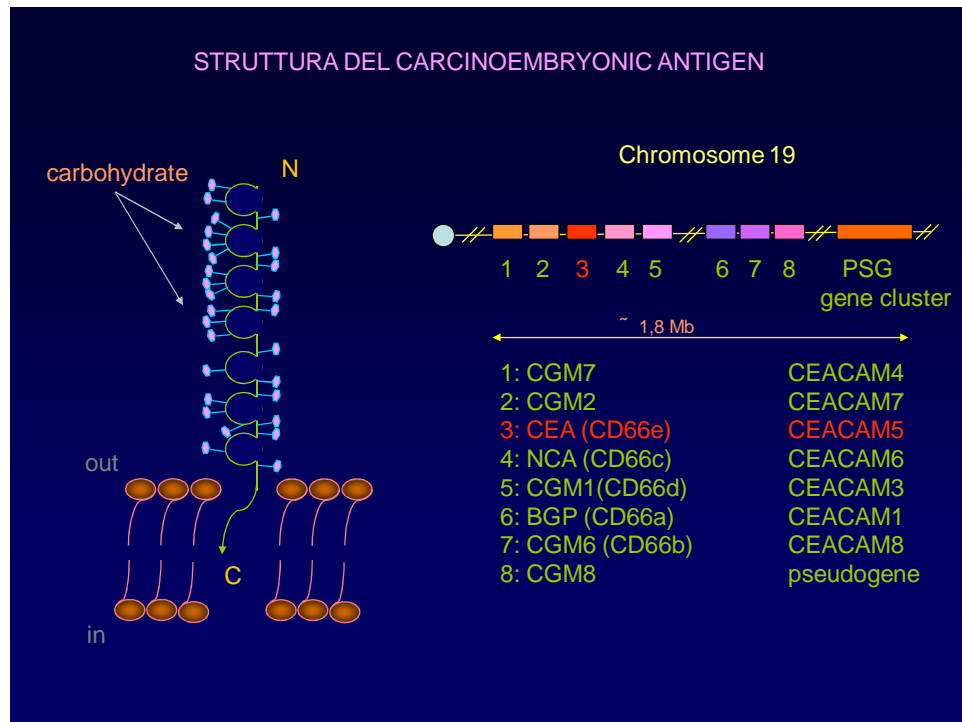
Figure 12. CEA structure and genome organization of the CEACAM genes

CEA is a member of "CEA gene family" clustered on Chromosome 19q, within a 1.8 Mb region. The deduced aminoacid sequence of CEA shows that CEA is synthesized as a precursor of 702 aminoacids, comprising a leader peptide of 34 aminoacids, and a mature CEA polypeptide of 668 aminoacids (Thompson et al., 1991). There is a close correlation between exon expression and the domain structure with the first exon encoding the 5'-untranslated region and the first two thirds of leader peptide. The mature CEA is divided into structural domains based on three internal repeat regions. These are referred to as N, A1B1, A2B2 and A3B3 domains (Thompson et al., 1991). It has long been recognized that CEA is a member of immunoglobulin gene superfamily. This diverse family includes major histocompatibility antigens, together with cell adhesion molecules such as ICAM-1, N-CAM and LFA1 (Johnson et al., 1991). Two types of immunoglobulin domain are found in CEA molecule: an N-terminal domain of

108 aminoacids homologous to the immunoglobulin variable domain (IgV-like) and six domain homologous to the immunoglobulin constant domain of the C-2 set (IgC2-like) (Williams and Barclay, 1988). The common structure of the immunoglobulin gene superfamily is thought to have evolved from a single ancestral unit which has undergone divergence and duplication (Piggot and Power, 1993).

4.2 CEA AND CEA FAMILY GENES

As a consequence of the development of molecular cloning techniques, 28 other genes/pseudogenes related to the CEA gene were discovered and are clustered on human chromosome 19q13.2. (Teglund et al.1994). The family is composed of three branches identified as the CEACAM (CEA related Cell Adhesion Molecule) subgroup, PSG (Pregnancy Specific Glycoprotein) subgroup and a third subgroup containing only pseudogenes (Teglund et al.1994). The genes have been assigned to the three branches of the CEA family on the basis of the homology of their primary sequences, characteristics of their membrane anchors and their patterns of expressions . The CEACAM subgroup contains 7 expressed genes CEACAM1 (or Biliary Glycoprotein, BGP), CEACAM3 (or CEA gene family member 1, CGM1), CEACAM4 (or CGM7), CEACAM5 (or Carcinoembryonic Antigen, CEA), CEACAM6 (or Non-specific Cross-reacting Antigen, NCA), CEACAM7 (or CGM2), and CEACAM8 (or CGM6)] and the PSG subgroup contains 11 expressed genes (PSG1-11) (Beauchemin et al., 1999) (Figure 12). Although CEA was the first gene cloned within this family, the assignment of CEACAM1 gene has been attributed to the BGP gene, owing to its high degree of conservation across the species. CEACAM2 is a mouse gene highly similar to

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CEACAM1 mouse genes, but absent in human and rat. The CEACAM subgroup members are attached to the cell surface membrane, while the PSGs are secreted molecules. The CEACAM subclass are generally anchored to the cell surface either by transmembrane domains (CEACAM1, 3 and 4 in human) or by glycophosphatidylinositol (GPI) lipid moieties (CEACAM5-8) (Beauchemin et al., 1999). Fair data on the tissue distribution are available for CEACAM5 (CEA), CEACAM1 (BGP) and PSG, while only limited information are available for CEACAM6 (NCA), CEACAM7, CEACAM8, and little is known about the tissue distribution of CEACAM3 and CEACAM4. CEACAM1 has the broadest distribution in normal tissues, being expressed in a number of different epithelia (oesophagus, stomach, colon, liver, kidney, cervix, endometrium and so on), in sweat and sebaceous gland, in granulocytes and perhaps in endothelial cells in some organs (Hammarstrom et al., 1997). CEACAM6 has probably also a fairly broad distribution, being present in epithelial cells in different organs and in granulocytes and monocytes (Hammarstrom et al., 1997). In contrast CEACAM5 shows a more limited tissue expression in normal adult tissue, being present in columnar epithelial cells and goblet cells in colon, in mucous neck cells and pyloric mucous cells in the stomach, in squamous epithelial cells of the tongue, oesophagus and cervix, in secretory epithelia and duct cells of sweat glands, and in epithelia cells of the prostate (Hammarstrom et al., 1997; Nap et al., 1988). It would seem that CEACAM7 has a similar distribution to that of CEACAM5, being expressed in certain epithelial cells notably in colon, but not in granulocytes. CEACAM3 and CEACAM8 are expressed in granulocytes but probably not in epithelial cells (Hammarstrom et al., 1997). Members of PSGs subgroup are abundantly expressed in the placenta during embryonic development, but also at others sites of expression

such as uterus, pancreas, testis, and foetal liver. PSGs may have immunomodulatory functions and they are also markers for some disorders of pregnancy (Zhou et al., 1997). A number of functions have been described for proteins derived for CEA family members (Stanners et al., 1998). CEACAM1, CEACAM5, CEACAM6 and CEACAM8 function as homophilic and heterophilic intercellular adhesion molecules in vitro. Most cell adhesion molecule interactions are accompanied by the transmission of signals regulating differentiation or proliferation, and allow the exchange of information mediated by other cellular components (Johnson, 1991). So it has been suggested that CEA family members play a role in cell recognition and in the regulation of cellular interaction. Because alterations in cell adhesion are involved in cancer invasion and metastasis, it was further suggested that some CEA family members may play a role in these processes (Duffy, 2001). Deregulation of some CEA related antigens has been confirmed in different types of tumors. Although in vitro data implicate CEACAM1, CEACAM5 and CEACAM6 in cell adhesions, their apical localization on polarized cells in normal physiology is difficult to reconcile with this role (Duffy, 2001). Human CEACAM1, CEACAM3, CEACAM5 and CEACAM6 are receptor for Neisseria gonorrhea and Neisseria meningitis (Bos et al., 1997). Moreover, in healthy colon CEACAM5 and CEACAM6 are found to bind certain strain of Escherichia coli (Leusch, 1990) by their complex multi-antennary carbohydrate chains. So, Hammarstrom (Hammarstrom, 1999) suggested that in humans CEACAM5 and CEACAM6 may play a role in innate immunity, in protecting the colon from microbial attack. These molecules are expressed and released in the apical glycocalyx facing the microbial environment in the gut. They may therefore bind and trap microorganism, preventing them from reaching down the microvilli of the epithelial cells and invading

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the epithelial cells. The expression and probable release of the molecules can be regulated by inflammatory cytokines. The dynamics of the system would assure that new glycocalyx is constantly formed at the apical surface of mature enterocytes replacing old glycocalyx with bound microorganisms. This process may be speeded up through signalling via CEACAM1, since CEACAM1 can associate with CEACAM5 and CEACAM6. CEACAM1 can then transduce a signal through phosphorylation of its cytoplasmatic part containing modified immunoreceptor tyrosine based activation/inhibition motifs (ITAM/ITIM motifs) (Obrink, 1997).

4.3 CEA AS TUMOR MARKER

The Carcinoembryonic antigen is one of the most extensively used clinical tumor markers. In fact, although CEA is also present in certain healthy tissues, its concentration in tumors were found expressed at high levels (Duffy MJ 2001, Hammarstrom S, 1999). CEA is normally expressed during oncofetal development, and it is expressed in some cells within normal colonic mucosa. It is overexpressed in nearly all colorectal cancers, 70% of non–small-cell lung cancers, and approximately 50% of breast cancers. It is not expressed in other cells of the body except for low-level expression in gastrointestinal epithelium. This expression profile makes it an attractive TAA for diagnostic and immunotherapeutic purposes (Berinstein N., 2002; Kass et al. 2002). Benchimol et al. proposed a model of role CEA in carcinogenesis of colonocytes: in the colon and elsewhere CEA is expressed along the apical border of normal epithelial cells. In tumour cell instead there is a reported increase in CEA expression along both lateral and basal surfaces, with cytoplasmic CEA expressed in

poorly differentiated cells. The overexpression of CEA leads to the disruption of normal intercellular forces at both the lateral and basal surfaces, owing to the reduction in strength of other adhesion molecules. So the overproduction of CEA tends to disrupt the normally operative intercellular adhesion forces, allowing more cell movement and the adoption of a less ordered tissue architecture (Benchimol, 1989) (Figure 13). In healthy individuals CEA is principally expressed in colon and there it is released from the apical surface of mature columnar cells into the gut lumen and disappears with the faeces. So very low levels are normally seen in the blood from healthy individuals ($\square$ 2 $\square$g/L). In colon cancer the malignant cells have no basal lamina and are multiplying in the tissue. Moreover, as it is described above, the tumor cells have lost their polarity and CEA is distributed around the cell surface. The components from plasma membrane are continually exfoliated from the surface as plasma membrane –derived vesicles (Taylor and Black, 1985), which through draining lymph and blood vessels can end up in the blood. As a consequence, CEA will accumulate in the blood in parallel with tumor size (Duffy, 2001) (Figure 13). For all this reasons CEA continues to be principally important marker in the diagnosis and prognosis of cancer (Goldstein and Mitchell, 2005). In colorectal tumors, preoperative serum CEA levels assist in the evaluation of Duke 's stage C carcinoma and when used in conjunction with flow cytometry, help to predict the prognosis of patients with Duke 's stage B2 and Duke 's stage C carcinoma (Scott NA 1987). Moreover very high preoperative serum CEA levels are highly indicative of liver metastases (Hammarstrom S, 1999) and non respectable tumors (Schneebaum S 1993). Serum CEA determination is also used as indicator in the post- surgical surveillance of colon cancer. Increased CEA level was the first indicator of recurrent disease in 89% of patients (Wanebo et al., 1989). It has

recently been shown that CEA measurement is the most cost-effective test in detecting potentially curable recurrent disease (Grahm et al., 1998). Cellular and tissue distribution pattern of CEA can also be used to predict metastatic potential and lymphonode status (Lorenzi et al., 1997). In this contest CEA can be useful in determining the primary site of metastatic adenocarcinoma (Lagendijk et al., 1998).
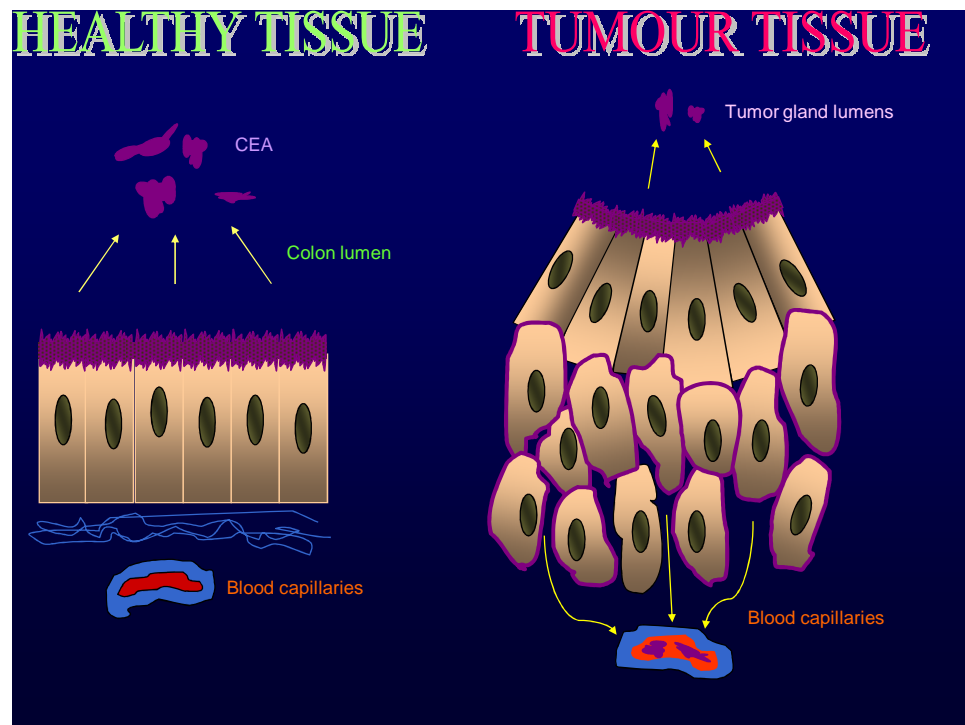

Figure 13. CEA distribution in healthy and tumour colon tissue epithelia

In several studies anti-CEA antibodies were used for tumor targeting. Murine (CEA)-specific monoclonal antibodies were successfully tested in animals for their capacity to localize accurately tumors formed by human colorectal carcinoma cell lines with various levels of CEA expression and therefore to be applicable in radioimmunoguided surgery (RIGS) (Kim et al., 2000). The immunoscintigraphy analysis with an anti-CEA monoclonal antibody fragment labeled with ^{99m}Tc in patients with colorectal carcinoma

recurrence was evaluated as effective method of early detection of pelvic and extrahepatic abdominal metastases (Fuster et al., 2003). In clinical studies is also known the application of recombinant phage-selected scFv anti-CEA antibody fragments in RIGS for primary or recurrent adenocarcinoma of the colon, rectum and pancreas (Mayer et al., 2000). Finally combined therapeutics strategies with humanized anti-CEA antibodies are currently in phase I/II clinical trials (Chester et al., 2004).

MATERIALS AND METHODS

Bacterial strain: TG1, *E.coli* strain (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F' [*tra*Δ36 *proAB*⁺ *lacI*�q*lacZ*ΔM15]) was used for phage antibody and yCD protein production

Cell lines. MelP5 human primary melanoma, LoVo human colon carcinoma

Antibodies and reagents. Recombinant human carcinoembryonic antigen (CEA) and recombinant glucose oxidase (GO, EC1.1.3.4) from *Aspergillus niger* were purchased from Sigma (St Louis, MO). The scFvE8 anti CEA antibody (Pavoni E et al., 2006), the scFvGO anti GO antibody (Ascione A et al., 2004), and the scFv anti yCD antibody were isolated from the same ETH-2 antibody phage library using an identical biopanning procedure (Pini A et al.,1998; Silacci M et al., 2005). 5-FC was purchased from Sigma, 5-FU (Fluorouracil Injection Solution) 2.5g 50ml⁻¹, Mayne Pharma, Naples Italy) was kindly provided by Dr.ssa A. Savarese , IRE, Rome, Italy.

Anti-Flag M2 and anti-polyhistidine antibodies were purchased from Sigma (St Louis, MO). The goat anti-mouse HRP-conjugated polyclonal antibody was purchased from Dako (Denmark).

Primers

CEASTU forward:

5'-CGTTATTAAGGCCTATGGCCGAGGTGCAGCTG-3'

LinCEA reverse:

5'TGAGCCGGAAGAGCTACTACCCGATGAGGAAGAGCCTAGGACGGTCAGCTTGGT -3'

LinyCD forward:

5'GGTAGTAGCTCTTCCGGCTCATCGTCCAGCGGCATGGTGACAGGGGGAATGG

CAA-3'

ESyCD reverse:

5'- ATCCGATATCGTCGACCTCACCAATATCTTC-3'

BamyCD

5'-CGAATTGGATCCATGGTGACAGGGGGA-3'

fdseq1:

 5'-GAATTTTCTGTATGAGG-3'

pelBback:

5'-AGCCGCTGGATTGTTATTAC-3

Genetic engineered constructs. The scFvE8 heavy and light chain variable regions were obtained by PCR amplification of pDN332 (Pavoni E et al., 2006). Oligonucleotide primers used in the PCR were *CEASTU forward primer*' contains *StuI* restriction enzyme sequence and 18-base pair (bp) sequence encoding for first 7 amino acid of scfvE8; *LinCEA* reverse primer contains 33 base pair sequences encoding for first 11 amino acid of linker fragment (SSSSG)$_3$, useful for joining yCD enzyme sequence, and the sequence encoding for the last 7 amino acid of scFvE8. The nucleotidic sequence of yCD was amplified by PCR from cDNA inserted in pQE30Xa (Qiagen; Madison, WI) with following primers *LinyCD* forward encoding for the last 11 amino acid of linker sequences and the first 7 amino acid sequence of yCD; the *ESyCD* reverse primer containing SalI restriction enzyme sequence and the last part sequence encoding for yCD. Both PCR fragments amplified with Pwo enzyme (Roche Diagnostics; IN), were agarose-purified (High Pure PCR Product Purification Kit,

Roche Diagnostics). Portion of the linker present in each PCR product have overlapping region necessary to assemble chimeric protein by filling reaction (5 min denaturation and 10 cycles 1 min 94°C, 4 min 65°C) Final amplification was carried out with primers *CEASTU and EsyCD* and the product was agarose-purified, digested with restriction enzymes *StuI* and *SalI*, and cloned into the plasmid pQE30Xa (Qiagen), containing 6×His tag sequence for protein purification. The same procedures were carried out for the construction of an irrelevant fusion protein made by the scFvGO, specific for glucose oxidase (GO) from *Aspergillus Niger*, genetically linked with the gene encoding for yCD enzyme. All genetic constructs were sequenced by Biofab research, srl, (Rome, Italy).

Expression and Purification. Plasmid pQE30Xa scFvE8:yCD and scFvE8:GO were transformed into the strain of E. coli, TG1 and the cultures were grown overnight in 2x TY broth containing 100 µg ml^{-1} ampicillin and 1% glucose in a 37°C shaker. The culture was diluted 1:100 in 1 L 2x TY broth lacking exogenous glucose and shaken at 37°C. When the culture attained A_{600} = 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 1 mM. Cells were harvested 3 h later. The culture was centrifuged at 10,000 rpm for 20 min at 4°C. The fusion proteins were purified by affinity chromatography on Ni-NTA resin (Qiagen), using native protocol according to the manufacture instructions (The QIAexpressionist^{TM}). Protein concentration was determined with Fernandez-Patron method. yCD enzyme was obtained by induction of expression of the encoding sequence inserted in pQE30Xa vector, in TG1 *E.Coli* strain. The proteins were purified by affinity chromatography on Ni-NTA resin (Qiagen), as above described.

SDS-PAGE and Western Blot Analysis[1] Purified scFvE8:yCD fusion protein was analyzed along with scFvE8 on 10 % SDS PAGE gel under reducing conditions. Gel was either stained with Fernandez-Patron method or transferred to nitrocellulose membrane in 1X TG buffer (Biorad; CA, USA) with 20% methanol, for 90 min at 140 Volt. The membrane was blocked in PBS 5 % powdered milk (M/PBS 5%) washed 3 times for 10 min in PBS and incubated for 2 h with anti-polyhistidine mouse mAb in 2% M/PBS (Sigma-Aldrich, 1:1000), washed 3 times as above, and incubated for l h in polyclonal goat anti-mouse HRP IgG (Dako,Denmark,1:1000) in M/PBS 2%. After 3 washings in 2% M/PBS, blot was developed with DAB buffer, one tabelet (10 mg) of 3,3'- diaminobenzidine (Sigma) in 20 ml of PBS and 3 µl of hydrogen peroxide 30%, for 3 min. The reaction is stopped with H_2O. ScfvE8:yCD was also detected with the supernatant scFv specific for yCD protein, developped in our laboratory. As secondary antibody was used anti antiflag Monoclonal Antibody M2 (Sigma) at the final concentration of 2 µg ml^{-1}.

ELISA [1] 96-well ELISA plate (Nunc, Maxisorp; Denmark) was coated overnight either with 50 µl/well of 10 µg ml^{-1} purified CEA or GO antigen in PBS. Next day a blocking solution 2% M/PBS was added, and after 2 hours the plate was washed with PBS. Then the plates was incubated for 2 hours a RT with 50 µl/well of 10 µg ml^{-1} of both fusion proteins and 50 µl/well of 5 µg ml^{-1} of scFvE8 or scFvGO. After 3 washes with PBS all well were incubated at RT with 50 µl of antibodies mixture composed by Mouse Monoclonal Anti-polyhistidine antibody (Sigma, 1:1000) and polyclonal goat anti-mouse HRP IgG (Dako,1:500) in M/PBS 2%, for 2 h. After 3 washes the reaction was

visualized using 3,31-5,51-Tetramethylbenzidine (soluble BM blue POD substrate, Roche Diagnostics), and read at 450 nm wavelength.

CEA binding specificity of the scFvE8:yCD. The specific recognition of the cell surface CEA antigen by the scFvE8 and the fusion protein scFvE8:yCD was determined by flow-cytometry and western blot analyses.

In the first study Mel P5 and LoVo cell lines in exponential phase of growth were trypsinized (we verified that CEA antigen is not sensitive to the enzymatic treatment), collected, washed in PBS 1% BSA and pelletted. About 2.5×10^5 cells were resuspended with 50 μL PBS, 1% BSA containing 5 μg ml^{-1} of scFvE8 or scFvE8:yCD primary antibodies and incubated for 1 h at RT. After several washings, cells were resuspended for 1 h at 4°C in PBS, containing an anti mouse polyhistidine antibody (Sigma Aldrich, 1:1000). At the end of this procedure, the cells were washed as usual and incubated again with 6 μg ml^{-1} of FITC-labelled goat anti-mouse IgG (Pierce, Rockford, IL) for 30 min at 4°C. In parallel experiment, an irrelevant human scFv antibody directed to glucose oxidase (Ascione A et al., 2004) was used as negative control. After staining, the cell samples were washed, maintained at 4°C and immediately analyzed by FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ) equipped with 15 nW argon laser. Fluorescence compensation was determined using samples stained with anti-glucose oxidase scFv and goat FITC-conjugated anti-mouse secondary antibody.

In the second study, Mel P5 and LoVo cell lines were harvested by trypsin (EuroClone, Milan, Italy), washed with cold PBS, resuspended and homogenated in AKT 150mM NaCl Buffer, 20mM Tris/HCl pH 7.4, 1% NP40, 10% glycerol, in presence of inhibitors

proteases (Sigma Aldrich). After centrifugation at 13000 rpm for 15 min, the supernatant was harvested and the protein total concentrations estimated with Bradford assay. 240 µg of total proteins were fractionated on 6.5% SDS PAGE under reducing conditions, transferred onto a nitrocellulose membrane and blocked in 5% M/PBS. After washing in PBS with 0.05% Tween 20 (T/PBS), the membrane was incubated with 10 µg ml^{-1} of scFvE8 or scFvE8:yCD fusion protein in 2% M/PBS for 1h, washed in T/PBS, and incubated for 1 h with the mouse mAb anti-polyhistidine antibody (Sigma-Aldrich) in M/PBS 2%. After washing in T/PBS, specific binding of scFvE8 or scFvE8:yCD was revealed with anti-mouse HRP IgG (Dako, 1:500) using ECL kit (Pierce, Rockford, IL).

Functional assays of the scFvE8:yCD on cells. The ability of the scFvE8:yCD fusion protein to convert far less toxic substrate 5-fluorocytosine (5-FC) to 5-FU was tested in two different investigations using a cell –based system. The cells used are Mel P5 derived from a human primary melanoma (Luciani F et al., 2004) and the human colon adenocarcinoma LoVo cells. Both cell lines were maintained in a basic medium (BM) constituted by RPMI 1640 (EuroClone) supplemented with 10% fetal bovine serum (EuroClone) and 1% penicillin-streptomycin in humidified atmosphere with 5% CO2 at 37°C.

In the first assay, Mel P5 and LoVo cells were seeded into 96-well microtiter plates (Corning Cable Systems srl, Turin, Italy) at 2500 cells/well in BM containing 3 µg ml^{-1} of scFvE8:yCD and different concentrations of 5-FC. The plates were incubated at 37°C for 4 days and cell viability estimated by WST-1 Assay (Takara, VinciBiochem, Vinci, Florence, Italy). In the second assay Mel P5 and LoVo cells were seeded as above and

allowed to adhere overnight .Then, medium was removed and a fresh BM containing 10µg ml^{-1} of scFvE8:yCD was added. After 4h incubation, the medium was changed and a fresh BM containing different concentrations of 5-FC, was added. Cell viability was determined after 4 days culture using WST-1 Assay (Takara).

Different concentrations of 5-FC and 5-FU alone were used respectively as positive and negative controls of cells vitality. yCD enzyme alone was not cytotoxic so that scFvE8:yCD alone (data not shown). Results were the mean of triplicate samples.

Vector construction. Complete yCD gene sequence (Erbs P et al., 1997) was amplified by PCR from cDNA inserted in pACCMV 115. The sense primer was : *BamyCD,* containing *BamHI* restriction site and the sequence coding for first five amino acid of yCD. The antisense primer was: *ESyCD* described before. PCR was performed using Pwo enzyme (Roche Diagnostics) and the resulting PCR fragment was agarose-purified using the High Pure PCR Product Purification Kit (Roche). Then it was digested with restriction enzymes *BamHI* and *SalI*, and cloned into the plasmid pQE30Xa (Qiagen), containing 6×His tag sequence for protein purification. The clone was sequenced by Biofab Research SRL (Rome, Italy).

Expression and Purification [2]**.** TG1 *E. coli* cells transformed with plasmid pQE30Xa yCD were grown in 100 ml 2x TY broth supplemented with 100 µg ml^{-1} ampicillin and 0.1% glucose in a 37°C shaker until $OD_{600} = 0.6$. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 1 mM. Cells were harvested 3 h later, centrifuged at 10,000 rpm for 20 min at 4°C and lysed with sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). The yCD protein was purified by affinity chromatography on Ni-NTA resin (Qiagen), using native

protocol according to the manufacture instructions. Protein concentration was determined with Fernandez-Patron method. The purified yCD protein was dissolved in PBS, aliquoted and stored at -80 ºC.

NMR. ^{19}F NMR analyses were performed on BRUKER AVANCE spectrometer (Bruker BioSpin GmbH – Rheinstetten – Germany) operating at 9.4 T. The spectra were acquired at 25 °C with a pulse angle of 60°, interpulse delay of 2 s and 64 transients. In order to compensate for partial magnetic saturation effect, the correction factors were determined by comparing the measured peak areas with those obtained at equilibrium (flip angle 90°, interpulse delay 30 s). At the end of reaction the concentration of 5-FU was determined by adding a known amount of the drug. Spectral analyses were performed utilizing the XWIN-NMR BRUKER suite. ^{19}F-MRS of 3,5 µmoles of 5-FC dissolved in 700 ul D_2O saline buffer was considered the time 0 of the reaction and after 70 µl of 25 µg/ml yCD enzyme were added. The reaction was followed during 1 h and 30 min. To verify the complete conversion of 5-FC to 5-FU the last spectrum was acquired at 3 h and 15 min.

ETH-2 library. The ETH-2 synthetic human recombinant antibodies library consists of a large array (more than 10^9 antibody combination) of scFv polypeptides displayed on the surface of M13 phage (Viti F et al., 2000). It was built by random mutagenesis of the CDR3 of only three antibody germline gene segments (DP47 for the heavy chain, DPK22 and DPL16 for the light chain). Diversity of the heavy chain was created by randomizing four to six position, replacing the pre-existing position 95-98 of the CDR3.

The diversity of the light chain was created by randomizing six position (96-101) in the CDR3 (Pini A et al., 1998).

Selection of yCD protein specific antibodies from ETH-2 library. Immunotubes (Nalge Nunc International; NY) were coated overnight (ON) at room temperature (RT) with purified yCD in PBS at the concentration of 10 µg ml^{-1}. After panning, performed according to Ascione et al., 2004, phages were eluted with 1 ml of 100 mM triethylamine, and the solution was immediately neutralized by adding 0.5 ml of 1 M Tris-HCl pH 7.4. Eluted phages were used to infect TG1 *E. coli* cells and amplified for the next round of selection. Briefly, 50 ml of 2xTY with 100 µg/ml ampicillin and 1% glucose (2xTY-amp-glu) were inoculated with enough bacterial suspension to yield an OD $_{600\ nm}$ $\cong$ 0.1. The culture was grown to OD $_{600\ nm}$ = 0.4-0.5 and infected with K07 helper phage at a ratio of around 20:1 phage/bacteria. The rescued phages were concentrated by precipitation with PEG 6000 and used for the next round of panning. For soluble scFv preparation, cloned E. coli cells were grown for 2 h at 37ºC in 180 µl of 2xTY-ampicillin (100 µg ml^{-1}) and 0.1% glucose in 96-well plates and induced with 50 µl of 2xTY-6mM IPTG. The following day the plates were spun down at 1800 g for 10 min at 4°C and the supernatants containing soluble scFv were recovered and tested for specific yCD recognition in ELISA.

ELISA [2]. 96-well ELISA plates were coated ON with 50 µl/well of 10 µg ml^{-1} purified yCD in PBS at 4ºC. Next day a blocking solution, 2% non-fat milk in PBS (2% MPBS) was added and after 2 h the plates were washed with PBS containing 0.05% Tween 20 (TPBS). Plates were incubated for 2 h at RT with 50 µl of supernatants containing

soluble scFv antibodies, anti-Flag M2 antibody and anti-mouse HRP-conjugated antibody. All antibodies were resuspended in 2% MPBS.

The reaction was developed using 3,3'-5,5'- tetramethylbenzidin BM blue and POD substrate soluble (Roche Diagnostics) and stopped by adding 50 µl of 1M sulfidric acid. The reaction was detected with an ELISA reader (Biorad) and the results were expressed as OD, i.e. the absorbance per unit length, were absorbance (A) is calculated as A=A (450 nm)- A (620 nm).

DNA characterization and sequencing. Plsmidic DNA encoding for selected scFvs were digested by specific endonucleases and CDR3 regions were sequenced with an automated DNA sequencer (M-Medical/Genenco, Pomezia Italy) using *fdseq1* and *pelBback* primers.

Soluble scFv purification. The clone scFvH5, was cultured for large-scale scFv production. TG1 *E. coli* infected cells were cultured at 30ºC in 2xTY containing 100 µg ml^{-1} ampicillin and 0.1% glucose up to OD $_{600}$ = 0.5. After induction of antibody expression by adding 1 mM IPTG to culture, cells were incubated ON at 30ºC. Then, the bacterial culture was centrifugated and antibody containing supernatant collected. Antibody fragments were precipitated with ammonium sulfate and dialyzed in PBS. His-tagged scFv fragments were purified by immobilized metal affinity chromatography using Ni^{2+}-nitriloacetic acid agarose (Qiagen). ScFv fragments were eluted with 250 mM imidazole in PBS, dialyzed, ELISA tested for specific antigen recognition, and stored at -80ºC.

SDS-PAGE and Western Blot Analysis [2]. Purified yCD protein was analyzed on 12 % SDS PAGE gel under reducing conditions. Gel was either stained with Fernandez-Patron method or blotted electrophoretically to nitrocellulose membrane, which was blocked in 5% MPBS and then washed three times for 10 min in PBS. For detection of yCD protein, the membrane was incubated either with anti-polyhistidine antibody or with soluble scFvH5. In the first case the membrane was incubated for 2 h with anti-polyhistidine antibody 1:1000 in 2% M/PBS and washed three times with PBS. In the other, the membrane was incubated for 2 h with soluble scFvs, washed with PBS containing 0.05% Tween 20 and incubated again with an anti-Flag M2 mouse antibody 1:1000 in 2% MPBS for 1 h at RT. In both cases specific binding was detected by HRP-conjugated Goat anti-mouse antibody 1:1000 in M/PBS 2% for l h at RT. After 3 washings in 2% M/PBS, the bound antibodies were visualized with DAB buffer obtained by dissolving one tabelet (10 mg) of 3,3'- diaminobenzidine (Sigma) in 20 ml of PBS and 3 µl of hydrogen peroxide 30%, for 3 min. The reaction was stopped with H_2O.

Determination of yCD activity. The deamination activity of purified yCD was measured by monitoring conversion of 5-FC to 5-FU in spectrophotometric studies. In 0.5 ml quartz cuvette, 250 µl of 1 µg ml^{-1} yCD was added to solution of 0.36 mM of 5-FC. The reaction was followed for 30 min by an UV/Vis spectrophotometer (Beckman DU-64, **Beckman Coulter S.p.A.**, CA, USA) which registered absorbance values every 30 seconds. The absorbance variation was measured at 265 nm, wavelength of the 5-FU maximum UV absorption according to Nishiyama et al., 1985, (Ascione A et al 2004). Absorbance values were calculated as A_{265} (t)- A_{265} (t$_0$), (t$_0$=0min); the values were

converted in concentration of formed 5-FU, dividing absorbance values by 5-FU molar extinction coefficient at 265 nm (ε_{265}). The calculated 5-FU ε_{265} was 7 mM^{-1} cm^{-1} . Initial velocity of the enzyme was calculated as ΔA_{265} min^{-1} or as Δ[5-FU] min^{-1} in the first 9 min when the reaction had linear trend.

The same procedures were used in order to examine eventual inhibition of yCD activity occurred in presence of scFvH5. Briefly, 5 µl of 200 µg ml^{-1} purified scFvH5 solution were added into the cuvette with yCD and 5-FC. Parallel experiments were performed in presence of the irrilevant scFvGO antibody.

Cytotoxic assay. The ability of purified yCD protein to convert 5-FC into 5-FU was tested in an vitro cell system. The human colon adenocarcinoma LoVo cells were maintained in a basic medium (BM) constituted by RPMI 1640 (EuroClone) supplemented with 10% fetal bovine serum (EuroClone) and 1% penicillin-streptomycin in humidified atmosphere with 5% CO_2 at 37°C.

In a cell growth inhibition assay 2500 cells/well were seeded into 96-well microtiter plates (Corning Cable Systems) in BM containing 2.5 µg ml^{-1} of yCD and different concentrations of 5-FC. The plates were incubated at 37°C for 4 days and cell viability was evaluated by WST-1 assay (Takara). As positive and negative controls different concentrations of 5-FC and 5-FU alone were used in identical in vitro conditions. A cell growth inhibition assay was also used in order to determine whether the binding with the specific scFvH5 antibody affects yCD enzyme function.

In this experiment LoVo cells (2500 cells/well) were seeded in 96- costar plates in BM containing 2.5 µg ml^{-1} of yCD and 10 µg ml^{-1} of 5-FC in presence of scFvH5 or

scFvGO antibodies at concentrations ranging from 0.1 to 10 µg ml^{-1}. All results were represented as the mean of triplicate samples.

RESULTS

Genetic engineering of the scFv antibodies for ADEPT.

To drive the enzymatic activity of yCD to CEA-expressing tumor cells a fusion protein was genetically engineered using the cDNA derived from CEA–specific antibody scFvE8 previously described (Pavoni E et al., 2006). As control for ADEPT studies an irrelevant fusion protein composed by glucose oxidase (GO) specific scFv antibody (Ascione et al., 2004), and yCD was genetically constructed. Antigen GO from *Aspergillus niger* is not present in mammalian cells. The cDNA corresponding to the open reading frame of yCD (Erbs P et al., 1997) was appended to the 3' end of the cDNA encoding for the antibodies scFvE8 or scFvGO by a linker of 45 bp $(SSSSG)_3$. The cDNA constructs were cloned into the pQE30Xa (Qiagen), containing $6 \times$His tag sequence for protein purification (see Figure 14A). PCR bands of the genetic constructs corresponding to 1300 bp, obtained after fill-in procedures are shown in Figure 14B. The clones isolated after transformation of TG1 *E.coli* bacterial strain, were characterized under genetic-molecular aspect. The complete aminoacid sequence of the genetic constructs scFvE8:yCD and scFvGO:yCD were shown in Figure 15.
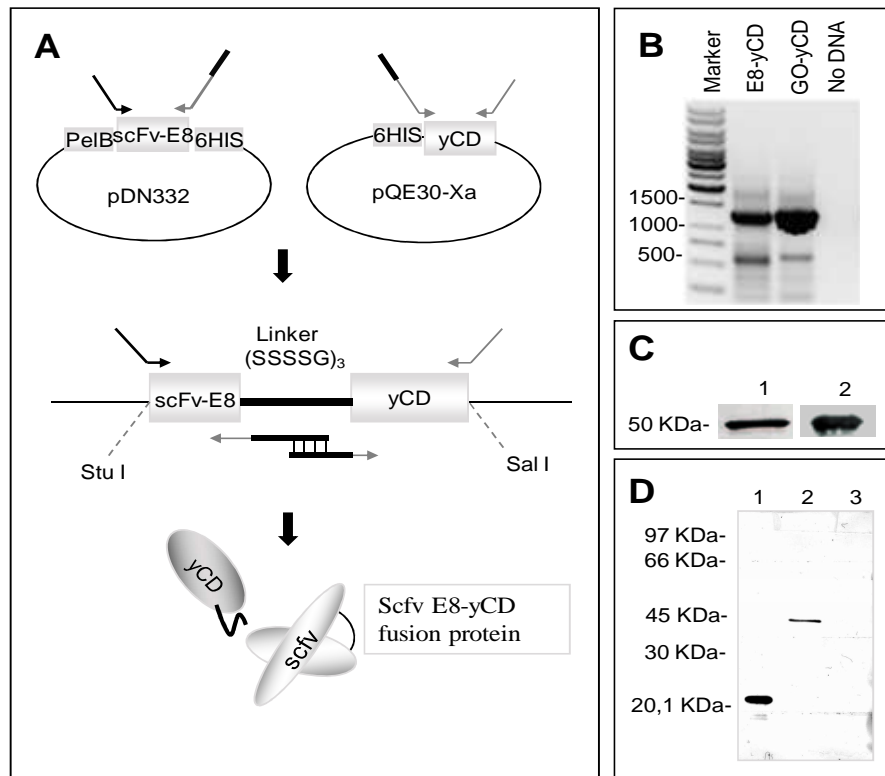
Figure 14. Schematic representation of the engineered scFvE8:yCD fusion protein.
In the panel (A), the genes encoding for the human scFv antibody to CEA (E8) and cytosine deaminase from yeast (yCD) were assembled by a (SSSSG)$_3$ linker, inserted in the prokariotic vector pQE30Xa and expressed in TG1 strain of E.coli to form a bifunctional protein. In the panel (B) the PCR-DNA fragments corresponding to the expected 1250 bases pair encoding for scFvE8:yCD and the irrelevant fusion protein scFvGO:yCD are shown. In (C), it is shown the result of western blot (WB) analysis of the purified fusion proteins scFvE8:yCD (lane 1) and scFvGO:yCD (lane 2). The 50 kDa-His tagged fusion proteins were detected with an anti-polyhistidine secondary antibody as described in the text. In the panel (D), the scFvE8.yCD protein was detected with WB as a band at approximately 50 kDa with an anti yCD antibody (in scFv format).

The fusion proteins scFvE8:yCD and scFvGO:yCD were purified from the pellet of TG1 strain E.coli by affinity chromatography. The yield was about 150 µg l^{-1} for each fusion protein. The expected 45-50 kDa size of the scFvE8:yCD and scFvGO:yCD

constructs was confirmed by western blot studies using an anti-polyhistidine antibody to detect the fusion proteins after SDS-PAGE migration (see Figure 14C).

In order to assess the exact expression of the yCD moiety of the scFvE8:yCD construct, the fusion protein was biochemically investigated by an antibody specific for yCD in scFv format. As it is shown in Figure 14D, the anti yCD antibody, reacts with yCD (lane 1) and scFvE8:yCD (lane 2) at the expected migration in SDS-PAGE and corresponding to 18-20 kDa (MW of yCD) or 45-50 kDa (MW of scFvE8:yCD). This specific antibody was selected and characterisez by us, below described.

ScFvGO:yCD Aminoacid sequence

MAEVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAIS
GSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK*WNNWRNFDY*W
GQGTLVTVSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYA
SWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSS
*ELPPVY*VVFGGGTKLTVLG**SSSSGSSSSGSSSSG**VTGGMASKWDQKGMDIAYEEAAL
GYKEGGVPIGGCLINNKDGSVLGRGHNMRFQKGSATLHGEISTLENCGRLEGKVYKDT
TLYTTLSPCDMCTGAIIMYGIPRCVVGENVNFKSKGEKYLQTRGHEVVVVDDERCKKIM
KQFIDERPQDWFEDIGE

ScFvE8:yCD Aminoacid sequence

MAEVQLAESGGGLVQPGGSLRLSCAASGFTFSSDAMSWVRQAPGKGLEWVSAIS
GSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK*SNEFLFDY*WGQ
GTLVTVSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSSYASW
YRQRPGQAPVPVIYGKNNWPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYW*NSSY*
*AWLPYVV*FGGGTKLTVLG**SSSSGSSSSGSSSSG**VTGGMASKWDQKGMDIAYEEAAL
GYKEGGVPIGGCLINNKDGSVLGRGHNMRFQKGSATLHGEISTLENCGRLEGKVYKDT
TLYTTLSPCDMCTGAIIMYGIPRCVVGENVNFKSKGEKYLQTRGHEVVVVDDERCKKIM
KQFIDERPQDWFEDIGE

Figure 15. Aminoacid sequences. The complete aminoacid sequences in a single letter code of the scFvE8:yCD and the scFvGO:yCD fusion proteins are shown. In red is shown Vh and Vl CDR3 sequences

The antigen specificity of the engineered fusion proteins

Flow-cytometry, immunobiochemical and ELISA investigations were performed in order to assess the specific antigen recognition of the engineered scFvE8:yCD. When tested in flow-cytometry the scFvE8:yCD shows weaker binding profiles on Mel P5 and LoVo cells in comparison with the original scFvE8 antibody (see Figure 16A). This difference in the antigen recognition may be due to an altered His-tag exposition in the fusion protein. To this regard, we observed a lower binding level of the original scFvE8 on CEA –expressing cells when the scFvE8 reactivity was detected with an anti-polyhistidine secondary antibody in comparison with an anti FLAG-tag secondary antibody (data not shown). Further, ELISA studies summarized in Table 4 demonstrate that the specific binding activity of the scFvE8:yCD is retained to a degree comparable to the parental scFvE8 and that the DNA recombinant procedures utilized for engineering scFvE8:yCD gave very effective fusion protein (i.e., scFvGO:yCD) as good as parental scFv antibody. Infact, scFvE8:yCD was very effective in the recognition of CEA specific antigen either in cellular total extract or in the purified version protein (see Figure 163B). To note that the high staining of Mel P5 lysates in western blot corresponds with the high reactivity of the scFvE8 antibody on melanoma cells.

scFv antibodies [a]	Antigens [b]			
	CEA	GO	yCD	scFvE8-
scFvE8	++		N	N
scFvE8-yCD	+	-	N	N
	-	+	N	N
scFvGO	-	+	N	N
scFvGO-yCD	N	N	++	+

Tale 4: (a)The scfv antibodies isolated by bio-panning and derived fusion proteins are the following: scfvE8 and scfvE8:yCD are specific for CEA; scfvGO and scfvGO:yCD are specific for GO; scfvyCD, is specific for yCD. (b) CEA and GO are purified proteins, yCD is expressed and purified as described in the text. (c) ELISA signals are indicated: (-) for equal or low background; (++) or (+++) more than three and four times the background, respectively. ND no disposible
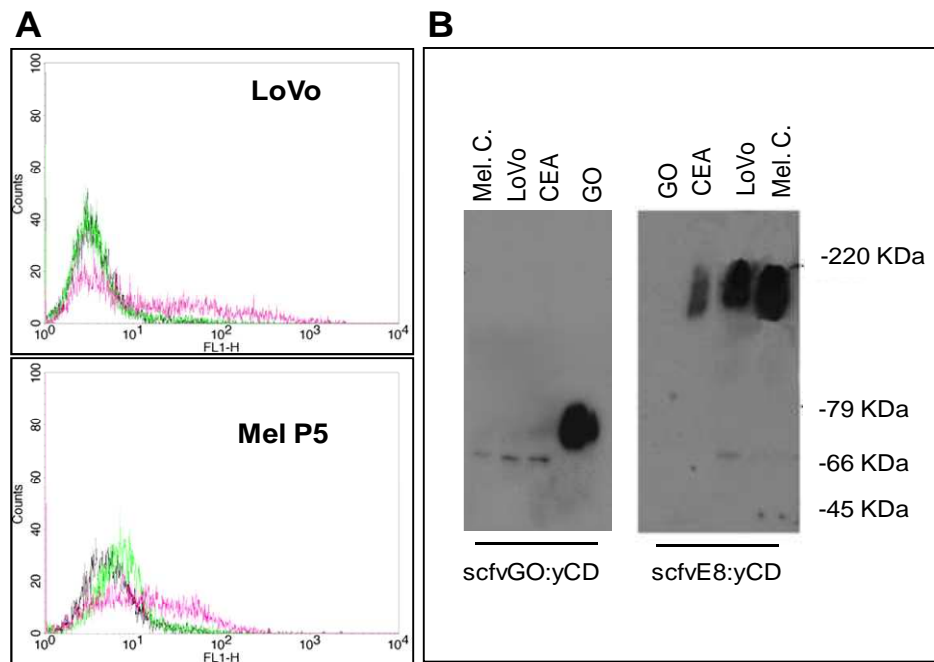
Figure 16. Specificity of the scFvE8:yCD fusion protein. In the upper part of the figure 3 (panel A), the flow-cytometry profiles representing the binding level of the scFvE8:yCD (red line), the irrelevant scFvGO:yCD (black line) and the parental scFvE8 (green line) on living intact/LoVo and Mel P5 cell lines are shown. In the panel (B) total extracts from LoVo and Mel P5 cell lines were runned on SDS-PAGE in parallel with CEA and GO. After incubation with scFvE8:yCD or scFvGO:yCD the specific reactivity was revealed by an anti-polyhistidine secondary antibody. Both fusion proteins retain the binding specificity for respective antigens.

Functional assay of the scFvE8:yCD

ScFvE8:yCD fusion protein was evaluated for the ability to control tumor cell growth of CEA–expressing cells by converting the antifungal agent 5-FC to the highly toxic 5-FU. Cell sensitivity to 5-FC, 5-FU, and selective cytotoxic effect mediated by ScFvE8:yCD in the presence of 5-FC was assessed on tumor cells Mel P5 and LoVo *in vitro*. These cell lines were cultured in BM for 4 days containing either 3 μg ml^{-1} of scFvE8:yCD or 1,5 μg ml^{-1} of yCD and different concentrations of 5-FC (see Figure 17).
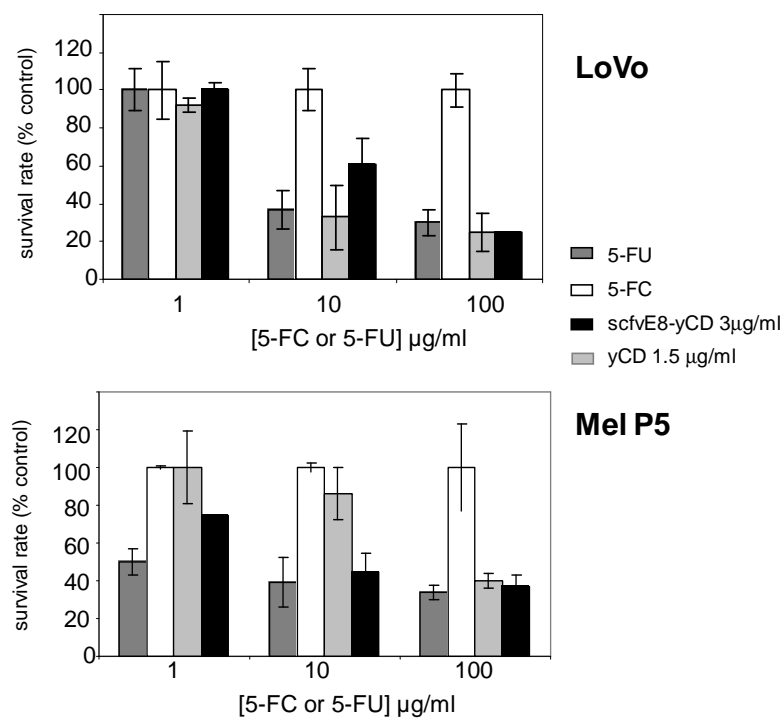
Figure 17. Functional assay of the scFvE8:yCD fusion protein. Cell sensitivity to 5-FC, 5-FU, and the selective cytotoxic effect mediated by ScFvE8:yCD was assessed on tumor cells Mel P5 and LoVo. Antifungal agent 5-FC was converted to the highly toxic 5-FU by ScFvE8:yCD or free yCD. The cell lines were seeded (2500 cell/well) and cultured in BM for 4 days containing either 3 µg ml^{-1} of scFvE8:yCD or 1,5 µg ml^{-1} of yCD (identical molar concentration for free yCD and yCD fused with scFvE8) in presence of the indicated concentrations of 5-FC. Cell cytotoxicity was evaluated by WST-1 assay and calculated as a percentage of survived cells. Values are reported as the mean of triplicate samples. The bars indicate SD.

Cell cytotoxicity evaluated by WST-1 assay shows that scFvE8:yCD and yCD exert a similar cell growth inhibition in the presence of 100 µg ml^{-1} of 5-FC while no effect was observed either in presence of 5-FC (100 µg ml^{-1}) or scFvE8:yCD fusion protein or yCD alone. When LoVo and Mel P5 cell lines, were pre-incubated with scFvE8:yCD at 10 µg ml^{-1} for 4h, washed and then cultured for 4 days with BM containing different concentrations of 5-FC, we observed that pre-treatment with scFvE8-yCD significantly

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inhibited cell growth in comparison with the same treatment in presence of the irrelevant scFvGO:yCD fusion protein (see Figure 18). The last observation it is of particular interest since the growth inhibition exerted by scFvE8-yCD in presence of 5-FC depends on the specific binding of the fusion protein on CEA–expressing cells and the subsequent conversion of 5-FC to 5-FU at the tumor cell level.
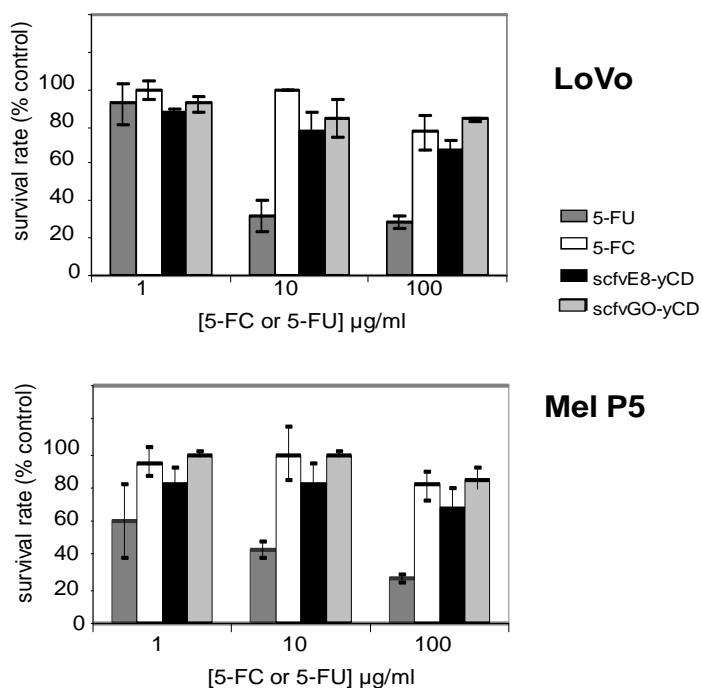

Figure 18. Cell specificity of the 5-FC activation driven by the antibody of fusion protein. The in vitro cytotoxic effect of the prodrug 5-FC on Mel P5 and LoVo cells was evaluated by pre-incubating the cells (2000 cells/well) with either scFvE8:yCD or scFvGO:yCD for 4h. After washing the cells were cultured for 4 days in BM and different concentration of 5-FC. Antibody binding dependent cell cytotoxicity, was evaluated by WST-1 assay and calculated as a percentage of survived cells. Values are reported as the mean of triplicate samples. The bars indicate SD.

Construction of scFv specific to yCD

A convincing demonstration that this strategy can be developed for clinical use requires knowledge of specific parameters which may include the in in vivo monitoring of the

CD complex. For this reason we have firstly constructed, expressed and produced a recombinant yCD functionally active. Subsequently a fully human antibody in scFv format not interfering with yCD activity was developed and analyzed.

Expression and purification of yCD protein.

A functionally active yCD was generated by recombinant DNA technology. The gene encoding for yCD was amplified and inserted into the pQE30Xa expression vector which contained the *lac* promoter for protein induction and 6×His TAG sequence for purification (Figure 19A). 500 base pairs band shown in Figure 19B corresponded to DNA fragment encoding for yCD obtained by PCR using specific primers. After TG1 E. *coli* bacterial strain transformation, several clones were isolated and proved suitable for yCD production.
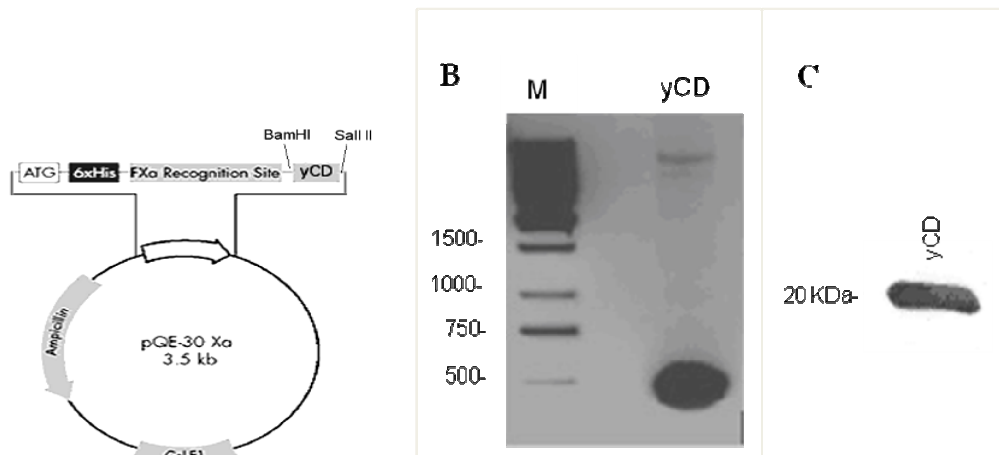

Figure 19. Expression of recombinant yCD. In (A), is depicted a schematic representation of yCD expression vector, constructed by inserting the coding sequence for yCD into pQE30Xa plasmid, and expressed in TG1 strain of *E.coli*. In (B) and (C) are shown respectively, the PCR-DNA fragment corresponding to the expected 500 bases pair encoding for yCD and the immuno-blot of the purified yCD protein.

The clone exhibiting the best protein induction was further characterized. The yield of purified protein was about 10 mg l^{-1}, using metal chelate affinity chromatography. The reliability of this novel expression system used for protein isolation and purification was confirmed by biochemical investigation showing that yCD migrated at the expected molecular weight of about 20 kDa (Figure 19C).

Selection and characterization of scFvH5 antibody specific for yCD.

To isolate phage-displayed specific antibodies, an aliquot of the human synthetic ETH-2 library containing approximately 1 x 10^{12} cfu phages was panned into Nunc-immunotubes coated with 10 µg ml^{-1} of purified yCD. Non-specifically absorbed phages were removed by intensive washing. Specific bound phages were eluted, amplified and used for next panning as previously described (Viti F et al., 2000). By using this protocol, we were able to isolate a phage-antibody population specifically recognizing yCD protein after only three rounds of selection. Plating on agar of TG1 cells infected with a pool of phage antibodies from third selection allowed individual clones harboring phagemid to grow. Soluble scFvs derived from IPTG inducted colonies, were screened by ELISA and several of them proved to be specific for yCD protein (Figure 20). One of the most reactive scFv antibody clone, named H5, was isolated and further characterized under biochemical and genetic aspects.

Western blot studies showed that scFvH5 recognizes a protein band of about 20 KDa corresponding to the expected molecular weight of the purified yCD protein (see Figure 20, inserted box). The genes encoding for variable regions of heavy (VH) and light (VL) chains of the scFvH5 were sequenced, and their CDR3 corresponding amino acid sequence are shown in figure 21
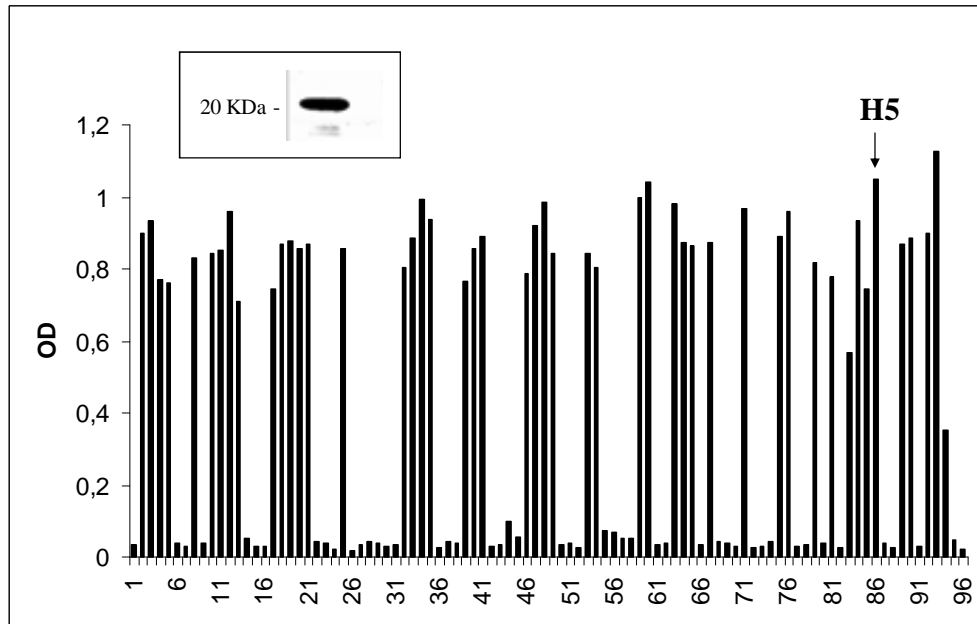
Figure 20. Selection of yCD -specific scFvs . IPTG inducted bacterial supernatants of individual colonies from the third round of the ETH-2 selection on yCD protein, were tested by ELISA in 96-well microtiter plates coated with the antigen. OD values higher than three fold the value of negative control are scored as positive. Negative and positive controls positioned in wells 1-4 reacted as expected. In the inserted box, the Western blot of yCD protein detected by scFvH5 (one of the most reactive clones) is shown.
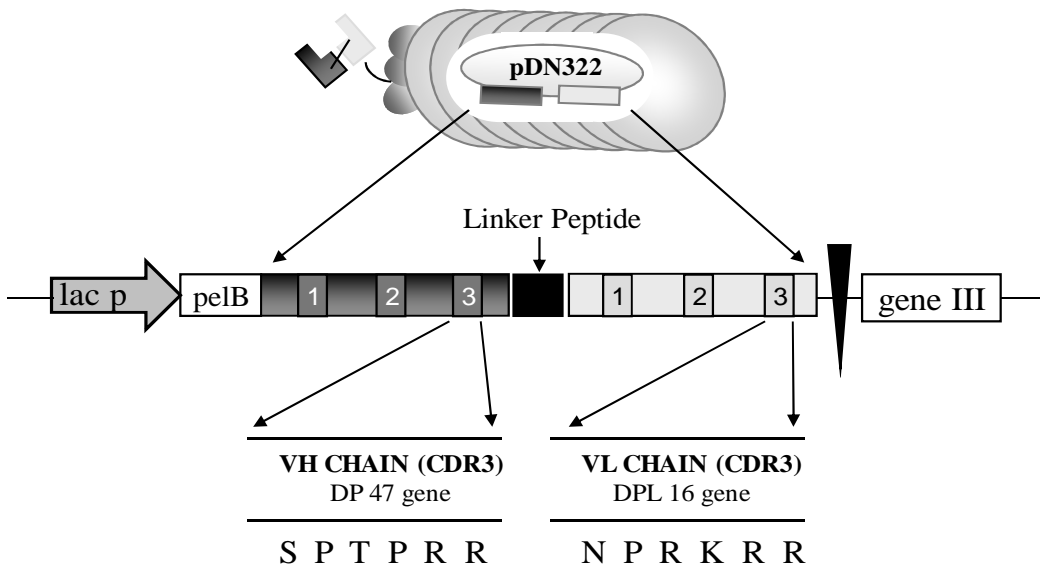

Figure 21 CDR3 aminoacidic sequence of Vh and Vl chains

Determination of yCD activity. In order to determine the functional activity of the recombinant yCD, the ability of the enzyme to deaminate 5-FC was assessed by fluorine NMR. This approach allowed simultaneous detection of the substrate and the product without interference by other compounds. Figure 22 shows that after 90 min 5-FC was completely converted into 5-FU in the presence of the yCD. Absolute quantification of the product was obtained by adding a known amount of 5-FU to the reaction mixture at the end of the experiment.
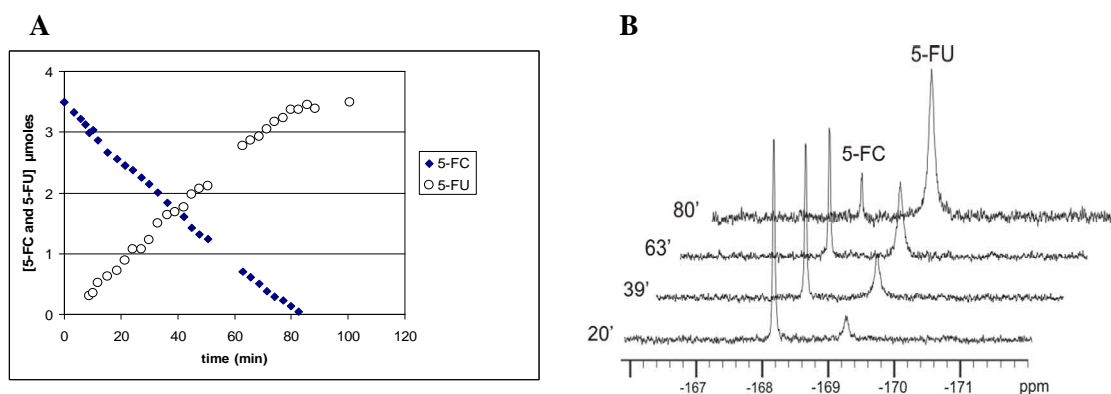

Figure 22. Functional analysis of yCD by ^{19}F NMR study. In (A) and (B) are shown respectively, the 5-FU formation (µlmoles) due to the conversion of 5-FC by yCD and representative spectra during the reaction at 20, 39, 63 and 80 min.

The specific yCD enzymatic activity was also assessed by spectrophotometric analysis in order to determine nanomolar concentrations of the reaction product. Figure 23A shows the initial velocity of the reaction which is represented by direction coefficient of the line plotted placing concentration of formed 5-FU versus reaction time.

In order to assess if the enzymatic activity of yCD was affected by the presence of the scFvH5 an identical experiment was performed in presence of the antibody. Figure 23B shows that the rate of product formation was similar to that with free yCD, suggesting that there was no apparent loss in enzyme activity as a result of binding with scFvH5. Identical results were obtained using the irrelevant scFvGO antibody (see Figure 9C).
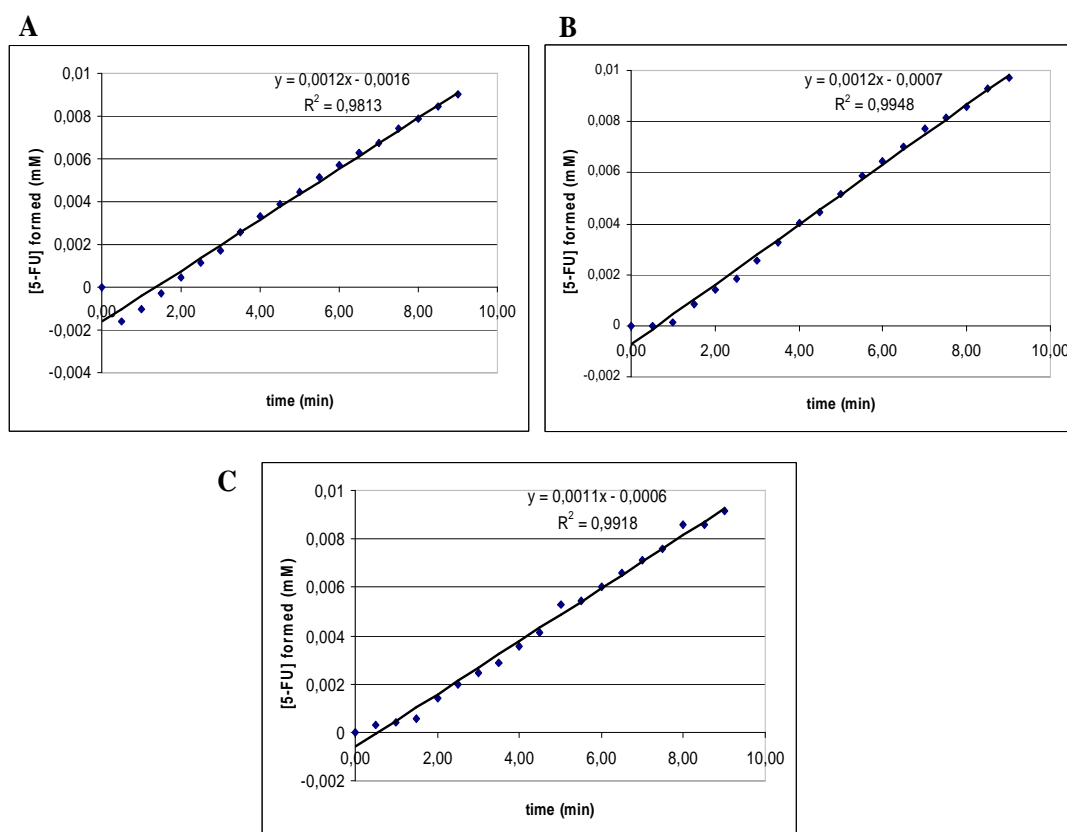

Figure 23. Spectrophotometry of yCD activity. In (A), are reported the values of de novo formed 5-FU (mM) obtained in presence of yCD (0.5 μg ml^{-1}) and 5-FC (0.18 mM) during the first 9 min of the reaction. In (B) and (C) are reported the 5-FU values obtained with identical reagents but in presence of 2 μg ml^{-1} of the specific (scFvH5) or irrelevant (scFvGO) antibodies. Slope of lines represents starting speed of the eaction. Correlation coefficient (R) indicates the strength and direction of the linear relationship between time and formed 5-FU.

Cytotoxic assay. Using an *in vitro* model constituted by human LoVo cells, we measured the enzymatic activity of the recombinant yCD protein in converting the antifungal agent 5-FC into the highly toxic anticancer compound 5-FU. In parallel we evaluated if co-incubation of the same reagents with scFvH5 affected yCD function. Figure 24A shows that 2.5 µg ml^{-1} of yCD exerted a significative cell growth inhibition of the human carcinoma LoVo cells in the presence of 5-FC concentration ranging from 1 mg ml^{-1} and 10 µg ml^{-1}. In contrast, the co-incubation of yCD and 5-FC with various concentration of scFvH5 did not interfere with the cytotoxic activity of *de novo* generated 5-FU (Figure 24B).
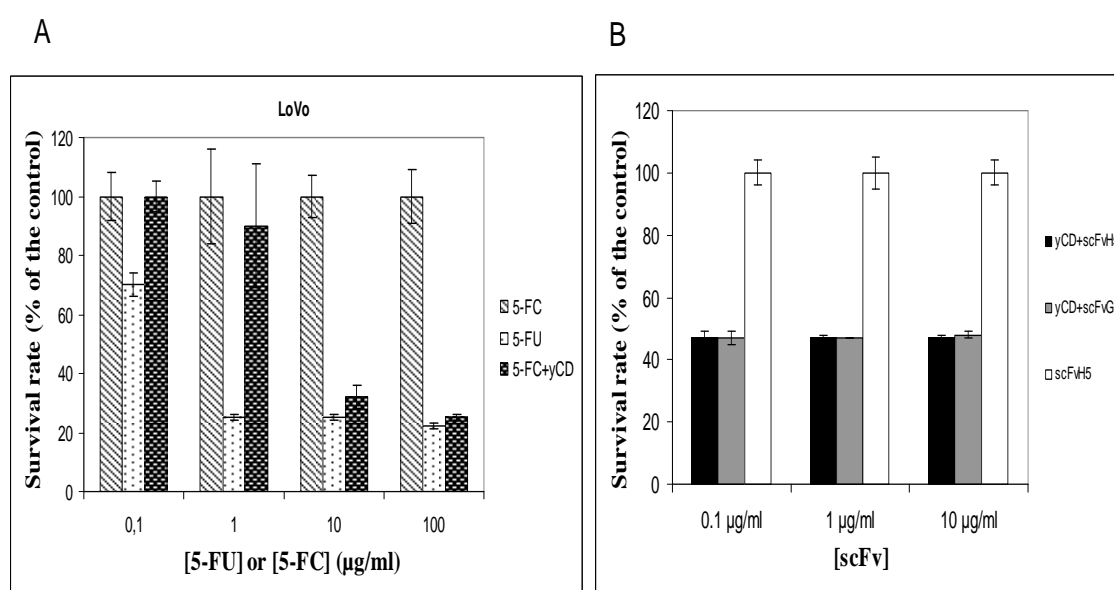
Figure 24. In *in vivo* assay of yCD protein. In (A), LoVo cells were seeded in 96-well plate (2500 cells/well) and cultured in BM for 4 days containing 2.5 µg ml^{-1} of yCD in presence of the indicated concentrations of 5-FC. In (B), the cells were culture at same conditions but in BM containing 2.5 µg ml^{-1} of yCD and 10 µg ml^{-1} of 5-FC in presence of different concentrations of scFvH5 or the irrelevant scFvGO antibodies. Cell cytotoxicity (due to *de novo* formed 5-FU) was evaluated by WST-1 assay and calculated as a percentage of survived cells. Values are reported as the mean of triplicate samples. The bars indicate SD.

DISCUSSION

Previous studies have demonstrated that significant *in vitro* and *in vivo* activities can be obtained using mAb-yCD conjugates to convert 5-FC into the antitumor agent 5-FU (Aboage EO et al.,1998; Wallace PM et al., 1994) In several works mAb-enzyme fusion protein was prepared using chemical cross-linking reagents that react with amino acid side chains on each individual protein of the conjugates (Bagshawe KD et al., 2006). In general, the utilization of chemical procedures that has been largely applied for ADEPT resulted in reagents having an inherent lack of specificity. The resulting conjugates are composed of highly complex mixtures presumably with varying degrees of binding and enzymatic activities. Differently, recombinant DNA technologies offer the opportunity to design and produce well defined molecules with bifunctional activities (Glennie MJ et al., 2003; Holliger P and Hudson PJ, 2005). Human monoclonal scFvs derived from phage display antibody library (Hoogenboom HR, 2005) afford the opportunity of developing a recombinant molecules maintaining the antigen-binding characteristics of the scFv parental antibody together with the competence of the desired enzyme (Carter PJ, 2006). Such scFvs antibodies have been shown to offer advantages over whole antibody with respect to tumor penetration and clearance from the circulation (Holliger P and Hudson PJ, 2005). Furthermore, it is possible to fuse genetically such single chain fragment variables to sequences encoding other functional domains, providing molecules having multiple activities with minimal size (Halin C et al, 2002; Ebbinghaus C et al., 2005). Another advantage of genetically engineering single chain molecules of minimal complexity is that such fusion proteins can be produced using prokaryotic expression systems which grow rapidly and inexpensively (Hudson PJ, 2005).

Recombinant fusion protein for ADEPT based on the humanization of rabbit gene encoding for scFv but retaining the rodent CDR's (Roder C et al., 2000) and catalytic activity of the monomer of CD has been already described (Dekert PM et al., 2003). In the present study, we describe that a fully human scFv (scFvE8) to CEA was genetically combined with yCD and expressed in E.coli system. This recombinant fusion protein could be readily purified in native conditions by cell lysates maintaining the functional activities of parental molecules. Figure 16 e table 4 show that CEA is identically recognized by scFvE8 and scFvE8:yCD both in ELISA and western blot analysis. In contrast, flow cytometry investigation shows a decrease in the binding level of scFvE8:yCD in comparison with scFvE8. This difference in the CEA recognition on the cell surface of living/intact cells may be due for technical reasons, very likely to different His tag exposition in chimeric proteins in comparison with scFv. Nonetheless, taken together, the data reported in Figure 16 and in table 4 demonstrate that the parental and the conjugated scFvE8 to CEA possess similar specificity. Thus, none of the genetic manipulation appear to have impacted its target antigen specificity. Then, we demonstrated that the simultaneous presence of scFvE8:yCD and 5-FC generated a toxic effect, resulting in increase in the 5-FC sensitivity of human cancer Mel P5 and LoVo cells *in vitro* compared with the incubation of scFvE8yCD and 5-FC alone. Also we observed that fusion protein maintained similar toxicity of yCD in presence of 5-FC. Further, pre-incubation of Mel P5 and LoVo cells with scFvE8:yCD followed by cell washing and 4 days exposure to 5-FC resulted in a cell growth inhibition if compared with the same treatment in presence of the irrelevant scFvGO:yCD fusion protein (see Figure 5). The yCD monomer of the fusion scFvE8:yCD is correctly expressed in the E.coli system since the specific monoclonal antibody to yCD recognize the enzyme both

in western blotting and ELISA studies. The functional activity of scFvE8:yCD fusion protein may depends either of the yCD monomer or dimers formed after antigen binding. While the homoexameric and dimeric structures of CD from bacteria and yeast respectively, supports monomer activity (Ireton GC et al., 2002), both hypotheses above reported would explain the lower catalytic activity of scFvE8:yCD alone. The data here reported and discussed indicate that this new scFvE8:yCD fusion protein meets several criteria for a potential anticancer compound: i) the scFv antibody is fully human thus substantially reducing the immunogenicity of the dual construct, ii) the low molecular weight (50 kDa) of the scFvE8:yCD may have favourable diffusion characteristics in solid tumors (Christiansen J and Rajasekaran KA, 2004), iii) it binds selectively and with good affinity to a CEA epitope shared by CEACAM1 and CEACAM5 isoforms which is expressed on several malignancies including melanoma (Ebrahimnejad A et al., 2004). This CEA epitope is a particularly attractive target for immunotherapeutic purposes because of its expression profile in solid tumors and low or absent presence in normal adult tissues and in various normal human cells, including distinct classes of lymphocyte subpopulations and neutrophils (Pavoni E et al., 2006). A convincing demonstration that such a complex system can be developed for clinical use requires evidence that each of the components of the antibody complex functions by the mechanisms proposed (Napier MP et al., 2000). This can be provided by well defined measurements including the concentration levels of the antibody-enzyme conjugate or *de novo* expressed enzyme, in plasma, tumour and healthy tissues (Connors TA et al., 1995; Yazawa K et al, 2002). To allow the detection of CD expression at the protein level, we raised a human monoclonal antibody in single chain fragment (scFv) format against a recombinant CD from yeast (yCD) through a human fagic library ETH2.

This antibody fragment proved to be functionally active in NMR and in *in vitro* studies to convert the antifungal drug 5-FC into the anticancer compound 5-FU. The specificity of the human scFv was confirmed by Western blot and ELISA analysis. With this antibody, yCD expression can now be monitored without interfering with its enzymatic function in ADEPT, in GDEPT (gene direct enzyme prodrug therapy) and other studies leading to the effect of the so called tumour amplified protein expression and targeting (TAPET) to localize in vitro and in vivo generation of the anticancer agent 5-FU (Hedley D et al., 2007). Since its particular genetic origin, the scFvH5 can be easily genetically engineered to construct a whole human antibody with a predefined IgG subclass, for selective removal of mAb-yCD conjugate from the circulation, without interfering with the enzyme function. Differently with other mAbs to CD generated by hybridoma (Coelho V et al., 2007) or recombinant DNA technologies (Kerr DE et al., 1993), the scFvH5 is the first fully human monoclonal antibody in scFv format so far described which is able to detect yCD protein in different routinary laboratory techniques. Hence, this antibody may represents an excellent candidate for in vivo detection and measurement of the CD complex in the future development of CD –based selectively guided tumor therapy. In conclusion, in this work we have described a novel immunoprotein exerting specific catalytic function on tumor target cells naturally resistant to chemotherapy. This recombinant fusion protein, scFvE8:yCD, conosts of the human scFvE8 to CEA and yCD enzyme relying on the ability to convert far less toxic substrate 5-FC (currently administrats for the treatment of opportunistic infections) to 5-FU (Schiel X et al., 2006). However further studies conducted in in vivo animal model are necessary to demonstrate the anti cancer potentiality of such strategy characterized by selective delivery of inert prodrug into potent antin tumor agent.

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