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# Nanobody, New Agent for Combating Against Breast Cancer Cells

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## 1. Introduction

Breast cancer (BC) is a major public health problem among women throughout the world. More than 1.1 million cases are diagnosed annually and more than 410,000 patients die of it worldwide (Ferlay et al., 2010). BC is a complex and intrinsically heterogeneous disease with different morphologies, molecular profiles and clinical behavior which require different treatments (Bosch et al., 2010). Cancer treatment has come a long way from surgery alone to combined therapies to control tumour growth. By introducing the new therapeutic agents, combinations of existing therapies and targeted therapies, there would be promising future to improve survival and life span of patients who deal with this disease.

## 2. Breast cancer and therapeutic approaches

Breast cancer remains a threatening health problem in both developed and developing countries. However, its mortality rates have declined in recent years because of the broad advancement in the treatment of BC; including modifications in surgical procedures that reduce the risk of surgical morbidity and improvement in the delivery of radiation using novel imaging techniques that allow enhanced dosing to specified locations with fewer side effects on normal tissues (Moulder and Hortobagyi, 2008). Also, developments in systemic therapies include chemotherapy, hormonal therapy and biological therapy or combinations of these supportive cares, hold great promise for future breast cancer therapy.

### 2.1 HER2 targeted therapy by clinically approved drugs

Receptor tyrosine kinases (RTKs) play great roles in the transmission of extracellular signals that lead to cancer cell growth, survival and differentiation. These proteins are localized within the cell membrane and contain three parts. An extracellular ligand-binding domain (domains I, II, III, IV), a transmembrane domain that anchors the receptor to the cell membrane and a kinase domain that contains the ATP-binding site that phosphorylated a number of downstream target proteins. Phosphorylation causes the activation of signaling

cascades such as the mitogen-activated protein kinase and phosphoinositol 3'OH kinase (PI3K) pathways (Kruser and Wheeler, 2010). The HER family consists of four RTKs with similar homology: HER1 (EGFR/ErbB1), HER2 (neu, C-erbB2), HER3 (ErbB3) and HER4 (ErbB4) (Moulder and Hortobagyi, 2008). HER2 is the preferred dimerization partner for activation of other HER receptors. Receptor activation via ligand binding leads to downstream signaling including mitogen-activated protein kinases (MAPK), PI3K and signal transducers and activators of transcription (Stats). Ultimately, cell proliferation, angiogenesis, invasion and metastasis can be promoted by these cascades (Kruser and Wheeler, 2010; Nielsen et al., 2009). Aberrant expression or activity of two members of this family HER1 and HER2 have been connected with the occurrence of breast cancer. Two key parts of HER1 and HER2 (ligand binding domain and tyrosine kinase domain) have attracted scientists to inhibit their activity.

After many endeavors, the dream for generating an antibody that could bind to HER2 and block it became true. Trastuzumab (Herceptin®) (Genentech Inc. San Francisco, CA, USA; Hoffmann-La Roche Ltd. Basel, Switzerland) is the only anti-HER2 antibody that approved by FDA. This antibody is a humanized monoclonal antibody that targets the extracellular juxtamembranal domain of HER2 (Park et al., 2010). Trastuzumab by binding to HER2 reveals therapeutic efficacy in HER2-positive early stage and metastatic breast cancer. (Emde et al., 2010; Nielsen et al., 2009).

## 2.2 Immunotherapy

The clinical purpose of cancer immunotherapy is to enhance immune responses against tumour cells with the lowest side effects on healthy tissue (Guinn et al., 2007; Leen et al., 2007). Two therapeutic strategies have been developed to stimulate anti-tumour immunity in patients with breast cancer; vaccination (active immunization) and antibody/ immune cell therapy (passive immunization) (June, 2007; King et al., 2008). Vaccination of patients with breast cancer has not showed satisfactory results (Stauss et al., 2007). Antibody/T cell therapy possess potential benefits, since two main arms of immune system participate in destroying tumours and prevention of cancer recurrence by developing the immunological memory (Guinn et al., 2007).

The advent of monoclonal antibodies (mAbs) revolutionized the treatment of cancer. Monoclonal antibodies have always been encouraging for scientist as if they have developed novel approaches to produce various antibody formations; recombinant mAbs (include antibody fragments), chimeric antibodies and more recently recombinant polyclonal antibodies (Elbakri et al., 2010). Monoclonal antibodies have emerged as a class of novel oncology therapeutics. The unique pharmacokinetic characteristics, high specificity and the ability to engage and activate the immune system have made them valuable therapeutic agents in breast cancer treatment (Yan et al., 2008). Monoclonal antibodies exert their effects through activating antibody dependent cellular cytotoxicity and complement-dependent cytotoxicity, triggering apoptosis and blockade of growth factor receptors (King et al., 2008). Several monoclonal antibodies against tumour-associated antigen (TAA) have been used as fascinating targeting agents in breast cancer therapy. Clinical success has been observed with passively acquired monoclonal antibodies directed against a number of targets including HER2, HER1, MUC1 and vascular endothelial growth factor (King et al., 2008). However, these molecules have shown some side effects, resistance, immunogenicity and toxicity that have limited their uses. Endeavors to find solutions for these drawbacks led to advent several alternatives (Elbakri et al., 2010).

### 2.2.1 Nanobody; an old concept and new tool for immunotargeting

The large molecular size and immunogenicity are some complications that explain why treatment of solid tumours by mAbs is so elusive. These problems in therapeutic efficacy of mAbs have been partly solved with applying novel approaches such as phage display in the development of new therapeutically effective antibody domains. Antibody domains which lack the Fc region such as Fabs, diabodies, single chain variable fragments (scFvs), bispecific antibodies and variable domains (VH) offer many therapeutic advantages for therapeutic applications (Elbakri et al., 2010). By serendipity, in 1993 the nicest substitutes, the new generation of magic bullets that called heavy-chain antibodies (HCAs) was found in *Camelidae* (Hamers-Casterman et al., 1993) and opened new window in breast cancer therapy (Rahbarizadeh et al., 2004b). HCAs have evolved to be fully functional in the absence of a light chain. The smallest antigen-binding fragment, harbouring the full binding capacity of the naturally occurring HCAs, is called VHH (variable domain of heavy chain antibodies) or single domain antibody. The crystal structure of an isolated single domain antibody is a particle of 2.5 nm in diameter and about 4 nm height, and is termed nanobody because of its size in nm scale and single domain structure.

### 2.2.2 Nanobody; structure and characteristics

Sequence analysis and studies on the crystal structure of nanobodies have revealed several structural features of nanobody domains (Hamers-Casterman et al., 1993; Muyldermans et al., 1994). These molecules contain four framework regions (FRs) that form the core structure of the immunoglobulin domain and three CDRs that are involved in antigen binding. When compared to human VH domains, the FRs show sequence homology of more than 80%, and their 3D structures can be superimposed (Desmyter et al., 1996). The genes of gamma 2 and gamma 3 chains of *Camelidae* HCAb show four amino acid changes at positions 42 (F, Y), 49 (E, Q), 50 (C, R) and 52 (F, G, L, W) according to the IMGT unique numbering, that are involved in forming the hydrophobic interface with VL domains. This co-evolution of the variable region (more hydrophilic) and of the constant region (absence of CH1 due to a mutation in the splicing site) is particularly remarkable. Occasionally, antigen-binding single domain antibody fragments that lack these characteristic FR2 substitutions are isolated from camelids. These fall into two categories:

- VHH-like conventional VHs: low-affinity binders isolated from a non-immune library which was originated from conventional antibodies, presumably because of the polymerase chain reaction crossover cloning artifact, as they were linked to the CH1 domain (Tanha et al., 2002)
- Conventional-like VHH domains: single-domain antibody fragments with conventional-like FR2 sequences that bind antigens with high affinity, isolated from immune libraries, which might have a hydrophobic residue at position 103 (mostly arginine) (Conrath et al., 2001; Harmsen and De Haard, 2007; Saerens et al., 2004).

CDRs of nanobodies are somewhat unique. The first amino acids of CDR1 are highly variable (Harmsen et al., 2000; Nguyen et al., 2000; Vu et al., 1997). VHH libraries generated from immunized camelids retain full functional diversity. High-affinity antigen binding domains can be isolated through screening a limited number of clones from immune libraries (Frenken et al., 2000; Harmsen et al., 2005).

This outstanding nature of nanobodies results in several advantages over the classical antibody families. The single domain nature of a nanobody makes molecular manipulation

easy and also facilitates the production of multivalent formats of them compared to conventional recombinant antibodies, in which the linking of specific length VH and VL domains often results in aggregation and reduced affinity due to mispairing of VH and VL domains. Nanobodies can be used readily for the production of such formats because they allow more flexible linker designs. This is important for simultaneous binding to multivalent antigens (Copier et al., 2009; Roovers et al., 2007).

The nanobody amino acid sequence closely resembles the family III of human variable heavy chain, with significant difference in FR2 and the CDRs (Harmsen et al., 2000; Vu et al., 1997). Nanobodies consist of three distinguished hyper-variable regions and CDRs. The CDRs of nanobodies have unique features when compared to mouse or human VH fragments. These are:

- Only three CDRs are involved in the binding surface of the nanobody (six in conventional antibody fragments) (Arbabi Ghahroudi et al., 1997)
- CDR1 and CDR2 loops are not canonical in structure (Decanniere et al., 2000)
- CDR1 loop might begin closer to the N terminus (Harmsen et al., 2000)
- A longer CDR3 loop with 17 residues on average (12 residues in human, 9 in mouse) (Wu et al., 1993). This long CDR3 results in new antigen binding modes, like binding the active site of the enzyme and also covers the hydrophobic interface that would be formed with the VL domain (Desmyter et al., 2002a; Desmyter et al., 1996)
- A second intra-domain disulfide bond connecting CDR3 with the CDR1, or a core residue between CDR1 and CDR2 (Muyldermans et al., 1994)

Although the amino acid sequences are similar between the nanobody and their classical VH counterparts, several hallmark changes occur in the frameworks encoded by the germ line V genes of the former. The most prominent change is seen in position 42 of nanobodies, which is exclusively occupied by either a F or Y adding to its hydrophobic character. In classical VH3 domains a smaller aliphatic residue, such as V or L, is seen in this position. Studies on crystal structures show that this change results in the packing of the residues from CDR3 against F/Y 42, forming a small hydrophobic core (Desmyter et al., 1996; Spinelli et al., 2001). Another change also contributes to this hydrophobic core, a substitution of an R at position 50 in place of the classical L or V. The long aliphatic side-chain of this R packs against F/Y 42, allowing the guanidium group to be present on the outer surface. A substitution of S for Y at position 52 and A for L/R at position 106 have been seen, but no clear consequences have been described (Bond et al., 2003). The presence of the additional intra-domain disulfide bond (connecting CDR3 and CDR1 or CDR1 and CDR2) may have two outcomes:

- Anchoring the CDR3 against the former interface
- Predispose the orientation of CDR3 for appropriate presentation to the antigen

Based on crystallographic studies the above mentioned substitutions of amino acids result in the conversion of a hydrophobic surface, seen in human and murine VH, into a more hydrophilic surface, thus resisting the VH-VL pairing (Chothia et al., 1985). These changes also seem to contribute to the high solubility of nanobodies compared to other single domain antibodies (sdAbs). Several studies have shown that the loss of the light chain binding partner in camelid nanobody is compensated through the interaction of CDR3 and the former light chain interface with CDR3 residues packing against F 42 (Decanniere et al., 1999; Desmyter et al., 2002b; Spinelli et al., 2000), forming a small hydrophobic core. It should be mentioned that these interactions vary considerably in a wide spectrum of

structural solutions. However, all result in the same outcome: sequestration of the hydrophobic framework residues from the solvent.

Nanobodies rapidly pass the renal filter and because of their small size (15 kDa); they are expected to clear rapidly from blood. This small size also is the reason for fast tissue penetration, an advantage for targeting tumours with nanobody coupled toxic substances (Cortez-Retamozo et al., 2004), *in vivo* diagnosis, imaging and treatment of snake bites.

### 2.2.3 Unique features of nanobodies

The unique intrinsic properties of nanobodies make them better options for medical and biotechnological applications, compared to antigen binding fragments of conventional antibodies. Nanobodies with a molecular weight of ~15 kDa have a size almost half of that for the scFv (30 kDa). Their smaller size also results in lower immunologic response and better pharmacokinetics. Nanobodies are highly soluble, and this is thought to be due to a tetrad of highly conserved hydrophilic substitutions compared to classical antibodies. As mentioned above, another typical feature of nanobodies is their long CDR3, enabling nanobodies to recognize alternative epitopes on an antigen. The lack of post-translational modifications makes the overexpression and production of regular antibodies in bacteria almost impossible. Due to nanobody being active without the modifications mentioned, and as single chains without the Fc domain are easily expressed in bacteria and yeast (Arbabi-Ghahroudi et al., 2005), expression in bacteria should be successful.

Nanobodies are highly stable to extremes of pH and can bind to their target at high concentrations of chaotropic agents (Dumoulin et al., 2002). They also have a remarkable resistance to high temperatures. Studies have shown that nanobodies regain their antigen binding property, even after prolonged incubation at temperatures of 80–92 °C. It should be mentioned that two other nanobody fragments elicited against a hydrophobic azo dye still exhibited activity in a binding assay at 90 °C. A possible explanation for this is that nanobodies do not aggregate during temperature denaturation, resulting in the reversibility of their folding to the native conformation upon cooling (Dumoulin et al., 2002; Ewert et al., 2003).

### 2.2.4 Nanobodies in cancer diagnosis and therapy

Based on their unique biophysical and pharmacological properties, nanobodies should be ideally placed to become a new class of cancer therapeutics. In addition to therapy, nanobodies are also expected to have a future as a tool for the diagnosis of cancer.

#### 2.2.4.1 Nanobodies as cancer diagnostic tools

The superior penetration potential of nanobodies, due to their high affinity target binding and fast clearance from the circulation of the excess of non-targeted nanobodies, represents an ideal basis for imaging purposes. Early detection and staging of prostate cancer is based on the detection of prostate-specific antigen (PSA) in the blood circulation. New nanobodies have been generated that can discriminate between different isoforms of PSA (Saerens et al., 2004). Nanobodies were also used as targeting probes for imaging the *in vivo* biodistribution of specific cell types, using a couple of nanobodies raised against mouse dendritic cells. The observed *in vivo* biodistribution for the two selected nanobodies with different cellular specificities nicely reflects the main *in vivo* locations of the cells that have been determined *in vitro* to be recognized by the nanobodies (De Groeve et al., 2010).

The successful selection and the characterization of antagonistic anti-EGFR nanobodies were shown by Roovers et al. (2007). These researchers isolated nanobodies from immune phage

nanobody repertoires, and showed that they specifically competed for EGF binding to the EGFR and were effective in delaying the outgrowth of A431-derived solid tumours in an *in vivo* murine xenograft model. Recently, llama single-domain antibody fragment was exploited for the *in vivo* radioimmunodetection of EGFR overexpressing tumours by means of a single photon emission computed tomography (SPECT) in mice. The nanobody (8B6) was then labeled with Technetium ( $^{99m}\text{Tc}$ -8B6) through its C-terminal histidine tail. The EGFR-specific nanobody investigated in this study showed high specificity and selectivity towards EGFR over expressing cells. Pinhole SPECT analysis with  $^{99m}\text{Tc}$ -8B6 nanobody enabled *in vivo* discrimination between tumours with high and moderate EGFR over expression. The favorable biodistribution further corroborates the suitability of nanobodies for *in vivo* tumour imaging (Huang et al., 2008). The high tumour uptake, rapid blood clearance, and low liver uptake of nanobodies make them powerful probes for noninvasive imaging of antigen expression. In other study pinhole SPECT/micro-CT was used in an experiment to evaluate *in vivo* tumour uptake and biodistribution of two specific anti-EGFR nanobodies. Uptake in EGFR expressing tumours was high for both compounds, whereas the EGFR negative tumours showed only minor uptake. This confirms the selective targeting of anti-EGFR nanobodies that have affinities in the nanomolar range (Gainkam et al., 2008).

In another study, for finding better antibody formats for *in vivo* imaging and/or therapy of cancer, three types of sdAb-based molecules directed against EGFR were constructed, characterized and tested. Eleven sdAbs were isolated from a phage display library constructed from the sdAb repertoire of a llama immunized with a variant of EGFR. A pentameric sdAb, or pentabody, V2C-EG2 was constructed by fusing one of the sdAbs, EG2, to a pentamerization protein domain. A chimeric HCAb (cHCAb), EG2-hFc, was constructed by fusing EG2 to the fragment crystallizable (Fc) of human IgG1. Whereas EG2 and V2C-EG2 localized mainly in the kidneys after *i.v.* injection, EG2-hFc exhibited excellent tumour accumulation, and this was largely attributed to its long serum half life, which is comparable to that of IgGs. The moderate size (80 kDa) and intact human Fc make HCAs a unique antibody format which may outperform whole IgGs as imaging and therapeutic reagents (Bell et al., 2010).

#### 2.2.4.2 Therapeutic applications of nanobody

Although the high specificity of antibodies makes them suitable in many applications and processes, antibodies fragments are mostly applied in human therapy and *in vivo* diagnosis. PCR and the development of powerful panning techniques led to the generation of large libraries of scFv from which several binders could be selected successfully. Gene cloning and expression in bacteria facilitate the mutagenesis of the antigen binding site and improve the immunological and pharmacokinetic qualities (Skerra and Pluckthun, 1988). However, the application of these techniques is quite complex. The expression yield, stability, and functionality of scFv often turn out to be problematic. The immunogenicity and unsatisfactory yield of functional and monomeric products in heterologous expression systems are still some drawbacks in the development of scFvs for therapeutic applications (Whitlow et al., 1993).

The discovery of nanobody raised new hopes to obtain soluble single domain antibodies with a small size. The high affinity and specificity of nanobodies, plus their small size make them suitable for targeting antigens in obstructed locations, such as tumours due to their poorly vascularized tissue. The delivery of toxins or radioisotopes to diseased tissues

(Carter, 2001) is another potential therapeutic use for nanobodies, ensuring targeted delivery of the toxin to specific tissues and minimizing the time of exposure of normal cells. The short half-life of these antibodies is well suited for certain applications where rapid clearance is essential.

Cancer diagnosis in early stages requires an imaging agent able to deliver a sufficient amount of label to the site through prompt tumour penetration and rapid clearance of the unbound conjugate (Rosebrough and Hashmi, 1996; Sundaresan et al., 2003; Zuckier LS, 1997). The small size and high affinity target binding of nanobodies and their fast clearance result in significant penetration of these agents into tumour tissues. There are several reports of successful isolation of nanobodies against tumour markers. MUC1 is a tumour-associated marker with an extensive extracellular domain. In the breast, ovarian, lung, prostate, colon and pancreatic cancer tissues, not only is MUC1 over-expressed, but the core protein is also aberrantly glycosylated, making the tumour-associated mucin antigenically distinct from the normal mucin. Anti-MUC1 antibodies are used for *in vivo* targeting of breast and ovarian tumours, and there is considerable interest in MUC1 as a possible target antigen for immunotherapy of breast cancer (Taylor-Papadimitriou et al., 2002). For the first time, we have reported immunization of one- and two-humped camels by cancerous tissues and tumour markers, preparation of VHH gene libraries from these lymphocytes and isolation of single domain antibodies against MUC1 tumour marker. Furthermore, this was the first report of the production of a nanobody against a tumour-associated peptide (Rahbarizadeh et al., 2004a). Nanobodies against this tumour associated antigen (TAA) showed good specificity toward the synthetic peptides and have been successfully expressed in *E. coli* (Rahbarizadeh et al., 2005), *P. pastoris* (Rahbarizadeh et al., 2006), Tobacco plants (Ismaili et al., 2007; Rajabi-Memari et al., 2006) and CHO cells (Bazl et al., 2007). Moreover, mouse models with breast cancer tumour originated from MCF-7 (human breast cancer cell line) were used for *in vivo* tumour targeting. We have labeled ER46-28, anti-MUC1 nanobody, with  $^{131}\text{I}$  and injected to these mice. The results of this assay confirmed our concept about nanobodies' effectiveness. Although short half life and rapid clearance of nanobodies are favourable in the case of cancer imaging, a longer serum half-life is more suitable for therapy. PEGylation (Chapman, 2002), conjugation and fusion to an Fc fragment of an antibody (Smith et al., 2001) successfully increase the serum half-life of nanobodies. Fusion of nanobodies with proteins such as albumin can provide multifunctional proteins with several binding sites (Bender et al., 1993; Harmsen et al., 2005).

The peculiar nature of nanobodies endows them more ability and they can be shared of immune-constructs. nanobodies act as a recognition site in chimeric T cell receptor, targeting vehicle for imaging and scanning of tumors and as a targeting agent on nanoparticles to drug or gene delivery to tumor associated antigens.

### 2.3 Combating against cancer with the multifunctional arms of immune system

CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are valuable components of adaptive immunity, which play pivotal roles in the elimination of tumours. The unique properties of T cells such as the capability of proliferation, homing, extravasation, and target rejection make them attractive candidates for adoptive immunotherapy (Cartellieri et al., 2010). Adoptive immunotherapy encompasses *ex vivo* manipulation and expansion of autologous T cells, followed by their re-infusion into tumour-bearing hosts. The *ex vivo* expansion of lymphokine-activated killer (LAK) cells or tumour-infiltrating lymphocytes (TIL) has attained some noteworthy



response rates in cancer patients. However, despite encouraging responses in patients with melanoma, response rates for several cancers such as breast cancer have remained low. This is partly because of the difficulty in isolating, expanding low frequent endogenous tumour-reactive T cells and their poor persistence after transfer (Berry et al., 2009; Hawkins et al., 2010). Meanwhile, tumours deploy strategies to persist and proliferate even if a large numbers of tumour-specific T cells exist. These strategies include; the low or absent expression of tumour-specific antigens, expression of antigens that are shared with normal cells at certain developmental stages, major histocompatibility complex I (MHC I) loss through structural defects, changes in  $\beta$ 2-microglobulin synthesis, defects in transporter-associated antigen processing or actual MHC I gene loss (allelic or locus loss), production of immunosuppressive molecules by the tumour itself or by the tumour microenvironment such as Interleukin-10 (IL-10), transforming growth factor  $\beta$  (TGF- $\beta$ ) and soluble Fas ligand, Indolamine-2,3-Dioxygenase, recruit regulatory T cells (Treg), impaired dendritic cell (DC) function via inactivation (anergy) and/or poor DC maturation through changes in IL-6/IL-10/VEGF/granulocyte monocyte-colony stimulating factor (GM-CSF) (Biagi et al., 2007; Brenner and Heslop, 2010; Copier et al., 2009; Leen et al., 2007).

To overcome these problems, many approaches have been developed. One of the most attractive is genetic engineering of T cells. It dates back to the middle of 1980, when scientists observed a spontaneous transcription of an aberrantly joined immunoglobulin variable heavy (IgVH) gene and a T cell receptor (TCR) JaCa gene resulting from site-specific Chromosome 14 inversion occur in human T-cell tumours (Gross et al., 1989). In another study in 1987, Gascoigne and co-workers reported a chimeric protein consists of T cell receptor variable domain and the immunoglobulin constant domain synthesized in myeloma cells. This protein with normal L chains formed a secreted tetramer (Gross et al., 1989). These reports have been inspirational for scientists to generate the supernatural T cells which possess both antibody and T cell abilities. These well-known T cells express T-bodies. In designing of T-bodies or chimeric antigen receptor (CAR), two humoral and cellular arms of immune system were exploited. CARs are typically composed of an extracellular antibody recognition domain (usually scFv) specific for tumour antigen that is linked by a hinge region to transmembrane and intracellular signaling domains. The use of antibody-binding regions in CARs enables T cell not only bind to TAA through their scFv in a non-MHC-restricted manner, but also respond to epitopes formed by protein, specially carbohydrate and lipid which are not recognized by conventional TCRs. After recognition site (scFv), second site belongs to hinge regions or spacers including the CH2-CH3 domains of the immunoglobulin heavy chain (IgG1, IgG3 and IgG4). Spacers lead to optimize function of CARs (by extending the distance between scFv and the T-cell membrane). The momentous parts of CAR, transmembrane and intracellular signaling domains, are derived from the cytoplasmic region of TCR complex (CD3 $\zeta$ ) or Fc-receptor- $\gamma$  chain. The signaling domains of CARs are so crucial and determine functionality of genetically engineered T-cells. Many studies have been done and examined several motifs such as protein tyrosine kinase (PTK); ZAP70 and LCK to increase the power of TCR signaling (Eshhar, 2010; Sadelain, 2009). Stancovsky and co-workers constructed two anti-HER2 scFv, N29CD3 $\zeta$  and N29CD3 $\gamma$ , chimeric genes that were expressed in cytotoxic T cell. Their study proved IL2 secretion and HER2-overexpressing cells lysis by redirected T cells (Stancovski et al., 1993). These simple structures were the first generation CARs with acceptable cytotoxicity but didn't show appropriate proliferation and prolonged survival after repeated exposure to Antigen. To achieve T cells with optimal activation and function, the signaling domain of co-stimulatory

molecules CD28, 4-1BB (CD137) and OX40 (CD134) were incorporated into the earlier structure and led to the second and third generations, respectively. In these tripartite constructs, in addition to a primary TCR-mediated signal, a secondary co-stimulatory signal is provided for T cells and results in highly efficient target cell lysis, proliferation, cytokine secretion, prolonged survival and rescue from apoptosis (Hombach and Abken, 2007; Zhong et al., 2010). The first generation of CAR have only been tested in phase I clinical trials in cancers such as ovarian, renal, lymphoma, and neuroblastoma, that have not shown significant results (Sadelain et al., 2003; Weisser and Hall, 2009). In the field of T cell therapy, scientists of ATTACK group (Adoptive engineered T cell Targeting to Activate Cancer Killing group) have focused on improving and optimizing the gene-redirected T cells activity (proliferation, secretion and cytotoxicity) in clinical applications. For these purposes, considerable progress in adoptive T cell therapy has been made in recent years.

### 2.3.1 Control of unwanted response of redirected T cells

Whereas usually retroviral or lentiviral gene transduction are utilized to constitutively express chimeric receptors in T cells, for the first time mRNA electroporation was applied to achieve transient immunoreceptor expression, to avoid of unintended auto-aggression. In this research, transfection of CD4 (+) and CD8 (+) T cells was efficiently performed with immunoreceptors specific for HER2 and carcino embryonic antigen (CEA). The immunoreceptor expression was transient with half-maximal expression at second day and no detectable immunoreceptor expression at nine days after electroporation. Immunoreceptor-transfected T cells were specifically activated upon co-incubation with HER2 (+) and CEA (+) tumour cells, respectively, resulting in secretion of interferon-gamma (IFN- $\gamma$ ), interleukin-2 (IL-2), and tumour necrosis factor-alpha (TNF-alpha). Furthermore, immunoreceptor-transfected CD8 (+) T cells specifically lysed HER2 (+) and CEA (+) tumour cells. The RNA-transfected T cells retained their cytotoxic function after two days of activation and exhibited cytolytic activities similar to T cells that have been transduced retrovirally (Birkholz et al., 2009).

Another approach to prevent unwanted response of redirected T cells on healthy tissues (Graft Versus Host), is incorporating the suicide genes such as herpes simplex viral thymidine kinase or caspase 9, into the CAR construct (Abken et al., 2003; Park et al., 2007). The herpes simplex viral thymidine kinase (HSV-tk) gene is widely used. Its product phosphorylates ganciclovir or acyclovir to the active moiety and interferes with DNA synthesis. Reprogrammed T cells with (HSV-tk) suicide gene are sensitive to the cytotoxic effects of ganciclovir, and even if GVHD (Graft Versus Host Disease) happened, by administering ganciclovir, cytotoxic T cells will be inactive. Unfortunately, there is a problem when HSV-tk is used in reprogrammed T cells in adoptive immunotherapy. Since the HSV-tk marker has viral origin and the viral antigens on transduced cells may be recognized by the host's native immune system, transferred T cells are eliminated before they have a chance to provide any therapeutic benefits (Berry et al., 2009; June, 2007; Leen et al., 2007).

### 2.3.2 Employing various strategies for reinforcing gene manipulated T cells against breast cancer cells

Several constructs have been designed to target three members of HER family HER2, HER3 and HER4, such as: scFv-CD3 $\zeta$  (Altenschmidt et al., 1997), scFv-CD3 $\gamma$  (Li et al., 2008), scFv-CD28-CD3 $\zeta$  (Moulder and Hortobagyi, 2008), heregulin-CD3 $\zeta$  (Muniappan et al., 2000), ScFv-CD28-CD3 $\zeta$  "influenza" (Dual-specific T cells were generated by gene modification of

influenza virus-specific mouse T cells with a chimeric gene-encoding reactivity against the HER2) (Murphy et al., 2007). In a study intravenously administration of primary mouse T cells with CAR against HER2 post tumour inoculation caused the rejection of established metastatic breast carcinoma (Berry et al., 2009; Moulder and Hortobagyi, 2008). Preclinical studies for investigating the engineered T cells with different constructs such as scFv-CD3 $\gamma$  and scFv-CD28- CD3 $\zeta$  against HER2 on breast cancer cells are ongoing.

In a different study, the ability of T cells expressing an anti-HER2 chimeric receptor in eradicating tumour in HER2 transgenic mice that express human HER2 as a self-antigen in brain and mammary tissues was evaluated. After the administration of T cells expressing CAR with anti-HER2 as a recognition domain, remarkable enhancement in the survival of mice bearing HER2 (+) - 24JK tumour was observed in comparison with control T cells. Prior to adoptive transfer of T cells with CAR against HER2, mice lymphodepleted and IL-2 administrated that led to further enhance survival. This study highlighted the therapeutic potential of using T cells as a safe and effective treatment of cancer (Wang et al., 2010). In another study two constructs with a scFv derived from the humanized mAb 4D5 Herceptin (Trastuzumab) were designed to generate a CAR against HER2. In the first construct, scFv derived from 4D5 was linked to CD28 and CD3zeta. Expression of it on human peripheral blood lymphocytes (PBLs) led to Ag-specific activities against HER2(+) tumours. Also, this study demonstrated that CD3zeta signaling caused the transgene decrease; T cells expressing 4D5 CARs with mutations in their CD3 immunoreceptor tyrosine-based activation motif (ITAM) were less prone to apoptosis. In the second structure, 4-1BB cytoplasmic domains were added to the CD28-CD3zeta signaling moieties that led to increased transgene persistence, cytokine secretion and lytic activity in 4D5 CAR-transduced T cells (Zhao et al., 2009). Also, T cells were engineered to target MUC1 on breast cancer cells and exhibited considerable results (Wilkie et al., 2008).

B7.1 (important receptor in co-stimulation process) by binding to cytotoxic T-lymphocyte antigen 4 (CTLA4) anergy in T cells. A monoclonal antibody that blocks CTLA-4 binding has been developed to break tolerance. CTLA-4 blockade has entered clinical trials for patients with breast cancer. OX40 signaling on T-cells results in increased survival. OX40 has been targeted in several preclinical tumour models. For example, an agonist monoclonal antibody for OX40 on T-cells showed therapeutic activity. Hence, in a phase I clinical trial of an agonistic anti-OX40 monoclonal antibody has been begun for patients with advanced breast cancers who have failed standard cancer treatments (King et al., 2008; Ward and Kaufman, 2007).

### **2.3.3 Nanobody in CAR receptor: new insight in designing of the extracellular domain of CAR**

As explained before, the recognition site of T-bodies is a scFv that includes the variable heavy (VH) and variable light (VL) domains of a specific antibody which are joined by a flexible linker. The scFvs specific for tumour antigens that are utilized in CARs, have murine origin and can be immunogenic in the host. Some studies were designed to develop chimeric receptor with a humanized scFv to reduce immunogenicity. An alternative approach has been explored that seems to be intellectual. scFvs with ideal properties, have still some drawbacks and must be improved in terms of stability, expression yield, protease resistance, and aggregation (because of its synthetic linker). Nanobodies with high affinity to a target antigen, small size and proper characteristics have been utilized to generate engineered T cells which express nanobody instead of scFv. Chimeric receptors with

nanobody as a recognition domain represent the fourth generation of chimeric T cell receptors. For first time, we replaced nanobody with scFv to target MUC1 on breast cancer cells. The final construct comprised of an anti-MUC1 nanobody as an extracellular domain which was linked via a hinge region to the intracellular domains of CD28 and CD3 $\zeta$ . The results showed the specificity of modified T cells to tumour cells, IL2 secretion, proliferation and toxicity against breast cancer cells (Bakhtiari et al., 2009). In other study, the insertion of intracellular domain of OX40 to the previous chimeric receptor, examined and resulting in IL2 secretion in higher level. Also, several other studies are ongoing such as generation of redirected T cell against HER2 and TAG72 (Rahbarizadeh et al., 2011).

#### **2.4 Nanoparticles for cancer therapy**

Among several drug carriers and drug delivery systems, nanoparticles are very attractive particulate carrier systems under investigation (Kreuter, 2001). The body distribution of these carriers can be controlled by size and surface properties (Stayton et al., 2000). The particulate drug carrier systems have got characteristics such as considerable payload, controlled release of the drug and protection of the drug from degradation (Li et al., 1997). Following intravenous application, nanoparticles accumulate in the tissues of the mononuclear phagocyte system (MPS) and also in tumour tissue, which is often characterized by badly formed and leaky vasculature. This process which is due to an enhanced permeability and retention effect is called passive targeting (Maeda et al., 2000). To enhance the targeting of nanoparticles to specific cells or tissues, target-specific ligands should be linked to the nanoparticle surface (active targeting). Antibody-coupled liposomes (immunoliposomes) were first described in early 1980s (Leserman et al., 1980). Among several coupled homing devices, antibody-coupled nanoparticles can be regarded as an attractive drug-targeting system due to their advantageous properties such as stability (Weber et al., 2000).

In recent years, a variety of nanoparticles (NPs) functionalized with cancer-specific targeting ligands have been investigated to image tumours and detect peripheral metastases (Gao et al., 2004). Most of different types of nanoparticles can be classified into two major groups; (1) particles containing organic molecules as a major building material and (2) those that use inorganic elements, usually metals, as a core. Liposomes, dendrimers, carbon nanotubes, emulsions, and other polymers are a large and well-established group of organic particles (Duncan, 2003; Lee et al., 2005; Tasis et al., 2006; Yezhelyev et al., 2006). Most inorganic NPs share the same basic structure, consisting of a central core that defines the physical properties including fluorescence, optical, magnetic, and electronic features of the particle, with a protective organic coating on the surface which is usually responsible for the biological recognition and improvement of the particle solubility, for protecting the core from degradation in a physiologically aggressive environment and for evading the clearance action of the host immune system.

With the increasing use of targeted therapies in oncology, there is the requirement for the methods of molecular profiling to be optimized. The success of many targeted treatments depends on the expression of specific proteins or genes present in tumour cells. In breast cancer cells, the level of hormone-receptor expression correlates directly with the benefit of endocrine treatments, and the presence of HER2 protein overexpression and/or gene amplification is a prerequisite to benefit from target specific monoclonal antibodies (You et al., 2008). Some breast cancers produce protein biomarkers (such as estrogen receptor, progesterone receptor, and HER2), on which therapeutic decisions are made. The design of

methods that can detect *in vivo* the expression of such markers and monitor them during treatment is a real challenge.

Nanotechnology can be applied for the design of multifunctional nanoparticles that will be able to detect and image tumours and their metastases and meanwhile, it is used for therapy and monitor treatment progression. The application and efficiency of these nanoparticles *in vivo* will help enormously the pre and post cancer treatments (Scott et al., 2008). To design a diagnostic approach to breast carcinoma using nanoagents in humans, it is necessary to conclude several points for their utilization. These include high resolution, accuracy and sensitivity of detection, which may be provided by using NPs coated with specific monoclonal antibodies against protein biomarkers overexpressed by breast cancer cells. In addition, they must ideally have no toxicity, and be able to interact in a physiological way with biological tissues. In particular, they should have a good safety profile and not aggregate when delivered to biological tissues. Finally, since membrane receptors are endocytosed as part of their normal response to ligand binding, functionalized NPs have to follow physiological pathways when internalized. The ultimate challenge is represented by the development of efficient strategies for the good conjugation of targeting biomolecules on the NP surface. In fact, a major issue is the reliable conservation of the biological activity of immobilized macromolecules.

Among the various molecular targets explored for the treatment of human breast carcinoma, NPs conjugated with the anti-HER2 monoclonal antibody (Trastuzumab/Herceptin) is explained here.

#### **2.4.1 Active targeting**

There are numerous investigations for finding efficient systems for site specific delivery of drugs. One strategy involves usage of tumour-specific antibodies against overexpressed tumour associated antigens or receptors as targeting moieties which can be conjugated onto the nanoparticulate surface for efficient delivery of drug. In active targeting of nanoparticles to specific sites in the body, targeting ligands are attached at the surface of the nanocarriers for binding specifically to appropriate receptors or exposed cellular biomolecules expressed with some degree of uniqueness at the target site (Mo and Lim, 2005). The ligand is chosen to bind to a receptor overexpressed by tumour cells or tumour vasculature and not expressed by normal cells. Moreover, targeted receptors should be expressed homogeneously on all targeted cells. Targeting ligands are either monoclonal antibodies (mAbs) and antibody fragments or non-antibody ligands such as, growth factors, transferrin, cytokines, folate and low-density lipoprotein (LDL) (Kocbek et al., 2007). Using tumour-specific antigens or antibodies as targeting moieties, cytotoxic drugs can be selectively delivered to tumour cells, thereby reducing the drug concentration in normal tissues and its toxic side effect (Smith et al., 2008).

#### **2.4.2 Nanoparticles under investigation for breast cancer**

Nanoparticles can be used to treat tumours in three different ways; (1) specific antibodies can be conjugated to the magnetic nanoparticles (MNPs) to selectively bind to related receptors and inhibit tumour growth; (2) targeted MNPs can be used for hyperthermia for tumour therapy; and (3) drugs can be loaded onto the MNPs for targeted therapy (Fernandez-Pacheco et al., 2007; Ghosh et al., 2008; Subramani et al., 2009).

Quantum dots (QDs) exhibit extraordinary photo-stable fluorescent signals and resistance to photo-bleaching. These NPs consist of a typical core/shell structure composed of heavy

metals (Lu et al., 2007). In many cases, QDs include a cadmium selenide or cadmium sulfide core, coated with a zinc sulfide shell. It is possible to modulate their size or change the nature of their metal core in order to vary their emission area in the range 450–850 nm. They are generally synthesized in high-boiling non-polar organic solvents. Thus, to be solubilized in aqueous buffers, their hydrophobic surface ligands must be replaced with suitable amphiphilic ligands.

Superparamagnetic iron oxide NPs are useful for molecular imaging and thermal therapy. They are typically composed of magnetite ( $\text{Fe}_3\text{O}_4$ ) nanocrystals; they have a spinel crystal structure with oxygen ions forming a close-packed cubic lattice and iron ions located at interstices. In recent years, several methods focusing on the synthesis of MNPs have been developed either in aqueous or organic phases (Peng et al., 2008). Many surfactants or polymers are usually employed in the synthesis to avoid aggregation and to reduce phagocytosis by macrophages and to increase circulation time in blood vessels. Among the most largely used MNP coating materials, PEG (poly-ethylene glycol) is highly water soluble and biologically inert, which renders MNPs immunologically stealth. Hence, significant efforts have focused on the possibility to functionalize their surface with ligands in order to create multifunctional NPs.

Gold nanoparticles (GNPs) and gold nanorods (GNRs) are under exploration in biomedicine since gold has been approved for optical detection and thermal therapy of tumours. These NPs are rapidly synthesized and their surface can be easily functionalized with targeting molecules and ligands by thiol chemistry (Chen et al., 2008). Many surfactants have been described, including citric acid and PEG, which are able to maintain the post-synthetic colloidal stability in aqueous physiological solutions.

### 2.4.3 Targeting of nanoparticle to breast cancer

One of the most commonly used strategies for targeted delivery of drugs to breast cancer utilizes the HER2, which is overexpressed in breast cancer. The surface of NPs may be coated with different functionalities, depending on the coating material and the functional groups present on the targeting ligand. Most commonly, amines or carboxylic acids are present on the NP surface. For this reason, the most largely employed method to attach Trastuzumab to NPs is the amide coupling involving carboxyl activation via the highly water soluble sulfo-NHS ester (Eghtedari et al., 2009). Two different targeting approaches have been reported by Nobs and co-workers for immunotargeting with Trastuzumab conjugated to nanoparticles (Nobs et al., 2006). One of the targeting procedure is a direct method using Trastuzumab-labeled poly lactic acid (PLA) nanoparticles and the other is a pretargeting method using the avidin-biotin technology. These experiments have shown that NPs covalently coupled with antibodies or neutr-avidin-rhodamine Red-X (NAR) can specifically and efficiently bind to cancer cells, suggesting that antibody conjugated NPs may be a useful drug carrier for tumour targeting. In other research nanoparticles based on human serum albumin (HSA) were developed. In this approach nanoparticles were covalently attached to thiolated trastuzumab (Steinhauser et al., 2006).

Anhorn and co-workers for the first time reported the specific targeting of HER2 overexpressing breast cancer cells with Doxorubicin-Loaded Trastuzumab-modified human serum albumin nanoparticles. HER2 overexpressing breast cancer cells showed a good cellular binding and uptake of these nanoparticles. The results indicate that these cell-type specific drug-loaded nanoparticles could achieve an improvement in cancer therapy (Anhorn et al., 2008).

Origin	Target	Fusion partner	Potential application
Immunized llama	EGFR	<sup>99m</sup> Tc	SPECT/micro-CT imaging
Immunized llama	EGFR	<sup>99m</sup> Tc	SPECT imaging
Immunized llama	Immunized or non immunized DC	<sup>99m</sup> Tc	<i>In vivo</i> imaging
Immunized llama	EGFR	PEG-liposome	EGFR down regulation
Immunized llama	EGFR	Pantabody-Fc	Tumour Targeting
Immunized llama	EGFR	(mPEG- <i>b</i> -p(HPMAm-Lacn)) core crosslinked thermosensitive polymeric micelles	Drug targeting
Immunized llama	EGFR	PEGylated quantum dots	Cancer imaging and detecting
Immunized dromedaries	CEA	<sup>99m</sup> Tc	Cancer imaging

Table 1. Some of nanobody-based fusions for diagnosis applications and therapy.

Cationic micellar nanoparticles were employed as carriers to co-deliver paclitaxel and Herceptin in order to targeted delivery of Paclitaxel to HER2 overexpressing human breast cancer cells. The co-delivery of Herceptin increased the cytotoxicity effect of Paclitaxel and this was dependent upon the level of HER2 expression on different cell lines used in this study. Targeting ability of this co-delivery system was demonstrated through confocal images, which showed significantly higher cellular uptake in HER2 overexpressing cells as compared to HER2 negative cells. This co-delivery system could be an important therapeutic tool against HER2 overexpressing breast cancers (Lee et al., 2009).

#### 2.4.4 Nanobody targeted nanoparticle as a cancer therapeutic tool

An important obstacle in the use of antibodies for therapeutic purposes is the immunogenicity of these molecules. In the challenge to reduce the size and immunogenicity of antibodies, different modifications have been performed on the existing antibodies.

In another study, an anti- carcinoembryonic antigen (anti-CEA) VHH was used for targeting the genetically fused  $\beta$ -lactamase to tumour cells. This enzyme then converts an injected nontoxic prodrug into a toxic drug in the vicinity of the targeted tumour cells, leading to their killing (Cortez-Retamozo et al., 2004). More recently researchers presented a multivalent platform, consisting of nanobodies recognizing the ectodomain of EGFR (EGa1) coupled to poly (ethylene glycol)-liposomes, and the *in vitro* and *in vivo* effects of this system on EGFR internalization and downregulation were investigated (Oliveira et al., 2010). In another study Talelli and co-workers developed poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-p(HPMAm-Lacn)) core-cross-linked thermosensitive biodegradable polymeric micelles suitable for active tumour targeting, by

coupling the anti-EGFR (EGa1 nanobody) to their surface (Roovers et al., 2007). In other research for the first time CdSe/ZnS quantum dots were biolabeled by a camelid single domain antibody (EG2), which is raised against epidermal growth factor receptor and these nanobody-conjugated quantum dot used as a specific labeling agent of EGFR expressing human breast cancer cells (Zaman et al., 2009).

#### **2.4.5 Nanobody targeted nanoparticle for targeted cancer gene therapy**

Cancer gene therapy is another approach in the treatment of breast cancer and it involves different strategies. An important issue for gene therapy is the choice of the delivery vehicle, which is able to successfully reach the nucleus of the tumour cells. In addition, the vector should be able to condense DNA from a large micrometer scale to a smaller nanometer scale suitable for endocytosis and to promote the escape of the gene from the endosomal compartment into the cytosol. Furthermore, the vector should be designed and synthesized to be recognized by specific receptors on the target cells and then, easily internalized. Following these concepts, nanoparticle-based DNA and RNA have been envisaged as advantageous delivery systems, using either viral or nonviral vectors for the gene transfection (Leuschner et al., 2006). In 2006, Hayes and co-workers published their preliminary results on using a DNA plasmid coupled with cationic lipids, to form lipid-nucleic acid-NPs, called Genospheres (Eghtedari et al., 2009). To increase the delivery of the nanosystem into the cells of interest, genospheres were immunotargeted to selectively transfect HER2 overexpressing cells, by insertion of an anti-HER2 human single-chain monoclonal antibody (scFv)-PEG conjugate. Developing cancer gene therapy constructs based on transcriptional targeting strategy of genes to cancer cells is a new and promising modality for treatment of cancer. Induction of apoptosis in cancer cells could be an endogenous mechanism for cell death. By cloning and targeted expression of the pro-apoptotic gene in cancer cells, an anti-cancer gene therapy approach could be achieved. In 2006, tBid (a 15kDa protein cleaved from the cytoplasmic protein Bid) was announced as a suitable pro-apoptotic gene because it doesn't need any modification to become fully active and also because of its small size (Kazhdan et al., 2006). In order to limit the tBid expression to cancerous cells transcriptional targeted pro-apoptotic gene strategies in combination with tumour microenvironment factors are considered as efficient ways in cancer gene therapy approaches. Hypoxia and estrogen are microenvironment features of breast cancer cells which limit the action of constructs only to cancerous tissues. In these circumstances cells express hypoxia inducible transcription factor (HIF) that activates several genes. HIF bound to hypoxia responsive elements (HRE) in promoter of these genes and caused transcription. Breast cancer cells also maintain the expression of intracellular estrogen receptors that act as a transcription factor to stimulate the expression of genes in the presence of estrogen and they bind to estrogen response elements (ERE) to activate transcription. We constructed two hybrid promoters which consisted of hypoxia responsive elements, estrogen response elements and MUC1 promoter (HEM) and also, Survivin promoter accompanied with hypoxia responsive elements and estrogen response elements (HES). tBid gene expression under the control of these two hybrid promoters were evaluated in normal and cancer cell lines with and without various treatments of hypoxia and estrogen. MUC1 promoter directs efficient expression of tBid gene under the control of the hybrid promoters which results in cell destruction. This study provides a significant advance in controlling lethal gene expression by using genetic characteristics and microenvironment elements in cancer cells



(Farokhimanesh et al., 2010). But the main hurdles in this type of treatment is finding appropriate vectors for the targeted delivery of genes *in vivo* and making sure that the apoptotic/killer gene is delivered to the target tumour cells. Polyplexes nanovectors are gaining more attention, mostly due to concerns regarding the safety and immunogenicity of vectors derived from viruses. Polycationic polymers, namely the highly cytotoxic poly-L-lysine (PLL) and polyethylenimine (PEI), are among the most widely used in gene therapy. PEI has shown to be efficient in gene delivery to eukaryotic cells both *in vitro* and *in vivo* (Boussif et al., 1995; Wightman et al., 2001). The high positive charge of the polyplexes (PEI/DNA complex) results in non-specific attachment of polyplexes to any negative charge surface, including plasma proteins and cellular membrane phospholipids. Free PEI, when administered systematically, precipitates in large clusters and adheres to cell surface which might result in destabilizing of the plasma membrane, inducing immediate toxicity. To overcome immediate toxicity, stealth nanoparticles were produced through coating the polyplexes with FDA approved polyethylene glycol (PEG) (Owens and Peppas, 2006). So we have used anti-MUC1 nanobody with high specificity for a MUC1 antigen to make PEGylated PEI conjugates for successful compaction of the tBid killer gene and its selective delivery into MUC1 expressing cell lines. Our attempt has provided a powerful proof of concept in combining nanobody-based targeting with transcriptional targeting as a safe way to deliver transgenes to specific cells.

### 3. Conclusion

With more than 20 therapeutic mAb products currently on the market and more than 100 in clinical trials, it is comprehensible that engineered antibodies have come of age as biopharmaceuticals (Reichert, 2008). In fact, in this decade, engineered antibodies are predicted to account for more than 30% of all revenues in the biotechnology market. Despite various beneficial characteristics of the conventional antibodies, the low inherent toxicity of the nanobodies together with their size characteristics and their high specificity and affinity for the antigen render them more promising candidates for delivery of pro-drugs, therapeutic genes (anti-angiogenesis, growth inhibitors and toxins) and chemotherapeutic agents. Moreover, nanobodies lack an antibody Fc tail, thus causing less immunological side effects. Nanobodies are anticipated to significantly expand the repertoire of antibody-based reagents against the vast range of novel biomarkers. Although few investigations have been conducted to use nanobodies against tumor markers, it is becoming increasingly clear that these potential immunotherapy nanosystems show omen in cancer therapy, and their successful use in treatment protocols is expected to be widely reported. As spelled out above, all these characteristics imply that there exists remarkable promise for nanobodies to be exploited as robust tumour diagnostic and cancer therapeutic reagents while presenting superior biological properties in comparison with conventional strategies.

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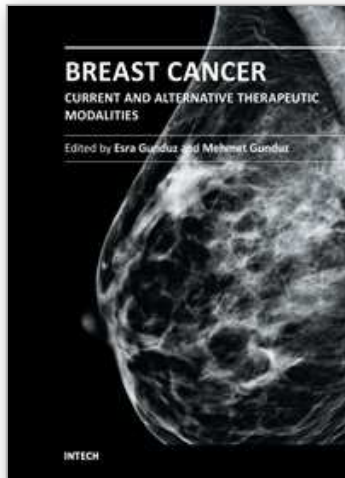
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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