Arch. Immunol. Ther. Exp. (2013) 61:75–83
DOI 10.1007/s00005-012-0206-x

ORIGINAL ARTICLE

Recombinant λ Bacteriophage Displaying Nanobody towards Third Domain of HER-2 Epitope Inhibits Proliferation of Breast Carcinoma SKBR-3 Cell Line

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Received: 22 February 2012 / Accepted: 20 August 2012 / Published online: 7 December 2012

Abstract Phage display of many nanobodies via filamentous phage in combination with helper phage has been reported by many scientists. The aim of this study was to produce lambda (λ) bacteriophage displaying high-affinity nanobody against HER-2 expressing breast carcinoma cells. Bacteriophage λ is a temperate phage with inherent biological safety in mammalian cells. Here we report the construction of a recombinant λ phage that efficiently expresses specific nanobody towards third domain of HER-2 target on SKBR-3 and MCF-7 cell lines in vitro. We constructed recombinant λ phage particles containing a mammalian expression cassette, C-Myc tagged, encoding VHH gene of camelid anti HER-2 third domain epitope using λ ZAP-cytomegalic virus (CMV) vector. The SKBR-3, MCF-7 and human endometrial stem cells were treated by the nanobody displayed recombinant λ phage. The cell growth inhibition assay was performed by MTT Cell Viability Assay Kit. After the fourth round of biopanning there was a significant enrichment in the phage specifically binding to the antigen. The ratio of targeted phage increased approximately 1,000-fold in the fifth round. The nanobody expressed by λ ZAP-CMV-VHH phagemid cloned in λ bioparticles significantly inhibited the proliferation of HER-2 positive SKBR-3 and MCF-7 cells. Recombinant bacteriophage λ ZAP-CMV-VHH-cDNA could be used efficiently for construction of nanobodies to mortify HER-2 positive breast carcinoma cells as a nanomedical therapeutic.

Keywords Nanomedicine · Nanobody · Nanobioparticle · Recombinant

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Introduction

Pharmaceutical discovery is based on the screening for desired pharmacological activities of large numbers of different molecules. The hunt for molecular targets expressed on diseased cells has recruited a number of new identification technologies. Progress in the field of antibody engineering has led to the production of improved humanized monoclonal antibody (mAb) constructs and recombinant antibody fragments such as single chain antibodies and nanobodies. Nanobody molecules have size in the nanometer range ~2.5 nm in diameter and 4 nm in height (Spinelli et al. 2000). In contrast to variable heavy domains of conventional antibodies, nanobodies are expressed as soluble proteins. These nanostructures are

highly stable against extremes of temperature (90 °C) and pH (3–11), also they are able to bind antigen with nanomolar affinity (Van Der Linden et al. 1999). Nanobodies recognize hidden epitopes that are normally not recognized by conventional antibodies (De Genst et al. 2002). Their small size results in a rapid tissue penetration (Presta 2006). In contrast, conventional antibodies stay in blood circulation for a long time and tend to accumulate in the liver and other organs (Burvenich et al. 2005; Goldenberg and Sharkey 2007). In addition, there is low affinity or stability problems, therefore new researches have focused to progress in high affinity and small size nanobodies that selectively target the tumors. The antigen binding parts of camel heavy chain antibody were proven to be an excellent choice to obtain nanobodies (Ahmadvand et al. 2009).

One efficient method to produce a high-affinity nanobody is the phage display technology. This technology allows ligand directed selection of peptides to corresponding receptors selectively expressed in tumor tissues. The expression of exogenous peptides on the surface of filamentous phage was described by George Smith in 1985 (Hajitou et al. 2006a, b).

In a phage display technique, the bioactive peptides are selected by biopanning against exclusive receptors (Sidhu et al. 2000). Bacteriophage (bacterial virus) is considered poor vehicle for transduction of eukaryotic cells. It has no tropism for mammalian cells (Barbas et al. 2001). Bacteriophage lambda (λ) is a temperate phage with a double-stranded DNA genome (~50 kb) and a 150-nm long tail. Its capsid is composed of glycoprotein (gp) E and gpD major coat proteins (Murialdo and Ray 1975). When the genome is packaged, the capsid expands in volume and about 405 copies of gpD are then added to fully populate and stabilize the capsid (Dokland and Murialdo 1993). λ phage have high production capacity, high degree of stability, rapid and inexpensive production or purification techniques and inherent biological safety in human cells (Catherine et al. 2004; Lankes et al. 2007; March and Clark 2004). Construction of phagemid vectors, which are packaged into phage particles, has made the manipulation of bacteriophage easy (Larocca et al. 2001). The coat protein of the λ phage can be engineered to incorporate targeting ligands without affecting structure significantly (Murialdo and Ray 1975).

It is well known that tumors express epitopes that are unique to tumor tissues. Tumor-specific peptides could be identified and exploited therapeutically. HER-2, a receptor tyrosine kinase, is highly expressed on epithelial cancer cells and remains a target for antibody-based detection and therapeutic applications. It is implicated in many cellular processes such as proliferation, differentiation and metastasis. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in the responsible gene are associated with cancers. Several studies have shown that HER-2 signaling is abnormal in many tumors of epithelial origin, such as breast cancer. Irregular HER-2 signaling is due to receptor over-expression, mutations or ligand over-expression. These changes lead to cancer initiation and progression, so this determinant is a target for anticancer therapy (Yarden and Sliwkowski 2001).

Phage display of many nanobodies via filamentous phage in combination with helper phage has been reported by many scientists. The filamentous phage particles mostly used for display purposes are known as Ff and include strains M13, f1, Fd and ft. So we decided to use another group of phage for this purpose. The aim of this study was to produce a bacteriophage λ that expresses specific nanobody against third domain of HER-2 antigen. Here we report the construction of a recombinant λ bacteriophage that expresses specific nanobody towards HER-2 desired epitope.

Materials and Methods

Strains and Media

Escherichia coli DH5α (Persian Type Culture Collection, Tehran, Iran) was used as a host for antibody production. RecA-*E. coli* host strain XL1-Blue MRF (Gigapack, USA) was used for λ phage amplification and titration purposes. The Luria-Bertani (LB; Sigma-Aldrich, USA) and 2YT media (Invitrogen) was supplemented with 100 μg/ml ampicillin (Sigma, USA) and kanamycin A (SantaCruz, USA) for the selection of transformants. The LB medium (supplemented with 0.01 M $MgCl_2$) and M9-medium (supplemented with ampicillin and IPTG) were used as electroporation and expression medium, respectively.

Cell Culture

The human breast carcinoma cell lines including SKBR-3 and MCF-7 (positive for HER-2) and human endometrial stem cells were obtained from Pasture Institute cell bank (Pasture Institute, Tehran, Iran) and cultivated in RPMI-1640 (Gibco, UK) and DMEM (Gibco, UK) supplemented by 10 % fetal bovine serum (FBS; Gibco, UK), respectively. All cells were grown at 37 °C in humidified atmosphere of 5 % CO_2.

Preparation of *Camelus dromedaries* VHH Library

The *C. dromedaries* immunization protocol followed the animal experimentation guidelines published by the Regional Canary Government and was approved by the Ethic

Commission of the Veterinary Faculty of Tehran University. Under the protocol, 1 mg of purified peptides from the third domain of HER-2 epitopes (the desired domain was a gift from Biologic Products Department, Pasteur Institute, Iran) was injected subcutaneously once a week for 6 weeks. Complete Freund's adjuvant (Pastuer Institute, Iran) was used for the first injection and incomplete Freund's adjuvant (Pastuer Institute, Iran) for the following injections. Blood was taken on EDTA (Merck, Germany) anti-coagulating medium 4 days after the last boost. Construction of the VHH library followed established steps as described previously (Conrath et al. 2009; Saerens et al. 2005; Tereshko et al. 2008). Total RNA from lymphocytes of immunized *C. dromedaries*, was isolated (Roche RNA purification kit, Roche, Germany) and used as a template for cDNA synthesis (Roche). DNA fragments encoding VHH genes were amplified by PCR with F (5′-A GCTTGAACCTCGCAGTC ATTTATTTC AAGAGAAT AAATGACTGCGAGGTTCTTTTTTG-5′) and R (5′-GAT CCAAAAAAGAACCTCGCAGTCATTTATTCTCTTGA AATAAATGACTGCGAGGTTCA-5′) primers containing *Bam*H1 (Promega) and *Hin*dIII (Roche, Germany) restriction enzymes sites using an ABI step one system (Applied Biosystems Inc., CA, USA). The VHH fragments with flanking *Bam*H1 and *Hin*dIII sites were cloned into pUC19 vector (AddGene, USA).

Construction of λ ZAP-Cytomegalic Virus (CMV) Vectors

The VHH gene was excised from the pUC19 vector using suitable restriction enzymes to be ligated into λ ZAP-CMV vector (Stratagene, USA). The ligation was performed into λ ZAP-CMV vector at a volume up to 2.5 µl using 2 U of T4 DNA ligase (Fermentas, USA) and 0.5 µl of 10 mM ATP (Invitrogen, USA). The amplified VHH gene and λ ZAP-CMV vector were digested using *Bam*H1 and *Hin*dIII, ligated and electroporated into DH5α strain electrocompetent cell and plated for separation of individual clones as described previously (Arbabi Ghahroudi et al. 1997; Sambrook and Russel 2001). The selected clone was grown at 37 °C until turbidity of 0.7 at OD_{600}. The culture was induced with different concentrations of IPTG (0.5, 1, and 1.5 mM) (MediaTech). For packaging of λ phage particles, the ligated DNA immediately was added to the packaging extract. Also, 1 µl of the control ligation was added to a separate tube of packaging extract. Then, the tube was stirred to mix well. The tube was incubated at room temperature for 2 h. One milliliter of SM buffer [included 5.6 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50.0 ml 1 M Tris–HCl (pH 7.5), 5.0 ml gelatin 2 % (w/v) in 1,000 ml distilled water] was added to the tube. Then, 20 µl of chloroform (Sigma-Aldrich, USA) was added and mixed the contents of the tube, gently. The tube was centrifuged to sediment the debris. The supernatant containing the phage was ready for titration. It was titrated by plating the infected DH5α on agar plates (Gibco, TM) according to the manufacturer's instructions. Large scale λ phage was conducted in 1 l of M9 medium (Sigma-Aldrich, USA) containing ampicillin (Sigma, USA). After 24 h the culture was induced by adding 1 mM IPTG and was incubated a further 72 h. Induced culture was centrifuged at 8,000*g* for 40 min. The nanobody expressing phage present in this extract was purified by immobilized metal affinity chromatography (Qiagen). The product was dialyzed against phosphate buffer 10 mM. The purification was confirmed using SDS-PAGE and Western blot analysis as described previously (Shoae-Hassani et al. 2009). The anti Her-2 antiserum was derived from rat ascitic fluid and was used at 1:100 dilution. The rat polyclonal antiserum was diluted by tris buffered saline containing 5 % bovine serum albumin (BSA). Finally, the horseradish peroxidase conjugated rabbit anti-rat antibody (Sigma, USA) was used for the detection of blotting product.

Biopanning of Phage Display Libraries

An overnight culture of DH5α *E. coli* in LB was grown until its absorbance reached a value equal to 0.6 at a wave length of 600 nm. After five rounds of biopanning against extracellular third domains of HER-2 epitope, the enriched phage was amplified. Plasmid profile of enriched library pools after each step of biopanning was monitored by electrophoresis on the agarose gel. SKBR-3, MCF-7 and endometrial stem cells were left to grow until being confluent. Both cells and phage ($\sim 10^{12}$ U) were first blocked in 4 % skimmed milk powder/PBS (MPBS) for 1 h on ice. To deplete the enriched library of non specific phage 10^6 third passage endometrial stem cells were incubated with phage antibody library for 1 h rocking at 4 °C. SKBR-3 cells (10^6) were incubated in a culture tube with the depleted phage for 1 h. Cells were washed three times with 4 % MPBS and bound phage was eluted by incubation of the cell pellet with 1 ml of 100 mM tri-ethylamine (Santa Cruz, USA). Solution was neutralized with 0.5 ml of 1 M Tris–HCl (pH 7.4) centrifuged for 2 min, and the supernatant was added to 10 ml of log phase DH5α *E. coli* ($OD_{600} = 0.6$). The infected bacteria was centrifuged at 3,500*g* for 10 min. The pellet was resuspended in 100 ml 2YT containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and was grown at 30 °C overnight. The phage was precipitated from the supernatant by the 40 % PEG diluted to NaCl (9 %) solution (1:1.5; Sigma-Aldrich, USA) as described previously (Kontermann and Dubel 2001). The cell selection procedure was repeated another two times.

Phage Clonal Amplification

Bacterial colonies were seeded into microplate wells containing 200 µL of 2YT medium with 10 µg/l ampicillin. After overnight incubation at 37 °C, microplates were centrifuged (200*g*, 4 °C, 15 min). The supernatant containing the phage was used for enzyme linked immunosorbent assay (ELISA).

Determination of the Apparent Affinity Constant

The apparent affinity constant of each phage library was determined by saturation experiments performed by the ELISA. Serial phage dilutions ranging from $\sim 10^8$ to 10^{12} M were prepared in calcium buffer (2 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, 3 mM NaN_3, pH 7). The quantity of bound phage obtained from the A_{450} values was plotted against the logarithmic concentration of total phage input, and the curve was fitted according to a sigmoidal profile.

Screening of Monoclonal Antibodies

Over 20 colonies were picked after the third round of biopanning. In the fifth round, we selected the phage clone that did have highest affinity to the SKBR-3 cells. It was the best clone for reaction with the HER-2 over-expressing cell line. To confirm the binding ability of selected phage clones towards the antigen, 10^{12} PFU of phage clone were tested by cell-ELISA.

Immunocytochemistry

The reactivity of phage displayed nanobody with SKBR-3, MCF-7 and human endometrial stem cells were examined. An unrelated antibody and 4 % MPBS were used as negative control. Each cell line was grown on microplates. The cells were left to grow until being confluent. Phage displayed nanobody at a concentration of 5 µg/ml were then added to each well and processed as explained previously (Rahbarizadeh et al. 2006).

SKBR-3 and MCF-7 Cells Proliferation Inhibition Assay

SKBR-3 and MCF-7 cell lines (10^4 cells/well) and EnSCs were plated onto 200 µl of medium without FBS, and incubated at 37 °C in a 5 % CO_2 overnight. Various amounts (0.25, 0.5, 1, and 2 µg/ml) of λ phage expressing anti HER-2 nanobody and another antibody as a negative control, were added to wells. Culture plates were incubated for 72 h. All assays were carried out in triplicates. During incubation, culture media were replaced with medium, containing phage displayed nanobody every 24 h. Cell proliferation was assessed by MTT Cell Viability Assay Kit (Biotium, Hayward, CA). After incubation, MTT reagent (10 µl) was added to the wells and incubated for 3 h. At the end of the incubation period, the medium was removed and 100 µl DMSO (Invitrogen) was added into each well. In order to dissolve the Formazan crystals the supernatant was pipetted several times. Absorbance on an ELISA plate reader at wavelength of 540 nm was measured.

Thermo-Stability and Serum-Stability of Phage Expressed Nanobodies

The purified construct samples were prepared in 50 mM sodium phosphate (pH 7.0) at 1 mg/ml of concentration. A volume of 100 µl of the sample was added to some microtubes and the tubes were put into the water bath, and heated from 30 to 55 °C at a constant rate (5 °C in 10 min). After every 10 min, one of the tubes was removed from the bath and subjected to cell-ELISA assay with SKBR-3 and MCF-7 cell lines. This process is continued to 55 °C. The activity of heat-treated phage expressed nanobodies was measured as a percentage activity of the not treated nanobodies. Also for the serum-stability test (Hmila et al. 2008), 1 µg samples of phage expressed nanobodies (at 1 mg/ml) were mixed separately in 100 µl sera. After different intervals of incubations (between 1 and 24 h) the samples were subjected to cell-ELISA assay with SKBR-3 and MCF-7 cell lines.

Statistical Analysis

Statistical analysis was performed using ANOVA with significant levels at $p < 0.05$.

Results

Lymphocytes from a *C. dromedaries* immunized with a desired part of human HER-2 antigen were isolated and the coding sequences of nanobodies were amplified by PCR. The PCR product (420 bp) was cloned into pUC19 vector between *Bam*H1 and *Hin*dIII restriction sites adapted for ligation into λ ZAP-CMV vector with the same excision sites. The vector was packaged into λ phage. This process resulted in the libraries of approximately 10^5 transformants. The nanobody expressed on the λ phage pool was screened by biopanning. Approximately 10^{12} phage were used in each round of biopanning. In biopanning 10^3, 10^5, 3×10^5, and 3×10^6 phage were eluted after the first, second, fourth and fifth rounds, in order (Fig. 1a). In each round, the bound phage was rescued and amplified for the

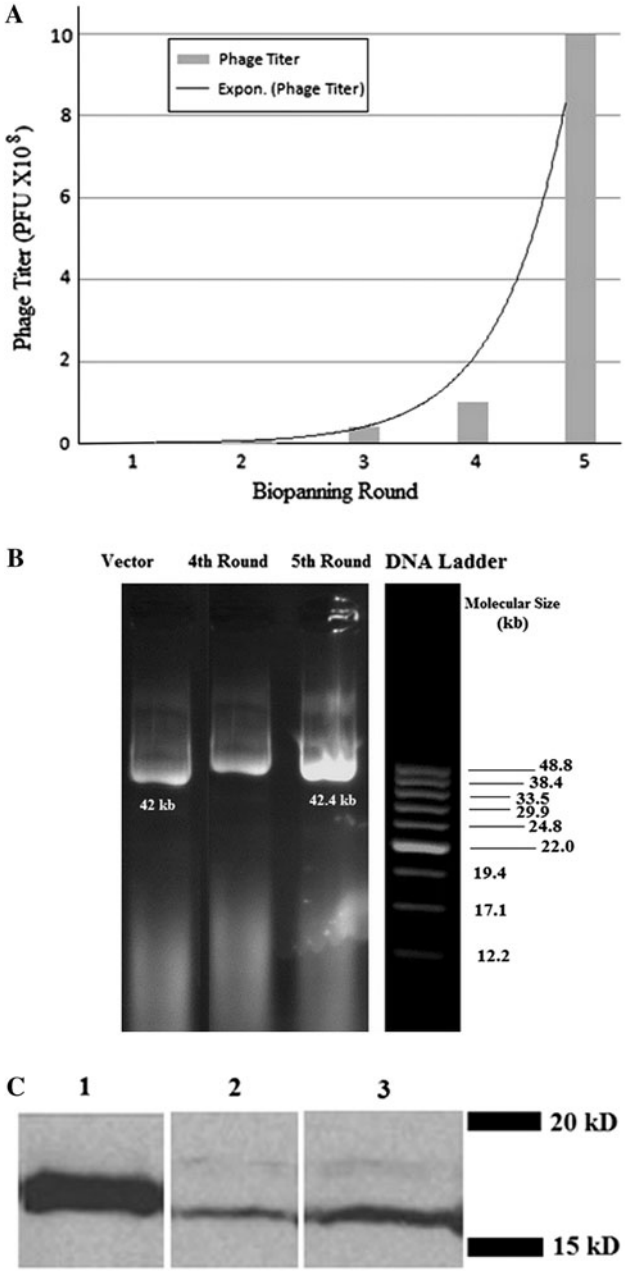


Fig. 1 Expression of nanobody after biopanning process. Specific enrichment of λ phage as noted when the fifth round of selection was finished. The titers of the recovered phage from each round were evaluated using a cell-ELISA assay (**a**). The phagemid DNA bands were visualized using ethidium bromide staining. From the *left corner*; *lane 1* ZAP-CMV expression vector, *lane 2* second round of biopanning, *lane 3* third round, *lane 4* fourth round, *lane 5* fifth round of biopanning (**b**). Western blot analysis: the *left corner*. *Lane 1* β-actin band as an internal control, *lane 2* cell lysate of IPTG-induced bacterial harboring phagemid, and *lane 3* λ bacteriophage expressing anti HER-2 nanobody (**c**)

following round of biopanning, while the unbound phage was removed by washing with TBST (Santa Cruz). After the fifth round of in vitro selection, the number of phage recovered was increased about 1,000-fold, as shown in

Table 1 ELISA results of biopanning for λ bacteriophage expressing nanobody at $OD_{600\ nm}$

Biopanning round	HER-2 on SKBR-3	Control on EnSC
First	0.3	<0.1
Second	0.4	<0.1
Third	0.5	<0.1
Fourth	0.7	<0.1
Fifth	1.2	<0.1

The results are expressed as OD. The experiments were repeated three times. The anti HER-2 antiserum was derived from rat ascitic fluid and was used at 1:100 dilution. The rats polyclonal antiserum was diluted by Tris buffered saline containing 5 % BSA. Finally, the conjugated rabbit anti-rat antibody (Sigma, USA) was used for the detection of recombinant protein. The data are presented as mean ± SD from triplicates experiments

Fig. 1a. From 10^5 obtained clones there were 20 clones isolated after the fourth round of biopanning that did have higher affinity in cell-ELISA assay. Finally, we selected the nanobody producing recombinant λ phage with the highest affinity towards SKBR-3 cell line after the fifth round of panning. The output/input ratio of λ phage recovered after each round of biopanning was used to determine efficiency. The results indicated an obvious enrichment of λ phage clones specifically binding to SKBR-3 cell line by ELISA (Table 1). To produce the bulk amount of anti HER-2 phage displayed nanobody, the encoding sequence was subcloned in the λ ZAP-CMV vector. Plasmid profile of the library after biopanning was detected on agarose gel. It is a good indicator of isolated clones' enrichment (Fig. 1b).

Disappearance of the smear of recombinant plasmids as random inserts size delivery and impression of discrete bands indicate enrichment of high affinity phagemids in the constructed library. The resulting plasmid was transformed into DH5α cells. The selected transformants were confirmed by PCR and digestion using *Bam*H1 and *Hin*dIII restriction enzymes. Induction of the recombinant protein studied by SDS polyacrylamide gel electrophoresis resulted to an IPTG concentration of 1 mM. The expression of the fusion protein containing anti HER-2 phage displayed nanobody was found to be 6 % of the total protein of bacterial lysate. The purification was confirmed using Western blot analysis (Fig. 1c).

Binding of nanobody to the HER-2 antigen at different concentrations is shown the reaction was completely inhibited in presence of 1 μg/ml of HER-2 molecule. The sensitivity rate of the phage displayed nanobody reaction against HER-2 is shown in Fig. 2a. The antibodies showed no significant cross reaction with BSA and ovalbumin by ELISA using the anti-C-myc-tag antibody and no reaction was seen for control phage that did not express nanobody (data not shown). The binding of selected phage displayed

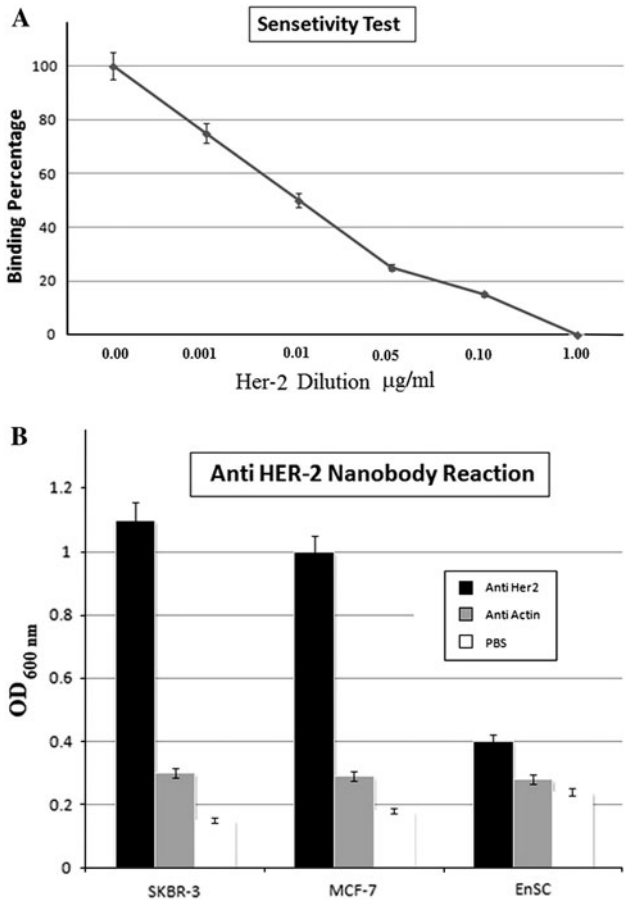


Fig. 2 Binding percent of nanobody towards HER-2. Reaction of anti HER-2 specific phage displayed nanobody against third domain of HER-2 antigens (**a**). Immunocytochemistry data of anti HER-2 phage displayed nanobody, anti β-actin antibody and 10 % PBS as negative controls performed with SKBR-3 and MCF-7 cell lines and endometrial stem cells (EnSCs) (**b**). The data are presented as mean ± SD from triplicates experiments

nanobody towards HER-2 molecule on the surface of SKBR-3 and MCF-7 cell lines has been shown in Fig. 2b. This nanobody showed no significant reaction towards endometrial stem cells as a control.

The effect of anti HER-2 phage displayed nanobody on the proliferation of SKBR-3 and MCF-7 cell lines was observed directly through crystal violet staining and investigated by MTT assay. λ Bacteriophage expressing nanobody also strongly suppressed SKBR-3 and MCF-7 proliferation in this assay. The suppressive effect of produced nanobody was higher for SKBR-3 cell in comparison with MCF-7 cell line (Fig. 3a). Herceptin (commercial mAb against HER-2/neu expressing cells) cytotoxicity on the HER-2 overexpressing cells was similar on both cell lines and its effect also was similar to our construct (Fig. 3a, b).

Nanobody expressed on recombinant λ phage that prepared in sodium phosphate used to determine the thermal stability (Fig. 4a). The highest binding percentage of

Fig. 3 Effect of nanobody on HER-2 overexpressing cell growth. ▶ MTT results of SKBR-3 and MCF-7 cell lines proliferation inhibition assay after incubation with λ bacteriophage expressing nanobody towards HER-2 and the commercial Herceptin mAb used in clinical therapy against HER-2 positive breast cancers. (*Nb* nanobody expressed by λ phage, *mAb* Herceptin mAb, *Vec* just vector as a control to see specific antiproliferative effects of Nb) (**a**). SKBR-3 (*1*), MCF-7 (*2*) and EnSCs (*3*) before proximity with nanobody. These cells after treatment (*4–6*) with nanobody expressed λ phage after 48 h and after treatment with Herceptin mAb (**b**)

nanobody after heat treatment in different temperatures was in 40 °C but it was functional even in 55 °C. The nanobody also tested in cell-ELISA examination for its activity after incubation in human serum (37 °C). The activity of samples incubated in serum for 24 h, was close to the first hours of incubation in serum (Fig. 4b). So the nanobody is still active in serum until complete attachment to the all in vivo targets.

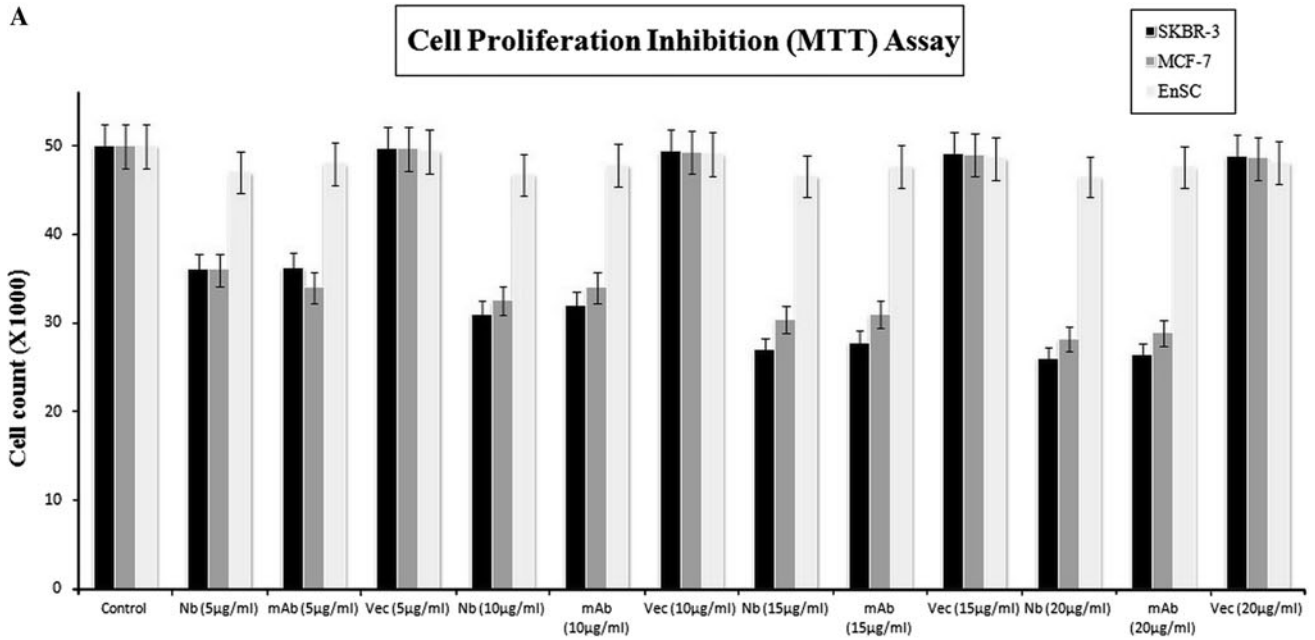
Discussion

This study presents targeted, nanobody expressing recombinant λ bacteriophage as a nanobody expression platform for targeting third domain of HER-2 antigen to inhibit the growth of HER-2 over-expressing cells. Our phage represents a targeted platform of nanometric dimensions. It is obvious that there are some limitations about mAb therapy, such as their molecular size, because these molecules are relatively large and do not penetrate solid tumors. The nanobody molecules are stable, highly soluble and are high affinity towards the antigens even in solid tumors like breast carcinoma tissues (Nguyen et al. 2001).

Nanobodies (VHH-based immunoglobulin single variable domains) are able to bind to cryptic antigenic sites like enzyme active sites that are normally not recognized by conventional antibodies (De Genst et al. 2006; Henderson et al. 2007). Nanobodies have reduced aggregation and they have low immunogenicity; because studies showed that repeated administration of nanobodies did not evoke an anti-nanobody response (Cortez-Retamozo et al. 2002).

Our research group used a recombinant λ phage to select nanobodies that bind to the third ectodomain of HER-2 antigen. In vitro studies on SKBR-3 cell line demonstrated these nanobodies block HER-2 binding epitopes. Also Hofman et al. (2008) showed that, receptor antagonism mediated by anti-epidermal growth factor receptor (EGFR) nanobodies has shown great potential by inhibiting the growth of human tumor xenografts.

The anti HER-2 nanobody was on the surface of recombinant λ phage to form a multivalent platform, and it may bind several HER-2 molecules on the cell surfaces to induce downregulation of HER-2.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Cell Proliferation Inhibition (MTT) Assay
SKBR-3
MCF-7
EnSC
Cell count (X1000)
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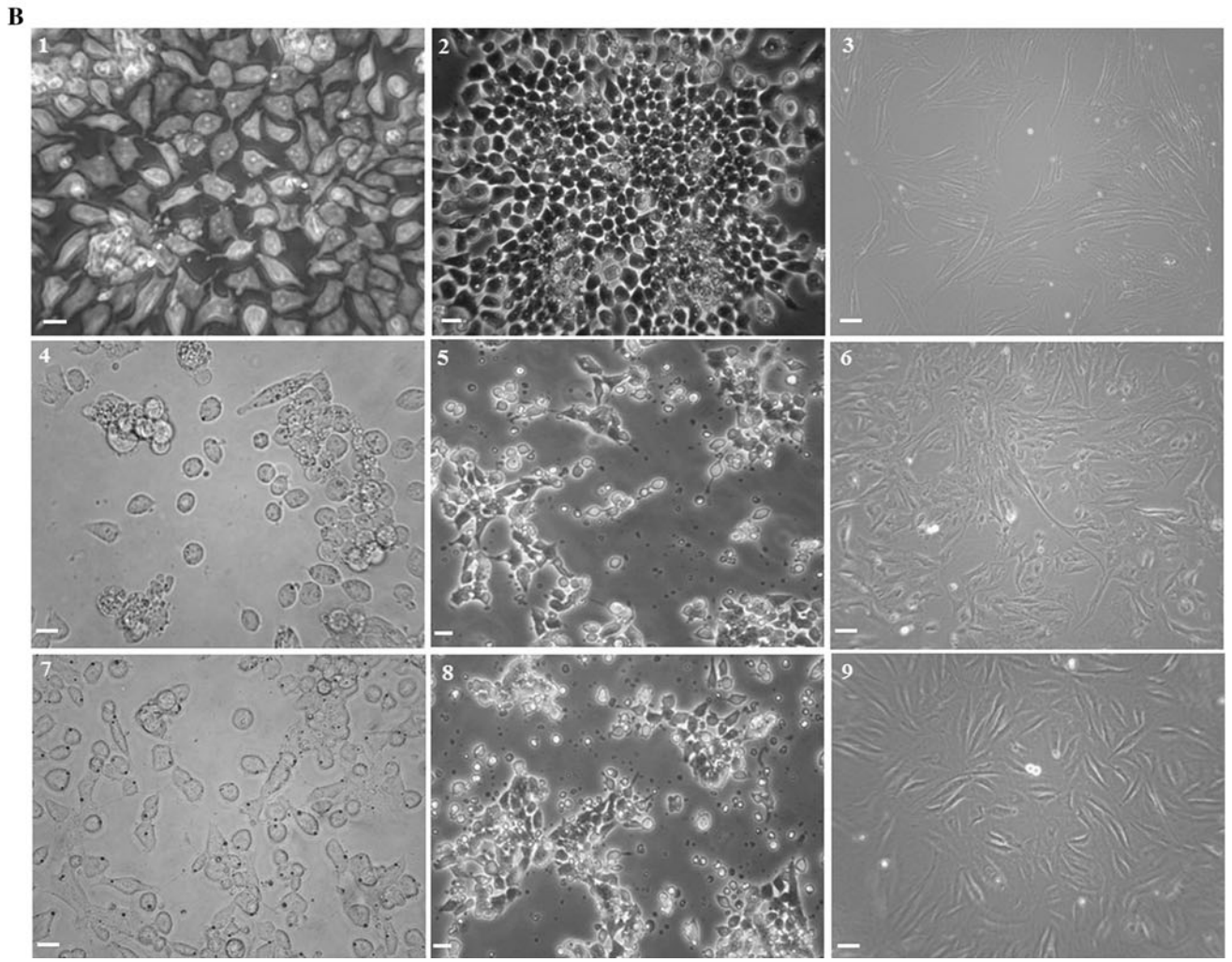
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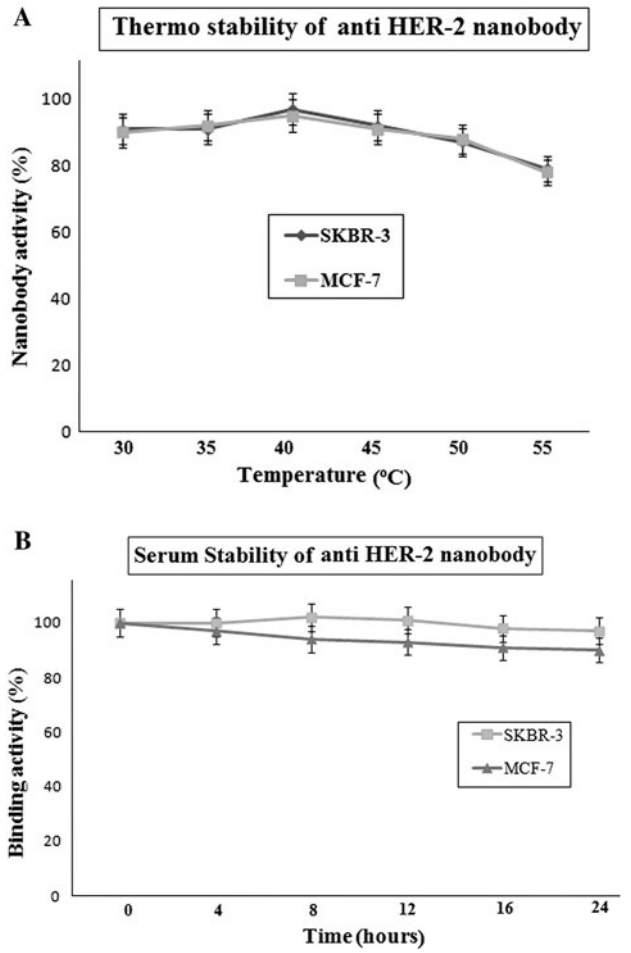

Fig. 4 The thermo-stability and serum-stability of nanobody construct. Thermo-stability of λ bacteriophage expressing nanobody towards HER-2 in temperatures ranged between 30 and 55 °C. The results are obtained from nanobody binding capacity towards SKBR-3 and MCF-7 cell lines and expressed as an activity percentage towards control (In vivo temperature: 37 °C) (**a**). Serum-stability of the nanobody incubated for various time periods (4, 8, 12, 16, 24 h) in human serum at 37 °C tested by ELISA on SKBR-3 and MCF-7 cells (*size Bar* is 10 μm) (**b**)

We firstly used a protocol of biopanning in which recombinant λ phage were selected for their binding affinity for the apical surface of the cultures. However, after two rounds of selection, no sequence had really shown a high affinity for the HER-2 epitope, but from the fourth round of biopanning there was a significant amount of VHH sequences were demonstrated by cell-ELISA (Fig. 1a). Our team successfully used cell-ELISA to confirm specific binding to SKBR-3 cell in vitro. We aimed to using cell-ELISA for the selection of the best clone with high affinity towards native HER-2 antigen. Individual clones from the fourth panning were screened for binding to SKBR-3 cell by cell-ELISA. The phage inserted vector (42 kb) with nanobody gene (430 b) detected by RT-PCR (Fig. 1b). The phage displayed nanobodies (16 kD) detected by Western blot analysis (Fig. 1c). The expression of the recombinant protein was found to be 6 % of total soluble protein of bacterial cell lysate. The screening of phage displayed peptide libraries on intact mammalian cells has proven to be a productive method to identify peptides that recognize certain cell types with high affinity and cell specificity (Ivanenkov and Menon 2000). The analysis of binding specificity with endometrial stem cells unrelated cell line showed that this nanobody exhibited significant specificity for SKBR-3 and MCF-7 cells, which express high levels of HER-2 epitope (Fig. 2b).

In other studies, several nanobodies binding human prostate specific antigen were characterized (Saerens et al. 2004). Gainkam et al. (2008) has been reported an anti-EGFR nanobody binding the EGFR by the 425 single chain variable fragment (425 scFv). Jaramillo et al. (2006) showed the fast removal of cell surface EGFR in association with increased intracellular degradation of EGFR. Also Perez-Torres et al. (2006) reported mAb dimerization mediated downregulation of EGFR. They showed downregulation was consequently not observed for monovalent Fab of commercial Cetuximab. Hmila et al. (2008) showed that dimeric bivalent nanobody has a toxin neutralizing capacity exceeding that of all previously scFv tested constructs. In our study, recombinant λ phage that expressed nanobody decreased the HER-2 positive cell growth efficiently as compared with Herceptin mAb that is used in clinical therapy. The therapeutic potential of this nanobody was clearly demonstrated in vitro due to presence of λ phage in conjunction with the nanobody (Fig. 3a, b). Thermal stability of our construct shows that the nanobodies have function even after recovery from 55 °C (Fig. 4a). According to the previous studies, nanobodies usually reach even higher thermo-stability (Dumoulin et al. 2002). The designed constructs were stable at 37 °C in human serum. We extended examination to 24 h and checked the results in cell-ELISA (Fig. 4b). Longer incubation in serum was not tested as these polypeptides would be cleared from the circulation via kidneys (Cortez-Retamozo et al. 2002). The obtained data that are in agreement with other reports (Conrath et al. 2001; Hmila et al. 2008) confirms the stability of the construct in serum microenvironment for many purposes including targeting and therapy of HER-2 over-expressing cells.

The reason for more efficient action of phage expressing nanobody rather than mAb is due to phage internalization into the target cells. The exact mechanism by which nanobody induces HER-2 downregulation is not understood. A different approach could be the designing of multivalent nanobody constructs, i.e., three or more nanobodies linked together on the recombinant λ phage surface that could recognize different epitopes on the same target.

In conclusion, in our study we demonstrated that HER-2 targeted λ ZAP-CMV-VHH cDNA phagemid cloned in the λ nanobioparticles represent an efficient therapeutic

approach for growth inhibition of carcinoma cells. Therefore, recombinant λ bacteriophage would be an attractive strategy for nanomedicine therapy.

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