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RESEARCH ARTICLE

# Efficient Inhibition of Pathologic Angiogenesis using Combination Therapy of Anti-Epcam and Anti-VEGFR2 Nanobodies

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ARTICLE HISTORY

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**Abstract:** ***Background*:** EpCAM and VEGFR2 play an important role in angiogenesis and tumorigenesis. It is currently of paramount importance to produce new drugs that can inhibit the angiogenesis and proliferation of tumor cells. Nanobodies are potential drug candidates for cancer therapy due to their unique properties.

***Objective*:** This study aimed to investigate the combined inhibitory effect of anti-EpCAM and anti-VEGFR2 nanobodies in cancer cell lines.

***Methods*:** Inhibitory activity of anti-EpCAM and anti-VEGFR2 nanobodies on MDA-MB231, MCF7, and HUVEC cells was investigated using both *in vitro* (MTT, migration, and tube formation assays) and *in vivo* assays.

***Results*:** Results showed that the combination of anti-EpCAM and anti-VEGFR2 nanobodies efficiently inhibited proliferation, migration, and tube formation of MDA-MB-231 cells compared to each individual nanobodies ($p < 0.05$). In addition, the combination of anti-EpCAM and anti-VEGFR2 nanobodies efficiently inhibited tumor growth and volume of Nude mice bearing MDA-MB-231 cells ($p < 0.05$).

***Conclusion*:** Taken together, the results indicate the potential of combination therapy as an efficient approach to cancer therapy.

**Keywords:** VEGFR2, EpCAM, angiogenesis, nanobody, target therapy, cancer.

## 1. INTRODUCTION

After cardiovascular disease, cancer is known as the leading cause of death [1]. Tumor cell proliferation in different cancers can exacerbate the disease in patients [2, 3]. The development of novel drugs, which can inhibit tumor cell proliferation with minimal side effects, has been of interest to researchers [3]. Angiogenesis is a physiological process through which new vessels are formed from old ones [4, 5]. Vascular endothelial growth factor (VEGF) is one of the factors involved in the formation of blood vessels. The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD, and PLGF [6, 7]. Among others, the VEGF receptors include VEGFR2/Kinase insert domain receptor (KDR) and VEGFR1 (flt-1) [8]; the former, which is much more stable, is overexpressed in many tumor cells, such as breast, colorectal, and lung, compared to normal ones [9-11]. Consisting of 1356 amino acids, VEGFR2 is the main VEGF receptor on the vascular endothelial cell surfaces [12, 13]. The anti-angiogenesis drugs for VEGF include kinase inhibitors (sorafenib and apatinib), and monoclonal antibodies (bevacizumab) which are used limitedly due to their side effects [14]. Another factor with a high expression in tumor cells is the Epithelial cell adhesion molecule (EpCAM), which was discovered as a tumor antigen on colorectal carcinomas four decades ago [15, 16]. EpCAMs are single transmembrane proteins containing 314 amino acids, of which 265 and 25 amino acids are extracellular and intracellular, respectively [17, 18]. The EpCAM molecule is a heart-shaped dimmer [19]. The EpCAM gene comprises six isoforms, called EpCAM-201, EpCAM-202, EpCAM-203, EpCAM-204, EpCAM-205, and EpCAM-206, with EpCAM-206 is more frequent [20]. EpCAM is considered a target and an anchor molecule due to its high expression on carcinomas and metastasis. The function and expression of EpCAM may vary depending on the type and site of the tumor [16]. It can generally be argued that the high expression of EpCAM is caused by the growth, proliferation, and increased metastasis of the tumor [21, 22]. High expression levels of EpCAM are seen at the early stages of breast [23], colorectal [24], prostate [25], gallbladder [26], ovary [27], bladder [28], and pancreas cancers, which may result from the proliferation and growth as well as the increased metastasis of tumor [29]. The development of inhibitors that can prevent VEGFR2 and EpCAM functions can be effective for the treatment of cancer. As a new generation of antibodies, nanobodies are of interest to researchers owing to their unique properties [30]. They are composed of 120 amino acids with a mean weight of 12-17 kDa and a size of 4 × 2.5 nm [31]. Nanobodies consist of four frame works (FRs) and three complementarity-determining regions (CDRs), among which CDR3 causes their specificity [30, 32, 33]. The high stability in different conditions, a simple structure, and lower production costs than antibodies have made nanobodies interesting to many researchers [31, 34]. The main drawback of nanobodies is their rapid elimination from the body after administration. This requires repeated injections, limiting practical clinical use [35-37]. However, the half-life of monoclonal antibodies vary from 7-21 days [38]. Caplacizumab (anti vWF nanobody) was the first nanobody approved by FDA in 2019 for treatment of acquired thrombotic thrombocytopenic purpura [39, 40]. This study is the first to investigate the simultaneous use of two nanobodies against VEGFR2 and EpCAM in the inhibition of angiogenesis compared to individual forms of nanobodies.

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## 2. MATERIALS AND METHODS

### 2.1. Nanobodies Targeting VEGFR2 and EpCAM

The nanobodies against VEGFR2 and EpCAM were developed using biopanning against immobilized antigens [41-44]. Four consecutive rounds of biopanning were performed using an immune nanobody library to select specific nanobodies against their targets. The sequence of selected nanobodies was sub-cloned in the pHEN6c expression vector using the restriction enzymes (*Pst* I and *BstE* II) and transformed into competent WK6 *E. coli* cells using heat-shock and $CaCl_2$. Expression of recombinant nanobody was induced by IPTG 1mM and incubated at 28°C overnight. Then, the culture was centrifuged at 5000 x g for 10 min. The periplasmic proteins were purified using 2 ml of TES (sucrose 20%, 0.5 M EDTA, 30 mMTris) and 18 ml of TES/4. Afterward, the resulting supernatant was purified by Ni-NTA affinity chromatography. Finally, the purified protein was dialyzed (cut-off 5 kDa) and stored at 4°C. The expression of the purified nanobodies was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The expression of target proteins was confirmed by western blotting in which the protein bands were transferred to nitrocellulose paper and blocked with a skim milk 4% solution at 4°C overnight. The nitrocellulose paper was incubated overnight with anti-His HRP conjugated antibody (1:500). Finally, a western blot was developed using ECL substrate [45].

### 2.2. Affinity Analysis

The affinity of nanobodies to the antigens was analyzed using the method described by Beatty *et al.* [46]. Briefly, 100 µl of two different concentrations (1 and 10 mg/ml) of VEGFR2 and EpCAM antigens, with BSA as control, were coated in a 96-well plate and incubated at 4°C overnight. It was then incubated with a skim milk 4% solution at RT for 1 h to block the wells. After washing the wells with PBST (Tween 20 0.05%, *v/v*), different concentrations (0-10 mm) of the nanobodies were added to each well and incubated for 1 h at RT. Then, the wells were washed and incubated with HRP-conjugated anti-His antibody (1:500). ELISA was developed with TMB and then stopped with 2N $H_2SO_4$. The peroxidase activity was measured at 450 nm [41, 47].

### 2.3. Specificity Assay

The specificity of anti-EpCAM and anti-VEGFR2 nanobodies to their targets was evaluated using the ELISA experiment. Briefly, 1 µg/ml of various antigens (EpCAM, VEGFR2, VEGF (vascular endothelial growth factor), NRP-1 (neuropilin-1), EGF (epidermal growth factor), PD-1 (programmed cell death protein 1). PD-L1 (programmed death-ligand 1), LIV-1 (zinc transporter protein) and BSA were coated onto a 96-well plate at 4°C overnight. The wells were blocked (skim milk 2%) and incubated for 2 h at RT. Then, anti-EpCAM and anti-VEGFR2 nanobodies were added to each well and incubated at RT for 1 h. After washing the wells with PBST (5 times) anti-His HRP conjugated antibody (1:500) was added to the wells and incubated for 1 h at RT. The wells were washed and incubated with TMB for 15 min [45].

### 2.4. MTT Assay

MDA-MB-231cells (expressing EpCAM and VEGFR2 as well), MCF7 (over-expressing EpCAM, no VEGFR2 expressing), HUVEC (Over-expressing VEGFR2, no EpCAM expressing) were obtained from the Pasteur Institute of Iran, cultured in the DMEM medium containing FBS 10%, and then incubated at 37°C. The required tests were conducted at a cell density of 90%. The cells (approx. $10^4$) with 1 ml of the media containing FBS 2% were transferred to a 96-well plate, and the cells were attached to the plate. Various concentrations of anti-EpCAM and anti-VEGFR2 nanobodies (as mixture or individual) (0-10 µg) were added to the wells and incubated at 37°C for 24 and 48 h. PBS and H39Nb (anti-scorpion nanobody, used as negative control nanobodies) were used as controls. Next, 10 µl of MTT (5 mg/ml) solution was added to the wells and incubated at 37°C for 4 h. Finally, MTT solution was removed from the wells, and each well received 10 µl of dimethyl sulfide (DMSO). After 30 min, the results were analyzed by spectrophotometer at 570 nm [41, 42].

### 2.5. Tube Formation Assay

The plate wells were coated with 50 µl of matrigel and incubated at 37°C for 45 min. MCF7, HUVEC, and MDA-MB-231 cells (approx. $10^4$) were seeded in the wells, followed by adding anti-EpCAM and anti-VEGFR2 nanobodies (as mixture or individual) (10 µg) and incubated for 6 h at 37°C. PBS and H39Nb (anti-scorpion nanobody, used as negative control nanobody) were used as controls. Afterward, the structure of the tubes was examined by the Image J software [33, 48].

### 2.6. Migration Assay

Considered an important assay in angiogenesis and metastasis, the migration assay examines the ability of cells to migrate toward the growth factor. In this assay, $3 \times 10^3$ MDA-MB231, HUVEC, and MCF7 cells cultured in 500 µl of the DMEM medium were added to the upper part of the Boyden chamber. 10 µg/ml of anti-EpCAM and anti-VEGFR2 nanobodies were added to the lower part of the Boyden chamber and incubated at 37°C for 12 h. PBS and H39Nb (anti-scorpion nanobody, used as negative control nanobody) were used as controls. Cells migrated toward the lower part of the Boyden chamber were trypsinized and counted afterward [37].

### 2.7. *In vivo* Assay

The animal study was conducted according to the ethical committee of the Pasteur Institute of Iran (IR.PII.REC.1398.35). About 200 µl of MDA-MB231 cells ($10^6$) were injected subcutaneously (s.c.) into the right flank of Nude mice (18-20 g). The tumor size was measured three times a week and tumor volume was recorded after each measurements (Tumor Volume = Length of the tumor × width$^2$ of the tumor × 0.52). When the tumor volume reached 100-150 mm$^3$, the mice received 10 mg/kg of nanobodies (mixture or individual) were injected intravenously (i.v.) three times a week for three weeks [36, 49].

### 2.8. Statistical Analysis

Statistical data analysis was performed using PRISM software (GraphPad, San Diego, CA, version 8.0). The *t*-test was performed for analysis between two groups and a $p$-value $< 0.05$ considered as statistically significant.

## 3. RESULTS

### 3.1. Expression and Purification of the Nanobodies

Anti-EpCAM and anti-VEGFR2 nanobodies were expressed with His tag at their C-terminal and purified from the periplasmic space of the WK6 cells using nickel affinity chromatography. The purity of purified nanobodies was checked by 15% SDS-PAGE (coomassie blue staining). In addition, the presence of His tagged nanobodies and their molecular weight were evaluated by western blot using anti-His HRP conjugated antibody. A band of 15 kDa was observed in SDS-PAGE and western blot analysis (Fig. **1**). The final yield of anti-EpCAM and anti-VEGFR2 nanobodies was about 5 mg/ml.

### 3.2. Affinity Analysis

Binding affinity ($k_{aff}$) of anti-EpCAM and anti-VEGFR2 nanobodies was measured to be 52 and 82 nM according to the modified formula of the Beatty *et al.* method, respectively [46, 47].

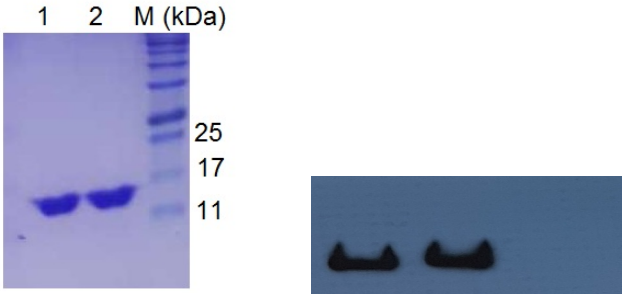


**Fig. (1).** Expression and purification of anti-EpCAM and anti-VEGFR2 nanobodies. (**A**) SDS-PAGE results after purification with Ni-NTA chromatography. 1; anti-EpCAM Nb, 2; anti-VEGFR2 Nb. (**B**) Western blot results. Western blot was performed with anti-His HRP antibody and developed by ECL. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

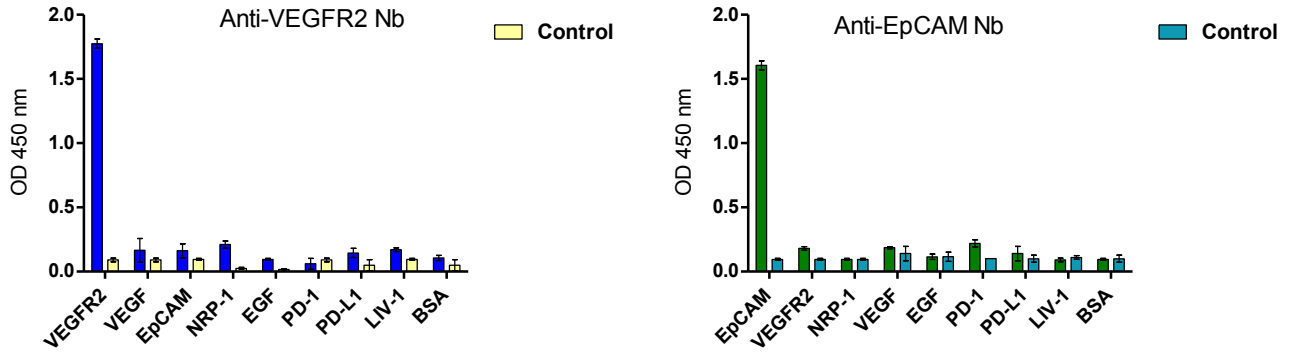


**Fig. (2).** Specificity analysis. Anti-EpCAM and anti-VEGFR2 nanobodies detected their own antigen in an ELISA experiment and no cross-reactivity with other antigens was observed. Error bar indicates for mean (duplicate assay) ± SD. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

### 3.3. Specificity Assay

Both anti-EpCAM and anti-VEGFR2 nanobodies specifically detected their target on ELISA and detection of other antigens was not observed even in the case of mixture use of anti-EpCAM and anti-VEGFR2 nanobodies (Fig. **2**).

### 3.4. Inhibitory Effect of Anti-EpCAM and Anti-VEGFR2 Nanobodies on Cell Proliferation

The cytotoxicity of anti-EpCAM and anti-VEGFR2 nanobodies on HUVEC, MCF7, and MDA-MB-231 cells was examined both individually and in combination using MTT assay. Results showed that a mixture of anti-EpCAM and anti-VEGFR2 nanobodies efficiently inhibited MDA-MB-231 cells (expressing EpCAM and VEGFR2), compared to the effect of each individual nanobodies ($p < 0.05$) (Fig. **3**). However, there was no significant difference between the inhibitory effect of anti-EpCAM and anti-VEGFR2 nanobodies in both states (mixture use and individual) on HUVEC (Over-expressing VEGFR2, lack of EpCAM expression) and MCF7 (over-expressing EpCAM, lack of VEGFR2 expression) cells as expected (Fig. **3**). In fact, the additive effect of anti-EpCAM and anti-VEGFR2 nanobodies was observed on MDA-MB-231 cells due to the presence of their target on the surface of this cell line (EpCAM and VEGFR2).

### 3.5. Anti-EpCAM and Anti-VEGFR2 Nanobodies Inhibited *in vitro* Tube Formation

The mixture of Anti-EpCAM and anti-VEGFR2 nanobodies significantly inhibited the formation of tube-like structures in MDA-MB-231 cells compared to each individual nanobodies ($p < 0.05$) (Figs. **4** and **5**). Whereas, the additive inhibitory effect of anti-EpCAM and anti-VEGFR2 nanobodies on tube formation of HUVEC and MCF7 cells was not observed (Fig. **4**).

### 3.6. Anti-EpCAM and Anti-VEGFR2 Nanobodies Inhibited *in vitro* Migration

Migration assay results showed that mixture of anti-EpCAM and anti-VEGFR2 nanobodies significantly inhibited the migration of MDA-MB-231 cells from upper part of the Boyden chamber to the lower part compared to each individual nanobodies ($p < 0.05$) (Fig. **5**). Whereas, there was no significant differences between migrated HUVEC and MCF7 cells from upper part to the lower part of the plate in both state (mixture or individual nanobodies) (Fig. **5**).

### 3.7. Anti-EpCAM and Anti-VEGFR2 Nanobodies Inhibited *in vivo* Tumor Growth

Animal study results showed that in agreement with *in vitro* results, the mixture of anti-EpCAM and anti-VEGFR2 nanobodies significantly suppressed tumor growth, and weight compared to individual nanobodies (Fig. **6**).

## 4. DISCUSSION

Angiogenesis is the formation of new blood vessels from the pre-existing vasculature [4]. Given the importance of angiogenesis, meticulous studies are focused on pathologic angiogenesis to investigate the safe and available drugs [50]. Proliferation, differentiation, and migration of cells are controlled by the protein tyrosine kinases (PTKs) [51]. Receptor tyrosine kinases (RTKs) mediate the transfer of signals to hormones, cytokines, and growth factors [51]. As one of RTKs, VEGFRs are VEGF receptors, with the subtypes VEGFR1and VEGFR2 [52]. Among VEGFs, VEGFA is a critical factor for angiogenesis during early embryogenesis [50]. Acting as an important receptor in the angiogenesis process, the VEGFR2 tyrosine kinase is more active in response to VEGFA [52, 53]. As a multi-functional transmembrane protein, the EpCAM antigen is

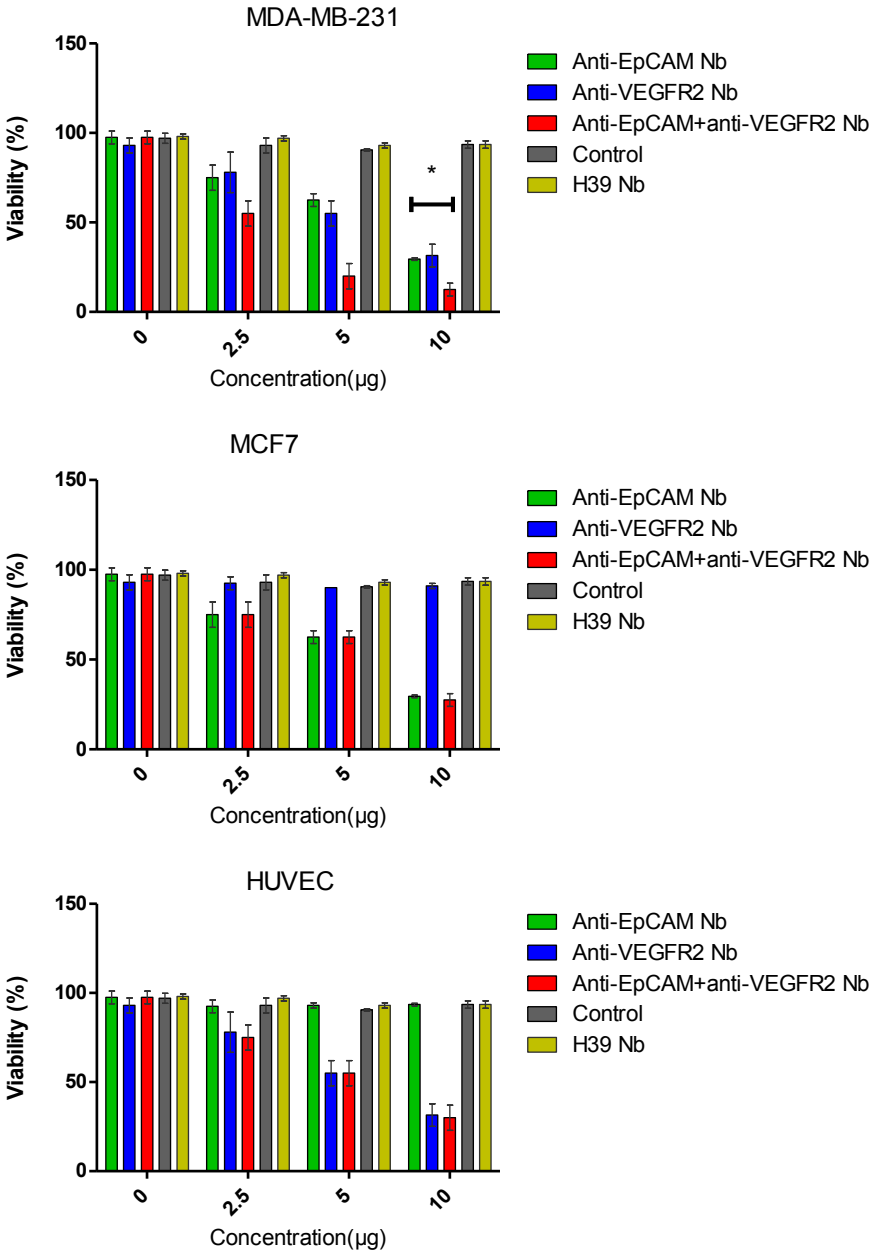


**Fig. (3).** Cytotoxicity results. Additive effect of anti-EpCAM and anti-VEGFR2 nanobodies was observed on MDA-MB-231 cells due to presence of their target on the surface of this cell line (EpCAM and VEGFR2). However, in HUVEC and MCF7 cells additive inhibitory effect of anti-EpCAM and anti-VEGFR2 was not observed due to absence of simultaneous presence of both targets. Error bar indicates for mean (duplicate assay) ± SD. *indicates for $p < 0.05$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

involved in the regulation of cell adhesion, proliferation, and migration [16]. Nanobodies are a new generation of monoclonal antibodies that are derived from the variable domain of the heavy chain antibodies [30]. Despite their simple structure, nanobodies are structurally stable and exhibit resistance to high pressure and pH. Compared to monoclonal antibodies, the easier production process makes nanobodies potential tools for the development of novel drugs [54].

The present study aimed to investigate the combined effect of anti-EpCAM and anti-VEGFR2 nanobodies on the inhibition of angiogenesis. Anti-EpCAM and anti-VEGFR2 nanobodies were expressed, purified, and their activity on MDA-MB231, HUVEC and MCF7 cells were examined by MTT, migration, and Tube formation assays. According to our results, combined anti-EpCAM and anti-VEGFR2 nanobodies showed efficient inhibitory activity on proliferation, migration, and tube formation of MDA-MB-231 cells than the individual forms of nanobodies. In addition, combined anti-EpCAM and anti-VEGFR2 nanobodies inhibited tumor growth of Nude mice bearing MDA-MB-231 cells more efficiently than each individual nanobodies. Combined treatment has recently received attention from researchers in many studies. For instance, the combination of two nanobodies against VEGF/VEGFR2 and NRP-1 was used to inhibit angiogenesis [47, 48, 55, 56]. In various studies, nanobodies against VEGFR2 and EpCAM were individually examined *in vitro* and *in vivo* [41-43]. Sadeghi *et al.* used a mono-specific anti-VEGF bivalent nanobody for the inhibition of

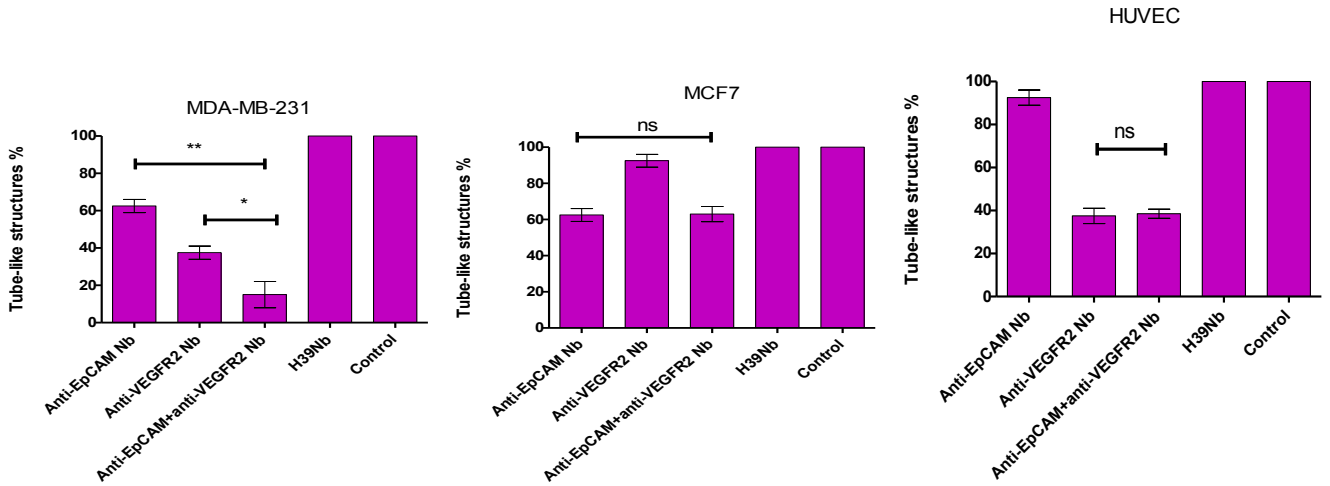


**Fig. (4).** Tube formation assay. Cytotoxicity results. Mixture of Anti-EpCAM and anti-VEGFR2 nanobodies significantly inhibited formation of tube like structures in MDA-MB-231 cells compared to each individual nanobodies. Error bar indicates for mean (duplicate assay) ± SD. *indicates for $p < 0.05$. **indicates for $p < 0.01$. ns indicates for no significant. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

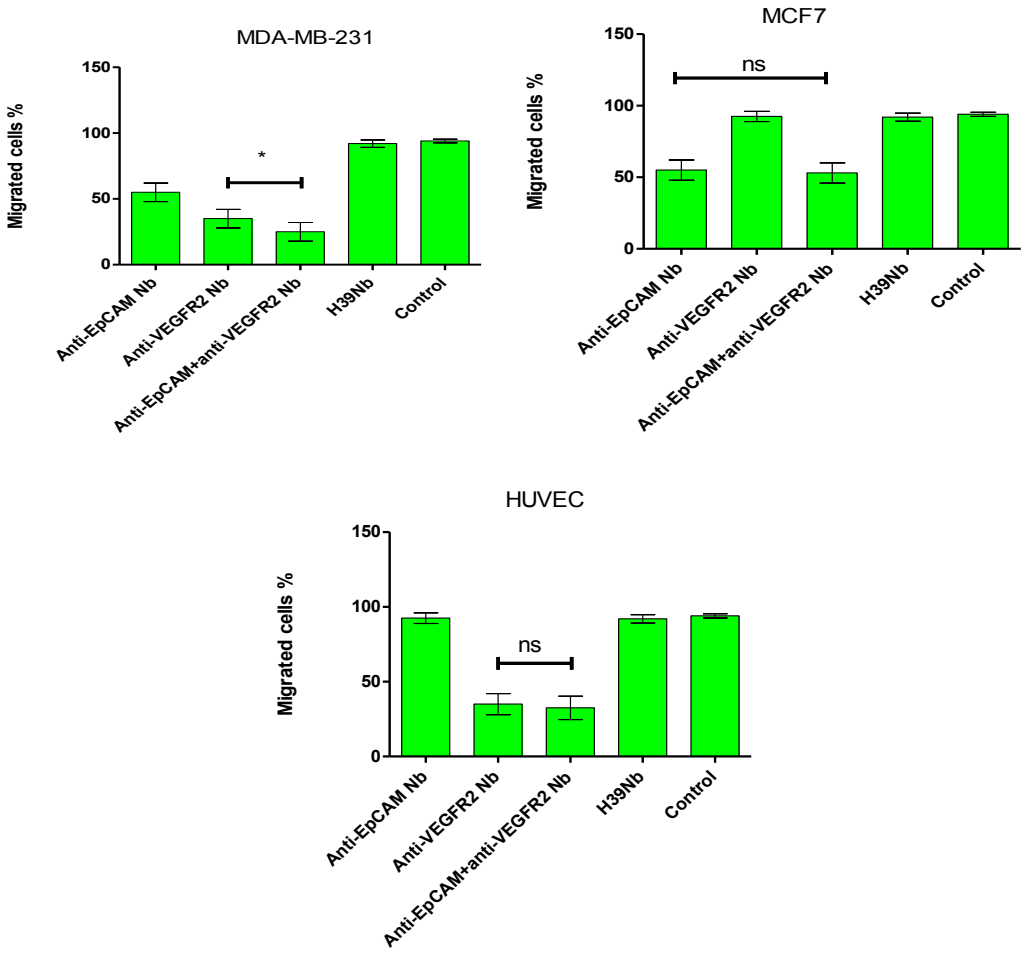


**Fig. (5).** Migration assay. Mixture of anti-EpCAM and anti-VEGFR2 nanobodies significantly inhibited migration of MDA-MB-231 cells from upper part of the Boyden chamber to the lower part compared to each individual nanobodies. Error bar indicates for mean (duplicate assay) ± SD. *indicates for $p < 0.05$. Ns indicates for no significant. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

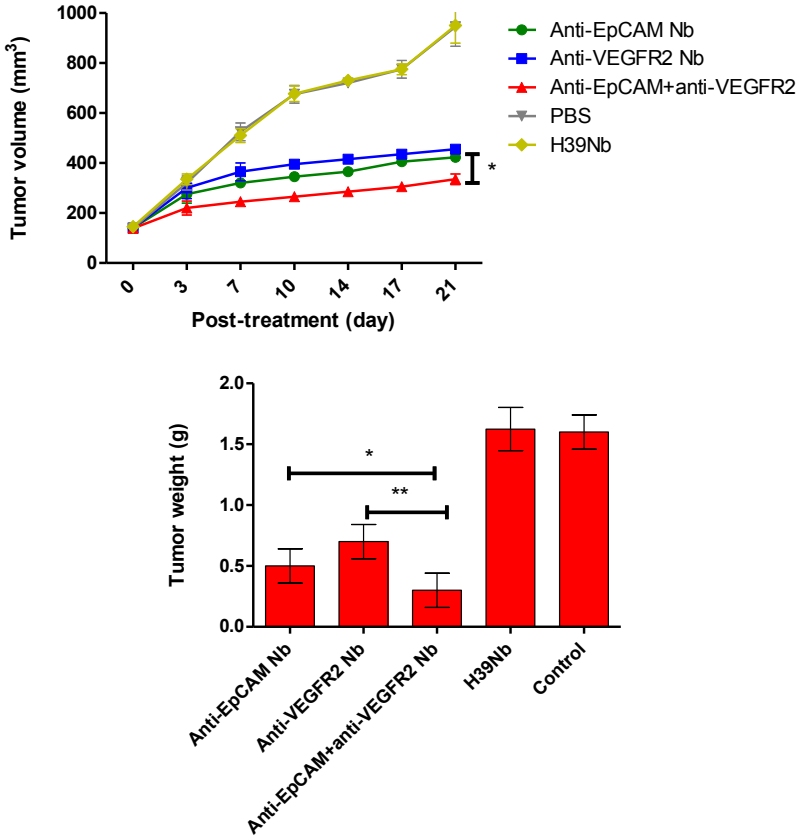


**Fig. (6).** Animal study results. Mixture of anti-EpCAM and anti-VEGFR2 nanobodies significantly suppressed tumor growth and volume of MDA-MB-231 cells compare to individual nanobodies. Error bar indicates for mean (duplicate assay) ± SD. *indicates for $p < 0.05$. **indicates for $p < 0.001$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

angiogenesis. Their results indicated that the mono-specific anti-VEGF bivalent nanobody could effectively inhibit angiogenesis compared to its nanobody form [37]. Huang *et al.* used an anti-VEGF agent together with doxorubicin to inhibit angiogenesis *in vivo* and *in vitro*. They reported that a combination of these two drugs could properly inhibit angiogenesis with an acceptable anti-tumor effect [57]. A combination of anti-cMET or anti-PLGF (Placental growth factor) monoclonal antibodies with bevacizumab was also reported to have a significant effect on the treatment of glioblastoma, and the combined drugs were more effective in the disease treatment than their individual forms [58, 59]. In the recent decade, the use of VEGFR1-3 angiogenesis inhibitor together with C-kit inhibitor has shown considerable antitumor activity [60]. Anti-EpCAM antibodies were developed and used for the treatment of colon cancer [61], metastatic breast cancer [62], and malignant ascites [63]. According to the reviewed articles, drugs in uncombined forms can be moderately effective in the treatment of tumors, sometimes making tumor cells resistant to the treatment. Combination therapy has been proposed to overcome limitations associated with monotherapy, such as drug-resistance of tumor cells and toxicity. In addition, combination therapy requires lower doses of therapeutic agents compared to monotherapy, thus reducing toxicity associated with high doses of therapeutic agents.

## CONCLUSION

According to the achieved results, the combination of anti-EpCAM and anti-VEGFR2 nanobodies showed efficient anti-cancer activity both *in vitro* and *in vivo*. These findings potentiate the combination use of nanobodies targeting two or more antigens as an efficient approach for the treatment of cancer.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| DMSO | = | Dimethyl Sulfide |
| PLGF | = | Placental Growth Factor |
| PTKs | = | Protein Tyrosine Kinases |
| SDS-PAGE | = | Sulfate-polyacrylamide Gel Electrophoresis |

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal study was conducted according to the ethical committee of the Pasteur Institute of Iran (IR.PII.REC.1398.35).

## HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All the animals were used in accordance with The US National Research Council's “Guide for the Care and Use of Laboratory Animals”, The US Public Health Service's “Policy on Humane Care and Use of Laboratory Animals”, and “Guide for the Care and Use of Laboratory Animals”.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

## FUNDING

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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