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1 Selection and characterization of specific nanobody against neuropilin-1 for inhibition of  
2 angiogenesis

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15

16 Abstract

17 Neuropilin-1 (NRP-1), non-tyrosine kinase receptor, was initially identified as axonal protein  
18 and later recognized as co-receptor for vascular endothelial growth factor (VEGF).  
19 Neuropilins (NRPs) are involved in vascular development and tumor angiogenesis. Over the  
20 last years, many studies have been accomplished to inhibit angiogenesis. In this study, the  
21 nanobody library was panned against immobilized NRP-1 antigen. High affinity and  
22 specificity nanobodies were selected through monoclonal ELISA. The selected nanobodies  
23 inhibited proliferation and tube formation of HUVEC and MCF-7 cells *in vitro* and *ex vivo*.  
24 The results highlight potential of anti-NRP1 nanobodies in inhibition of angiogenesis both *in*  
25 *vitro* and *ex vivo* and promises development of novel therapeutics against pathologic  
26 angiogenesis.

27 Key words: VEGF, NRP-1, Nanobody, Angiogenesis.

28

29

30 1. Introduction

31 Neuropilin (NRP), as cell surface receptor, plays important role in cell survival, migration  
32 and angiogenesis. NRPs with 40% identity at the amino acid level, are conserved in all

33 vertebrates(1). NRP-1 is a type I transmembrane receptor with molecular weight of 120–140  
34 kDa. NRP-1 is a homodimer receptor which is involved in binding to extracellular ligands  
35 including some isoforms of vascular endothelial growth factor (VEGF), fibroblast growth  
36 factor (FGF) , transforming growth factor beta (TGF- $\beta$ ), hepatocyte growth factor (HGF) and  
37 class 3 semaphorins (2). The most studied interaction of the NRP-1 (which works as co-  
38 receptor with VEGFR1/2) is with VEGF<sub>165</sub> that result in angiogenesis, invasion, and  
39 metastases of tumor cells. NRP-1 enhances transduction of biological signals in endothelial  
40 cells by binding of VEGF-A to VEGFR2(3). In addition, NRP-1 mediates tumor  
41 development and angiogenesis. It has been considered that increased expression of NRP1  
42 correlated with more invasive and advanced stage of cancer. Several inhibitors including  
43 small molecules, siRNA, and monoclonal antibodies, have been evaluated against NRP-1 (4,  
44 5). For instance, in a study, it has been found that blocking of NRP-1 function enhanced the  
45 antitumor effects of anti-VEGF-A antibody in mouse xenograft models by preventing VEGF-  
46 A binding to NRP-1(6). In addition to the VEGF-mediated pathway, other growth factors like  
47 FGF, platelet-derived growth factor (PDGF), HGF and receptors may interact with NRP-1 on  
48 tumor cells to stimulate angiogenesis and tumor progression(7). During the last decades,  
49 monoclonal antibodies (mAbs) have been developed against cancer-associated membrane  
50 proteins, or their ligands. Nevertheless, therapeutic application of mAbs especially in  
51 blocking tumor angiogenesis, have been limited by their complexity and large size in clinical.  
52 To overcome this obstacle, attractive strategies such as miniaturization of the antibodies have  
53 been conducted (8, 9). Discovery of antibodies devoid of light chains, VHHs or Nanobodies<sup>®</sup>,  
54 in *dromedaries* have been revealed several advantages for biotechnological applications.  
55 Nanobodies have small size (~15 kDa), a high stability and solubility and express  
56 comfortably in microorganisms. Furthermore, they have high penetration rate into tissues that  
57 make them appropriate candidate for therapy (10, 11). The purpose of this study was to  
58 establish anti-NRP-1 nanobodies in order to inhibit angiogenesis. In this study, anti-NRP-1  
59 nanobodies have been isolated using phage display and their characteristic and functionality  
60 have been evaluated.

61

## 62 2. Materials and methods

### 63 2.1. Materials

64 Monoclonal anti-hemagglutinin antibody(anti-HA), anti-mouse HRP conjugated antibody,  
65 and anti-M13-HRP antibody were purchased from Roche, Switzerland. HRP-conjugated anti-  
66 His tag antibody was purchased from cytomatingen.VCSM13 helper phage was purchased  
67 from Amersham-Pharmacia. NRP-1-Fc chimera antigen and other antigens used in this study  
68 were obtained from R&D Systems. The pHEN-4 and pHEN-6c vectors gifted by Serge  
69 Muyldermans (Laboratory of Cellular and Molecular Immunology, Vrije University Brussels,  
70 Brussels, Belgium). BSA, Casein, and Skim milk were purchased from Merck. Maxisorp 96-  
71 well plate was purchased from Roskilde. Protein marker was purchased from Sinaclon, Iran.  
72 Immobilized- metal affinity chromatography resin was purchased from Qiagen, Germany.  
73 HUVEC, MCF-7 and HEK cells were purchased from cell bank of Pasteur Institute of Iran.  
74 All of reagent for cell culture and GeltrexTMLDEV-Free Reduced Growth Factor Basement  
75 Membrane M were purchased from Gibco. All other reagents used in this study were  
76 purchased from Sigma.

## 77 2.2. Preparation of nanobody library

78 The nanobody library preparation method for anti-NRP-1 was similar to protocol used for  
79 previous work by our team (12). Briefly, a young camel (*Camelus dromedarius*) was  
80 immunized with NRP-1 antigen. Immune reponses against NRP-1 was checked by ELISA  
81 and peripheral blood mononuclear lymphocytes (PBMCs) were isolated and total RNA was  
82 extracted. The cDNA was synthesized and VHH gene was amplified by two consecutive  
83 PCR. The VHH gene was digested with *Pst* I and *Not* I restriction enzymes. The digested  
84 VHHs were ligated into linear pHEN-4 vector which was digested by the same restriction  
85 enzymes. Therefore, the recombinant phagemids were transformed into electrocompetent *E.*  
86 *coli* TG1 cells (12).

87

## 88 2.3. Biopanning of the nanobody library

89 An amplified nanobody library containing  $10^7$  clones was infected with  $10^7$  colony forming  
90 units (c.f.u) of VCSM13 helper phage. In this way, phage particles displaying nanobodies  
91 were rescued and biopanning was performed against immobilized NRP-1 antigen. To capture  
92 NRP-1 specific phages, four subsequent rounds of biopanning were performed. Briefly, a 96-  
93 well plate was coated overnight at 4 °C with 100  $\mu$ l of NRP-1(0.5  $\mu$ g/mL) as target antigen  
94 and phosphate-buffered saline (PBS) as negative control. The next day, the plate was washed  
95 with PBS and blocked with 4% skimmed milk in PBS (MPBS) at RT for 1h. About  $10^9$  cfu of

96 phage particles were incubated for 1h with the human- full Fc (1 mg/ml in PBS) with gentle  
97 mixing to eliminate Fc-bounded phage particles. The next, supernatant transferred to a  
98 negative well containing PBS at RT for 1 h. Then, supernatant was transferred from the  
99 negative well to antigen-coated well and incubated at RT for 1 h. The wells were washed 5  
100 times with PBST (0.05% (V/V) Tween 20 in PBS). The bounded phages eluted by adding of  
101 100µl triethylamine(TEA, pH 10.0, 100 mM) for 10 min, and then neutralized by 100µl Tris-  
102 HCl (pH 8.0, 1M). Eluted phages (output) were used for titration and for infection of 5 ml of  
103 *E. coli* TG1 (OD<sub>600nm</sub> 0.4- 0.6). By adding of VCSM13 helper phage, phages were rescued  
104 and subsequently precipitated in PEG/NaCl (PEG/NaCl (20% polyethylene glycol 6000/2.5  
105 M NaCl in water). Then phages were centrifuged and the pellet was resuspended in PBS  
106 (input) and used for subsequent rounds. The next rounds of panning were performed as  
107 mentioned above with more stringent washing by enhancing the amount of Tween 20 in  
108 PBST solution (0.05%, 0.1%, 0.2%, and 0.4%). To evaluate enrichment of panning rounds,  
109 TG1 cells (OD<sub>600</sub> 0.4-0.6) were infected with serially diluted of input phages and then  
110 infected cells were plated on a 2XTY plate containing 100 µg/mL ampicillin. Colonies were  
111 counted at each round and the result of each round was identified.

112

#### 113 2.4. Polyclonal phage ELISA

114 The maxisorp 96-well plate was coated with the NRP-1 antigen (1 µg/ml) overnight at 4 °C.  
115 In addition, for detection of non-specific binding, BSA and human- full Fc was used as  
116 control wells. The next day, coating solution was discarded and the plate was washed with  
117 PBST and then blocked with MPBS for 1h at RT. Then wells were washed and 10<sup>12</sup> cfu of  
118 output phages were added to the wells and incubation was performed at RT for 1 h. After  
119 washing, the anti-M13-HRP antibody (1:2000) was added and incubated at RT for 1 h.  
120 Finally, TMB (3,3', 5,5'-tetramethyl benzidine) was added and the reaction was stopped by  
121 adding 2 N H<sub>2</sub>SO<sub>4</sub>. The absorption was measured by ELISA reader at 450 nm.

122

#### 123 2.5. Periplasmic extract ELISA (PE-ELISA)

124 Individual *E. coli* TG1 colonies were randomly selected by infecting of bacteria with eluted  
125 phages as above mentioned and grown in 1 ml of TB medium to reach log phase (OD<sub>600</sub> 0.4-  
126 0.6). Production of nanobody containing a haemagglutinin (HA) tag in the periplasme of *E.*  
127 *coli* was induced with 1 mM isopropyl D-1-thiogalactopyranoside (IPTG). Expression of

128 nanobody was identified with ELISA. The bounded nanobodies to coated antigen in 96-wells  
129 plate, were detected using anti-HA antibody (1:2000) followed by anti-mouse HRP  
130 conjugated antibody (1:5000). Then TMB substrate was added and absorbance was measured  
131 at 450 nm. Finally, positive clones were then sequenced to identify the specific nanobody  
132 genes. The 3D structures of nanobodies predicted by Iterative Threading ASSEmbly  
133 Refinement (I-TASSER) method(13). Interaction between nanobodies and NRP-1 was  
134 evaluated using Hex software (9, 14-16).

135

## 136 2.6. Expression and purification

137 The specific nanobody genes were amplified using forward;  
138 5\_GATGTGCAGCTGCAGGAGTCTGGGGGAGG\_3 and reverse primers;  
139 5\_GGACTAGTGCGGCCGCTGGAGACG-GTGACCTGGGT \_3. Amplified nanobody  
140 gene sub-cloned into pHEN-6c bacterial expression vector containing c-terminal His6 tag  
141 using the *BstE* II and *Pst* I restriction enzymes. The recombinant pHEN-6c construct was  
142 transformed into *E. coli* WK6 cells. The transformants were confirmed by colony-PCR.  
143 Transformants were grown in 330 ml of 2XTY medium in baffled flask. Nanobody  
144 expression was induced in periplasmic space of the *E. coli* cells by 1 mM IPTG. Periplasmic  
145 expressed proteins were released using osmotic shock. Recombinant proteins then were  
146 purified by immobilized metal affinity chromatography resin with 500 mM imidazole.  
147 Ultimately, the eluted protein was dialyzed against PBS with 5 kDa cutoff(17).

148

## 149 2.7. Polyacrylamide gel electrophoresis analysis

150 The SDS-PAGE was carried out under reducing condition on 12% gel. After then, Coomassie  
151 Blue stain was used to stain the protein bands in polyacrylamide gel. The next, identification  
152 of nanobodies were evaluated by western blot using HRP-conjugated anti-His tag antibody  
153 (1:2000). Finally, the nanobody bands were exposed by 3, 3'-diaminobenzidine substrate  
154 (DAB). The concentration of nanobodies were determined by assessment of UV absorption at  
155 280 nm(18, 19).

## 156 2.8. Specificity of nanobodies

157 The specificity and cross-reactivity of selected nanobodies were examined with the following  
158 antigens by ELISA. One  $\mu\text{g/mL}$  of antigens such as BSA, casein, skim milk, epithelial cell

159 adhesion molecule (EpCAM), programmed cell death protein 1 (PD-1), programmed death-  
160 ligand 1 (PDL-1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) , and zinc  
161 transport protein LIV-1 were coated onto 96-well ELISA plates. In this experiment, bounded  
162 nanobodies to the wells were identified by HRP conjugated anti-His antibody followed by  
163 HRP substrate.

164

## 165 2.9. Affinity of nanobodies

166 Affinity of nanobodies were estimated by the method described by Beatty et al(20). Briefly,  
167 100 µl of two different concentrations (1 and 10µg/ml) of NRP-1 antigen and BSA as  
168 negative control was coated on 96-well plate. After blocking, wells were incubated for 1 h  
169 with increasing concentrations (0–100 nM) of nanobodies. Detection of binding was  
170 evaluated by anti-His HRP conjugated antibody (1:1000) followed by TMB substrate.  
171 Finally, The affinity constant ( $K_{aff}$ ) of the nanobodies to the NRP-1 was calculated using the  
172 following equation:

173

$$174 [Ag]/[Ag^*] = N$$

$$175 k_{aff} = N - 1/2(N[Nb] - [Nb^*])$$

176

## 177 2.10. Competitive ELISA assay

178 To examine ability of NRP-1 specific nanobodies to detect the solution form of NRP-1, a  
179 competitive ELISA was designed. In this experiment, 1µg/ml of NRP-1 antigen or BSA as  
180 negative control were coated in 96 well microplate and incubated at 4 °C overnight. Then,  
181 1µg/ml of nanobodies were incubated with increasing concentrations (0-1 µg/ml) of NRP- 1  
182 for 1 h at 37 °C and then added to the wells coated with NRP-1. The wells incubated for 1 h.  
183 The next, the wells were washed and bounded nanobody to immobilized NRP-1 was detected  
184 by ELISA.

185

## 186 2.11. Cell lines

187 MCF-7 (human breast cancer cell line) and HEK293 (human embryonic kidney) were  
188 cultured in Dulbecco's Modified Eagle's Medium (DMEM). Human umbilical vein  
189 endothelial cell line (HUVEC) was grown in HAMS-F12. All of the culture media were  
190 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin

191 and 100 µg/ml streptomycin. Cells were detached by a one or three- min exposure to 0. 05%  
192 w/v trypsin –EDTA.

193

## 194 2.12. *In vitro* assay

### 195 2.12.1. MTT assay

196 To investigate the anti-proliferative effect of nanobodies, colorimetric assay was performed  
197 using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, MCF-  
198 7, HUVEC and HEK cells (as negative control) were seeded ( $3 \times 10^3$  cells/well) in a 96-well  
199 plate. Then cultures were grown for 24 and 48 h and then treated with culture medium  
200 containing different concentrations (0 – 20 nM) of nanobodies, or PBS as control. The next,  
201 10 µl MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The  
202 medium was removed and MTT-formazan crystals solubilized by 100 µl of  
203 dimethylsulfoxide (DMSO). Finally, the absorbance was determined by spectrophotometer  
204 at 570 nm using a reference wavelength of 630 nm.

205

### 206 2.12.2. Tube formation assay

207 In this experiment, Geltrex TMLDEV-Free Reduced Growth Factor Basement Membrane M  
208 solution was thawed on ice. Fifty µl of Geltrex were transferred into 96-well plate. The plate  
209 was incubated in 37 °C for 30 min. About 100 µl HAMS-F12 medium supplemented with  
210 FBS containing  $10^4$  HUVEC cells were added on Geltrex. Then, diluted nanobodies was  
211 added to the wells. In addition, cells treated with BSA was considered as control. After  
212 incubation at 37 °C, capillary like structures and endothelial tube formation was observed  
213 during 1 to 6 h under inverted microscope. Branching or capillary like structures of the cells  
214 were analyzed by Image J software.

215

## 216 2.13. *Ex vivo* assay:

### 217 2.13.1. Chicken chorioallantoic membrane model

218 To investigate anti-angiogenesis activity of selected nanobodies *ex vivo*, chicken  
219 chorioallantoic membrane (CAM) was performed. Briefly, fertilized chicken eggs incubated  
220 at 37 °C with 70% humidity. Six days after, the contents of the eggs were transferred into the  
221 petri dish under sterile condition. The embryo and the yolk vessels located on top of an  
222 undamaged yolk. Then, discs containing concentrations of nanobody (at the IC50) or 10 µl



223 PBS as negative control were placed on the top of the embryo and petri dish was transferred  
224 to the incubator for 48 h. Finally, inhibition of vessel growth was observed macroscopically.

#### 225 2.14. Statistical analysis

226 Statistical analyses were performed by Prism 8.0 Software (GraphPad, San Diego, CA). *P*  
227 values < 0.05 was considered statistically significant.

### 228 3. Result

#### 229 3.1. Selection of anti-NRP-1 phages

230 Four rounds of panning were performed to identify NRP-1 specific nanobodies. To evaluate  
231 the enrichment of NRP-1 specific phages, the number of output and input phages were  
232 compared at each round. The increasing ratio was observed in third and fourth round of  
233 biopanning that was 6.3 and 19.4 fold, respectively (Table 1). In addition, to confirm success  
234 of selection process, specific bounding of each eluted phage from different round of  
235 biopanning was performed by polyclonal phage ELISA. The highest signals were observed in  
236 the third and fourth round of panning (Fig. 1). To screen monoclonal phages, over 100 clones  
237 were infected with output phages of the third and fourth round of biopanning. Monoclonal  
238 phage ELISA results showed that four clones specifically reacted with NRP-1 (Fig. 2).  
239 Finally, selected clones were sequenced and alignment was performed by NCBI BLAST  
240 tools and the results showed at least 89% homology with VHH sequence of *Camelus*  
241 *dromedaries*. The amino acid sequences of selected nanobodies are shown in Table 2. The  
242 predicted 3D structures of nanobodies are shown in Fig 3. Docking was performed to identify  
243 protein-protein interaction between nanobodies and NRP-1. In docking, NRP-1 considered as  
244 receptor and Nanobodies were to be ligand (Table 3).

#### 245 3.2. Expression and purification of soluble nanobodies

246 To generate soluble nanobodies, the nanobodies-encoding genes were sub-cloned into the  
247 pHEN-6c expression vector and transformed into *E. coli* WK6 cells. The His-tagged  
248 fusion nanobodies were purified by nickel affinity chromatography (Ni<sup>+</sup>-NTA). Fractions  
249 containing each nanobody independently was pooled and dialyzed against PBS1×.  
250 The purity of the nanobodies was verified using SDS-PAGE (Fig. 4 A). Western blot analysis  
251 was performed using anti-His conjugated HRP and successful expression was confirmed with  
252 a band with approximately 17 kDa (Fig. 4 B). The yield of purified nanobodies depending to  
253 clones was variable from 0.04–0.07 gram per liter of culture.

254

### 255 3.3. Binding specificity

256 To determine the specificity of nanobodies, binding of anti-NRP-1 nanobodies to NRP-1 and  
257 different proteins were investigated by ELISA. The results indicated that four nanobodies  
258 showed significantly high signal in ELISA in comparison with the control proteins (Table4).

259

### 260 3.4. Affinity results

261

262 The affinity constant of nanobodies were determined by ELISA, according to the Beatty et al.  
263 method (Fig. 5). The  $K_{aff}$  of nanobodies are shown in Table 5.

264

### 265 3.5. Competition assay

266

267 The results of ELISA showed that binding of selected anti-NRP-1 nanobodies to immobilized  
268 NRP-1 was inhibited by binding of nanobodies to solution NRP-1. At increasing  
269 concentration of NRP-1, and in the presence of 1  $\mu$ g/ml NRP-1, nanobodies bounded to  
270 soluble antigen and inhibited binding to immobilized antigen. As the result show in Figure 6,  
271 the OD values of, Nb16, Nb48 and Nb53 nanobodies were decreased considerably when the  
272 concentration of soluble antigen NRP-1 was increased in solution.

273

### 274 3.6. Inhibition of cell proliferation by NRP-1 specific nanobodies

275 To evaluate concentration-dependent cytotoxic effect of NRP-1 specific nanobodies, MTT  
276 assay was performed using MCF-7, HUVECs and HEK cells. It has been shown that after 1  
277 and 2 days incubation period with NRP-1 specific nanobodies, the rate of cell viability  
278 decreased significantly ( $P < 0.05$ ) in HUVECs and moderately in MCF-7 cells in a  
279 concentration-dependent manner whereas the HEK cells remained unaffected (Fig. 7). The  
280 IC50 value in HUVEC cells was 4 and 48 nM in Nb48 and Nb53, respectively and >50 nM  
281 in Nb2 and Nb16. In addition, the IC50 value in MCF-7 cells was 18 and 69 nM in Nb48 and  
282 Nb53, respectively and >70 nM in Nb2 and Nb16.

283

284

### 285 3.7. Tube formation assay

286 To study whether the NRP-1 is involved in progress of angiogenesis, we used selected  
287 nanobodies toward NRP-1. Results showed that incubation of HUVECs with Nb 48 (at IC50  
288 concentration), significantly decreased tube length of the cells (Fig. 8). The Nb48 attenuated  
289 tube formation by ~46% compared to HUVECs untreated as control (Fig. 9). The other Nbs  
290 showed a slightly effect on total length. These results demonstrated that NRP-1 receptors  
291 might be involved partially in tube formation of HUVECs.

292

### 293 3.8. CAM assay

294 To evaluate the anti-angiogenic responses of the selected nanobodies, CAM assay was  
295 performed. After 48h, the Nb48 significantly inhibited the development of angiogenesis. The  
296 other nanobodies, Nb2, Nb16 and Nb 53 slightly inhibited growth of the vessels (Fig. 10). In  
297 the control group treated with only PBS there was not observed any anti-angiogenic effect.

298

## 299 4. Discussion

300

301 The purpose of the present study was to generate and characterize specific nanobodies against  
302 NRP-1. Previous studies have been demonstrated that NRP-1 plays an essential role in tumor  
303 angiogenesis, and metastasis and is considered as a promising candidate for cancer therapy  
304 (21). Currently, a few anti-angiogenic agents have been approved by FDA that could inhibit  
305 VEGF pathway. However, resistance to anti-angiogenic drugs may limit therapy in patients  
306 and lead to disease progression(22). In this study, anti-NRP-1 nanobodies were isolated and  
307 successfully characterized by phage display method. Phage display technology is a  
308 fundamental drug discovery technology in identification of antibodies(23). To obtain  
309 nanobodies with high affinity and specificity(24) toward NRP-1 antigen, and eliminate the  
310 nanobodies that cross-react with BSA and human full- FC, consecutive rounds of panning  
311 was performed with increased stringency. To isolate specific clones to NRP-1, periplasmic  
312 extract ELISA was performed. More than 100 randomly selected clones from round 3 and 4  
313 of panning were chosen. Four clones showed strong signals on ELISA. Colony PCR  
314 showed that four selected colonies contained an inserted fragment of the nanobodies  
315 proper size. Docking simulation results showed strong binding of selected nanobodies to  
316 NRP-1. Selected colonies were successfully sub-cloned into expression vector and then  
317 soluble nanobodies were investigated further by ELISA. Each clone produced on large scale

318 and then purified through immobilized metal affinity chromatography (IMAC).  
319 Immunoblotting indicated the successful production and purification (>95%) of nanobodies  
320 for *in vitro* experiment. Binding affinity for nanobodies was different from 5 to 80 nM. The  
321 different binding affinities of the nanobodies to NRP-1 suggest that they may recognize  
322 different epitopes on same antigen (12). The most NRP-1 inhibitors mainly targeted VEGF  
323 binding to NRP-1. In a study by Pan, Q *et.al*, monoclonal antibodies, anti-NRP1<sup>A</sup> and anti-  
324 NRP1<sup>B</sup>, obtained by naive-antibody phage library and targeted the NRP-1 a1-a2 (Kd= 0.9  
325 nM) and b1-b2 domains (Kd= 0.4 nM), respectively. Both antibodies could inhibit VEGF-  
326 dependent endothelial cell migration and angiogenesis *in vitro* (6). The binding specificity of  
327 nanobodies were exhibited their desirable reactivity to NRP-1. To determine binding  
328 capability of nanobodies in two different phases (solid and solution), competitive ELISA was  
329 performed. As immobilized antigens undergo some conformational changes, it may be  
330 disturbed antigen recognition in soluble phase (12). In addition, we shown in proliferation  
331 assay, Nb48 effectively inhibited HUVEC and MCF-7 proliferation in a dose-dependent  
332 manner. However, it had no significant effect on HEK cell proliferation. In a study by Zeng  
333 F and *et.al*, it was found that a monoclonal antibody against NRP-1, significantly reduced the  
334 proliferation of MCF-7 cells at 400 µg/ml(25). In addition, we demonstrated that Nb48  
335 effectively inhibited tube formation of HUVECs. Tube formation assay is one of the potential  
336 *in vitro* assays for angiogenesis assessment and can be easily perform and quantitative on  
337 matrigel(26). Based on our data, we found that Nb48 -treated HUVEC cells significantly  
338 decreased the total tube length. Thus, Nb48 may be considered as a functional nanobody  
339 candidate to inhibit angiogenesis. Our results suggest that Nb48 have such relative functions.  
340 In other study, by phage display screening method, an A7R peptide (ATWLPPR) identified  
341 that could bind to NRP1 and partially reduced endothelial cell tube formation (with 500 µM  
342 of A7R ) (27). In Borriello *et al* study, a potent small molecule could inhibit tubulogenesis by  
343 blocking of VEGF-NRP-1 interaction *in vitro* with an IC50 of 0.2µM. The antagonist was  
344 able to decrease HUVEC viability by 68% at the IC50 concentration(28). Recent studies have  
345 shown that targeting of VEGF-independent NRP-1 may possible a therapeutic potential. It is  
346 consider that NRP1 could improve angiogenesis in the absence of VEGFR-1 or VEGFR-2. In  
347 a study, targeting with micro RNAs (miRNAs) could regulate NRP-1 expression and reduce  
348 angiogenesis *in vitro*(29).  
349 The CAM assay is a frequently applied technique to study angiogenesis. It is cost effective,  
350 and is an easily reproducible *ex vivo* model (30). We performed CAM assay to assess the

351 inhibitory function of nanobodies on tube formation of CAM. Results exhibited the lack of  
352 vessel formation in response to anti-NRP-1 nanobody. In a study, development of peptide  
353 pTM-NRP1 toward the transmembrane domain of NRP-1 results in maximum inhibition of  
354 tube formation (in  $10^{-7}$ M of peptide). In addition, anti-angiogenic effect of peptide was  
355 confirmed using *ex vivo* CAM assay(31).

356 In conclusion, two high affinity and specificity nanobodies were selected from the immune  
357 nanobody library by phage display technique. Selected nanobodies efficiently inhibited *in*  
358 *vitro* and *ex vivo* angiogenesis of endothelial cells. The results indicate for potential of  
359 selected NRP-1 nanobodies as novel anti-angiogenesis agent.

360

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- 444
- 445
- 446

447 Tables

448 Table 1: Enrichment results of phage particle after biopanning on NRP-1 antigen.

Rounds of biopanning	No. of colonies in NRP-1 coated well (cfu) (A)	No. of colonies in PBS coated well (cfu) (B)	Enrichment ratio (A/B)
1	201	112	1.8
2	192	96	2
3	182	29	6.3
4	608	31	19.6

449

450

451 Table2: Amino acid sequences of the selected nanobodies. Similar residues are colored as the most  
452 conserved one (according to BLOSUM62).

453 FR1 CDR1 FR2 CDR2

454 Nb16 QVQLVQSGGGSVQAGGSLRLSCvAS-GDTSITVGS GWFRQAPGKEREGVAI-RYMIGVML

455 Nb48 QVQLVQSGGGSVQAGGSLRLSCAASAGLRAGPIHYGWFRQAPGKEREGVAV--ESDDTAL

456 Nb2 QVQLVQSGGGSVQAGGSLRLSCAAS-GHERNNYCVGWFRQVPGKEREGVAIFRAGITAVS

457 Nb53 QVQLVQSGGGSVQAGGSLRLSCAAS-GYERNNYCVGWFRQAPGKEREGVAIFNSGGVSAI

458

459 CDR2 FR3 CDR3

460 Nb16 AAQTYTDSVKGRFTISQDNTKNTVYLQMSSLKPEDTAIYYCAAGCVQVRSV-TVPSVY--

461 Nb48 GFATYADSVKARFTISQDNTKNTVYLQMNSLKPEDTAIYYCAASI--RRCTWYHPTGYNY

462 Nb2 GTTYYADSVKGRFTISQDNTKNTVYLQMNSLKPEDTAIYYCTAAkIYPPCT-GISRTYDY

463 Nb53 QVPY YADSVKGRFTISQDNTKNTVYLQMNSLKPEDTAIYYCCAkIYPPCT-GISRTYDY

464

465 FR4

466 Nb16 WGQGTQVYVSS

467 Nb48 WGQGTQVYVSS

468 Nb2 WGQGTQVYVSS

469 Nb53 WGQGTQVYVSS

470

471

472 Table 3: Results of docking simulation of nanobodies and NRP-1.

	E. Total	E. Shape	E. Air	Bmp (Bit)	Rms (Root)
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	(kcal/mol)			maps)	mean square)
Nb2	-334/15	-334/15	0	-1	-1
Nb16	-362/13	-362/13	0	-1	-1
Nb48	-347/5	-347/5	0	-1	-1
Nb53	-343/7	-343/7	0	-1	-1

473

474

475 Table4: Specificity of nanobodies. Data display the mean  $\pm$  standard deviation (SD) of three  
476 experiments.

477

Nanobodies	NRP-1	EpCAM	CTLA-4	LIV-1	PD-1	PDL-1	BSA	Skim milk
Nanobody 2	1.56 $\pm$ 0.12	0.46 $\pm$ 0.07	0.9 $\pm$ 0.17	0.46 $\pm$ 0.07	0.61 $\pm$ 0.12	0.32 $\pm$ 0.08	0.11 $\pm$ 0.12	0.12 $\pm$ 0.08
Nanobody 16	2.01 $\pm$ 0.12	0.51 $\pm$ 0.15	0.51 $\pm$ 0.12	0.47 $\pm$ 0.14	0.42 $\pm$ 0.10	0.43 $\pm$ 0.10	0.12 $\pm$ 0.10	0.13 $\pm$ 0.10
Nanobody 48	2.83 $\pm$ 0.10	0.32 $\pm$ 0.10	0.44 $\pm$ 0.15	0.38 $\pm$ 0.13	0.31 $\pm$ 0.11	0.40 $\pm$ 0.08	0.10 $\pm$ 0.11	0.10 $\pm$ 0.08
Nanobody 53	2.69 $\pm$ 0.1	0.26 $\pm$ 0.08	0.40 $\pm$ 0.11	0.26 $\pm$ 0.08	0.22 $\pm$ 0.09	0.26 $\pm$ 0.11	0.13 $\pm$ 0.09	0.12 $\pm$ 0.11

478

479 Table 5: Binding affinity of the selected nanobodies to the immobilized NRP-1. The affinity  
480 of NRP-1 specific nanobodies were acquired based on the Beatty method. BSA was used as a  
481 negative control. The experiments were performed in triplicate, and the data was represented  
482 with mean  $\pm$  SD.

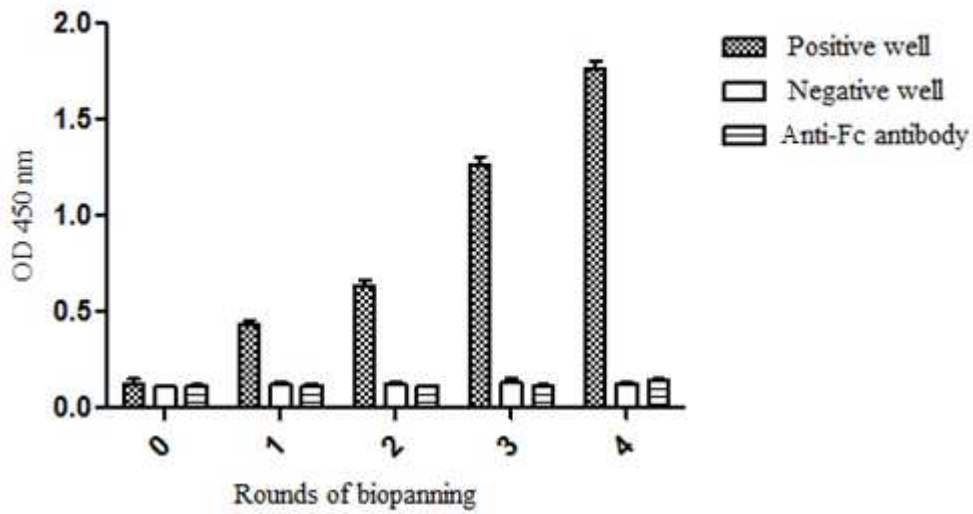
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Nanobodies	Affinity constant ( $k_{aff}$ ) nM
Nanobody 2	50
Nanobody 16	5
Nanobody 48	82.5
Nanobody 53	70

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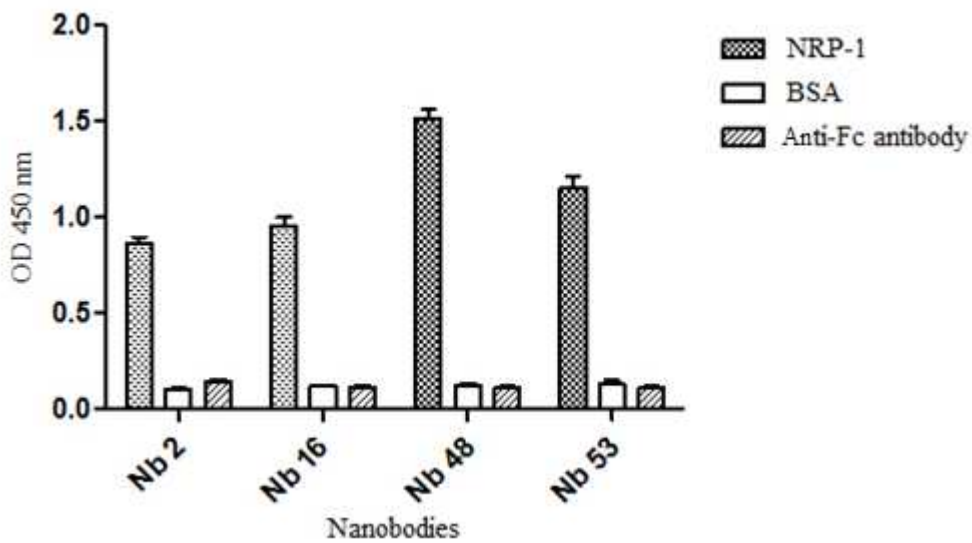
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488

489 Figure 1. Results of polyclonal phage-ELISA. The assay was performed in triplicate and error  
 490 bar was considered for mean ± standard deviation.



491

492 Figure 2. Results of Periplasmic extract ELISA (PE-ELISA). The PE-ELISA showed that  
 493 four clones specifically reacted with NRP-1 antigen. The graphs display the mean ± standard  
 494 deviation (SD) of three experiments.

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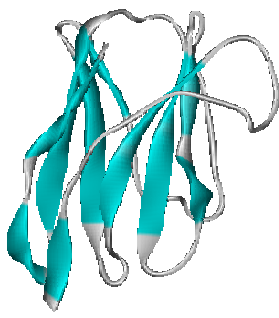
A

B

498

499

500



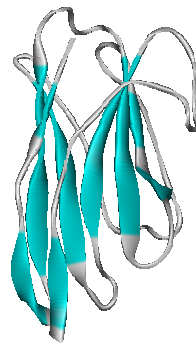
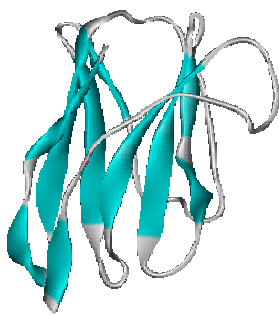
C



D

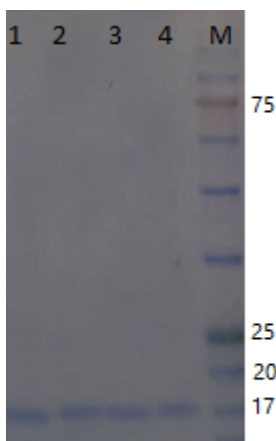
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503 Figure 3. Predicted 3D structure of nanobodies. The 3D structure of nanobodies were  
 504 predicted by I-TASSER. A; Nb2, B; Nb16, C; Nb48, D; Nb53.

505



A



B

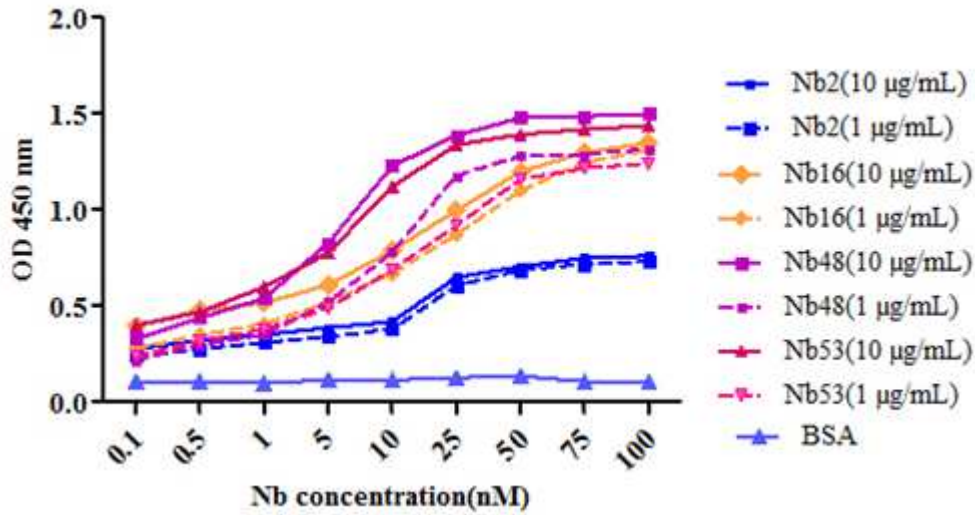
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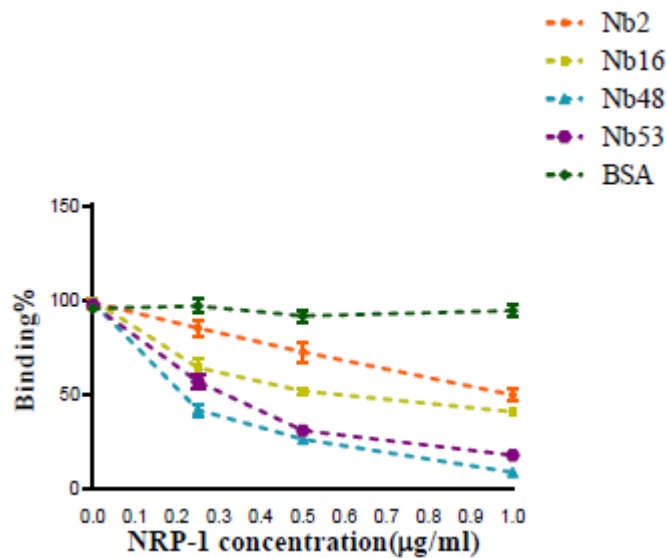
508

509 Figure 4. Results of expression and purification of the selected nanobodies. A). SDS-PAGE  
 510 of purified nanobodies. B) Immunoblotting. M: molecular weight marker, lane 1: Nb2; lane

511 2: Nb16; lane 3: Nb48. lane 4: Nb53. Detection was done by the HRP conjugated anti -His  
 512 antibody.  
 513



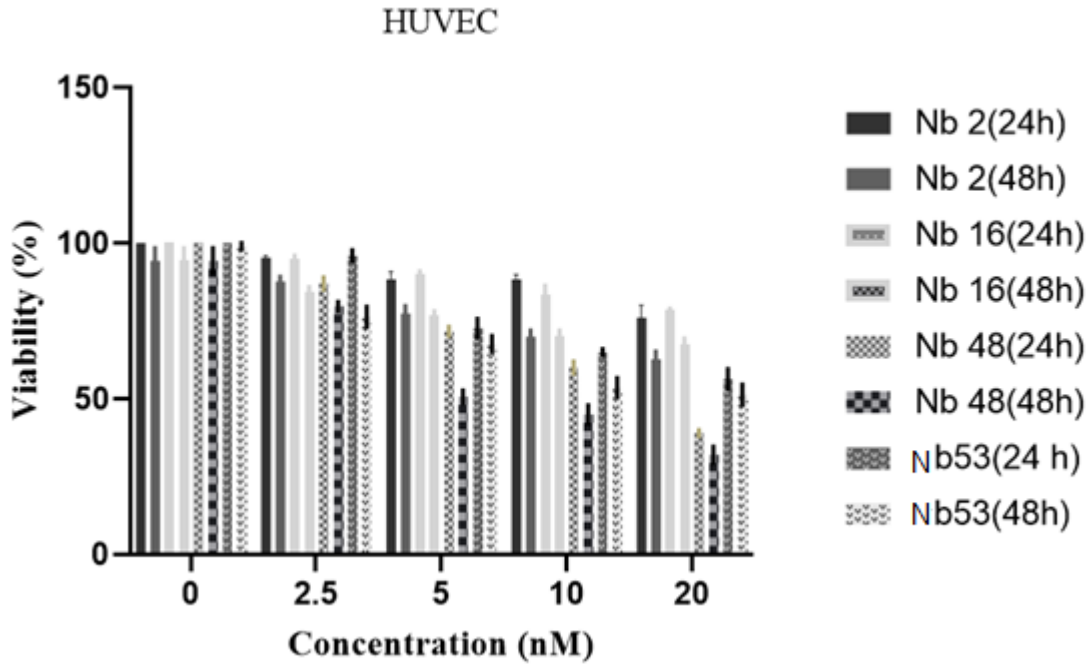
514  
 515 Figure 5. Affinity measurement. Affinity graph was represented based on Beatty ELISA  
 516 method.



517  
 518 Figure 6. Competitive ELISA assay. Wells were coated with NRP-1 antigen, and the  
 519 competitive binding of soluble nanobodies to immobilized NRP-1 and different amounts of  
 520 soluble NRP-1 was examined. BSA used as a negative controls. The data are presented as  
 521 mean  $\pm$ SD.

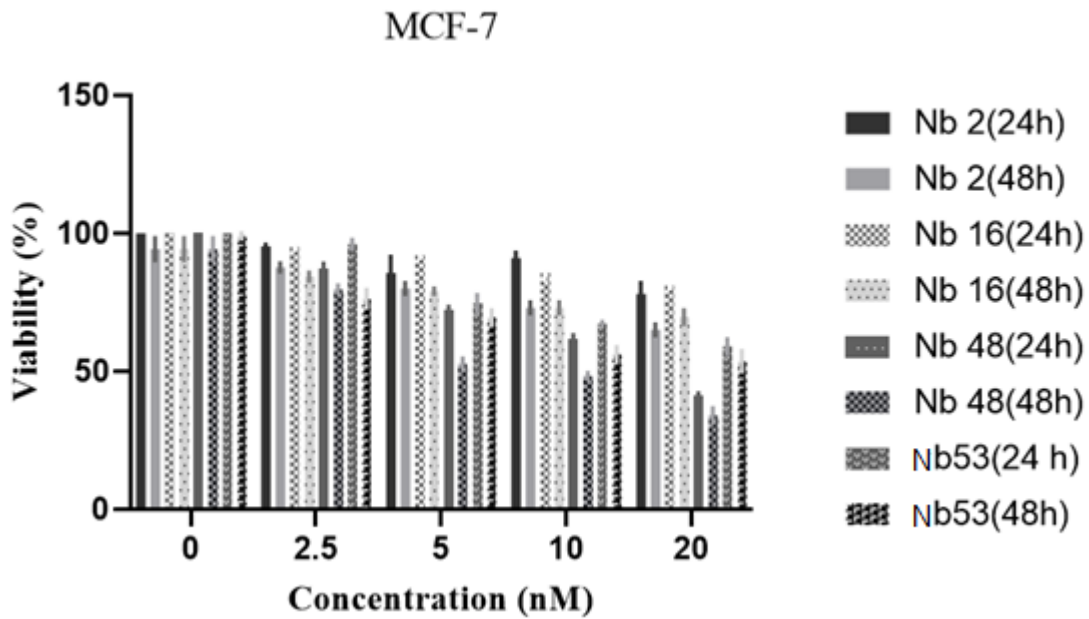
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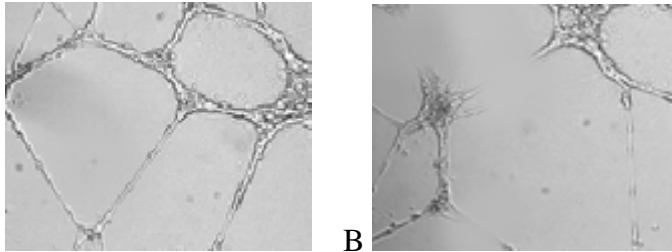
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529 Figure 7. MTT Cell Proliferation Assay. MTT assay was performed for proliferation assay.

530 According to the figure, more than half of the HUVECs proliferation were inhibited in

531 presence of Nb48. Viability of MCF-7 was reduced by approximately 50% in case of Nb48  
532 treatment. Data are expressed as mean  $\pm$  SD

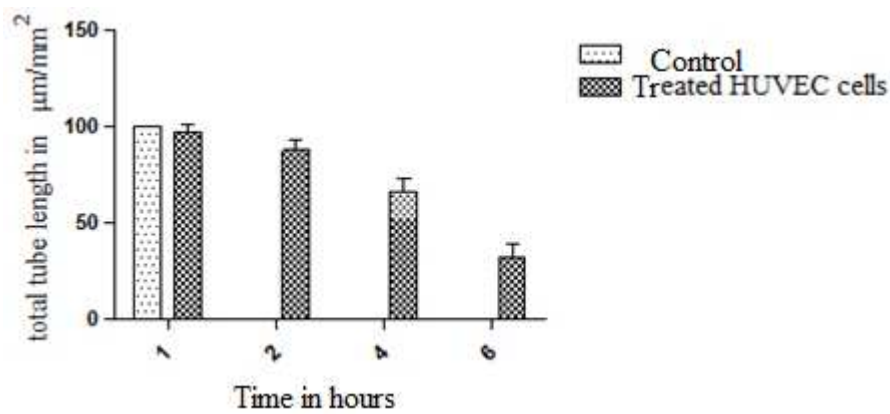
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534

535 Figure8. Inhibition of NRP-1 receptor attenuates tube formation in HUVEC cells. Cells were  
536 incubated in the absence (A) or presence (B) of the Nb48. The data represent the means of  
537 three replicate  $\pm$  SD ( $p < 0.05$ ).

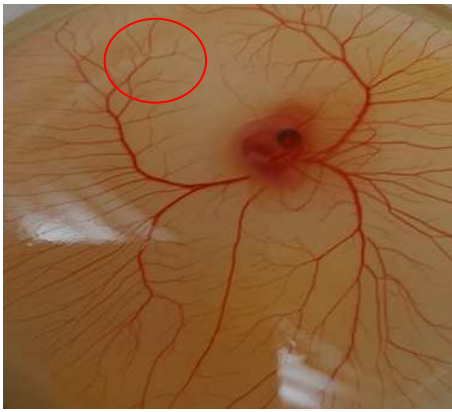
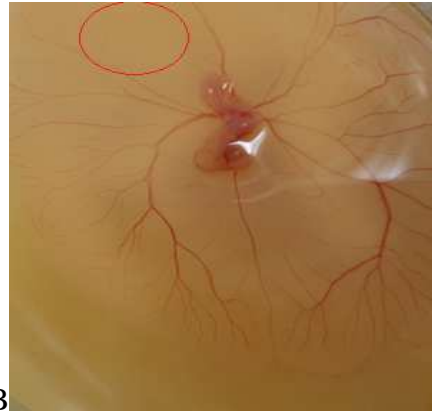
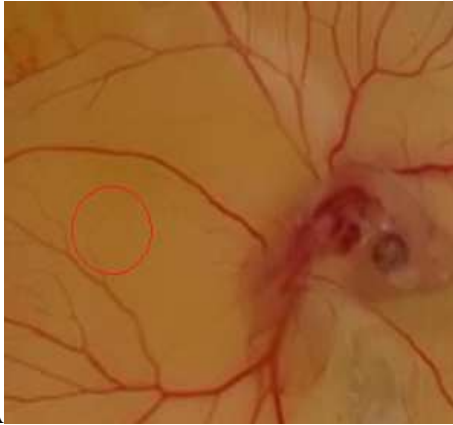
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539

540 Figure9. Tube formation was inhibited by  $\sim 46\%$  after 6h in presence of the 4nM of Nb48.  
541 Analysis was examined using Image J software.

542



543

A

B

544

C

545

546

547

548 Figure10. CAM assay, (A): chick embryo in presence of 4nM/disc (after 24h) and (B)

549 4nM/disc (after 48h) containing of Nb48. (C) control (disc PBS).

550

551