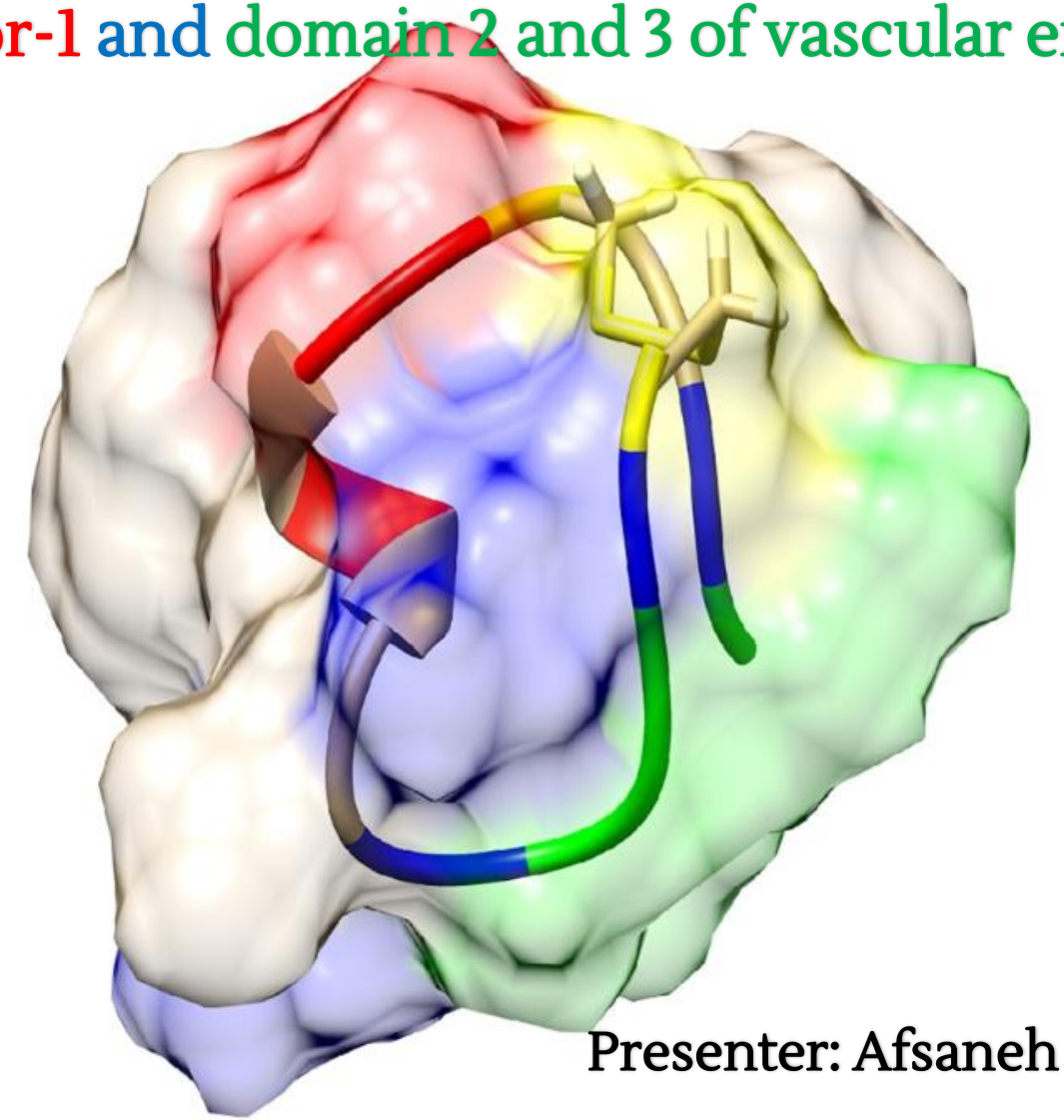
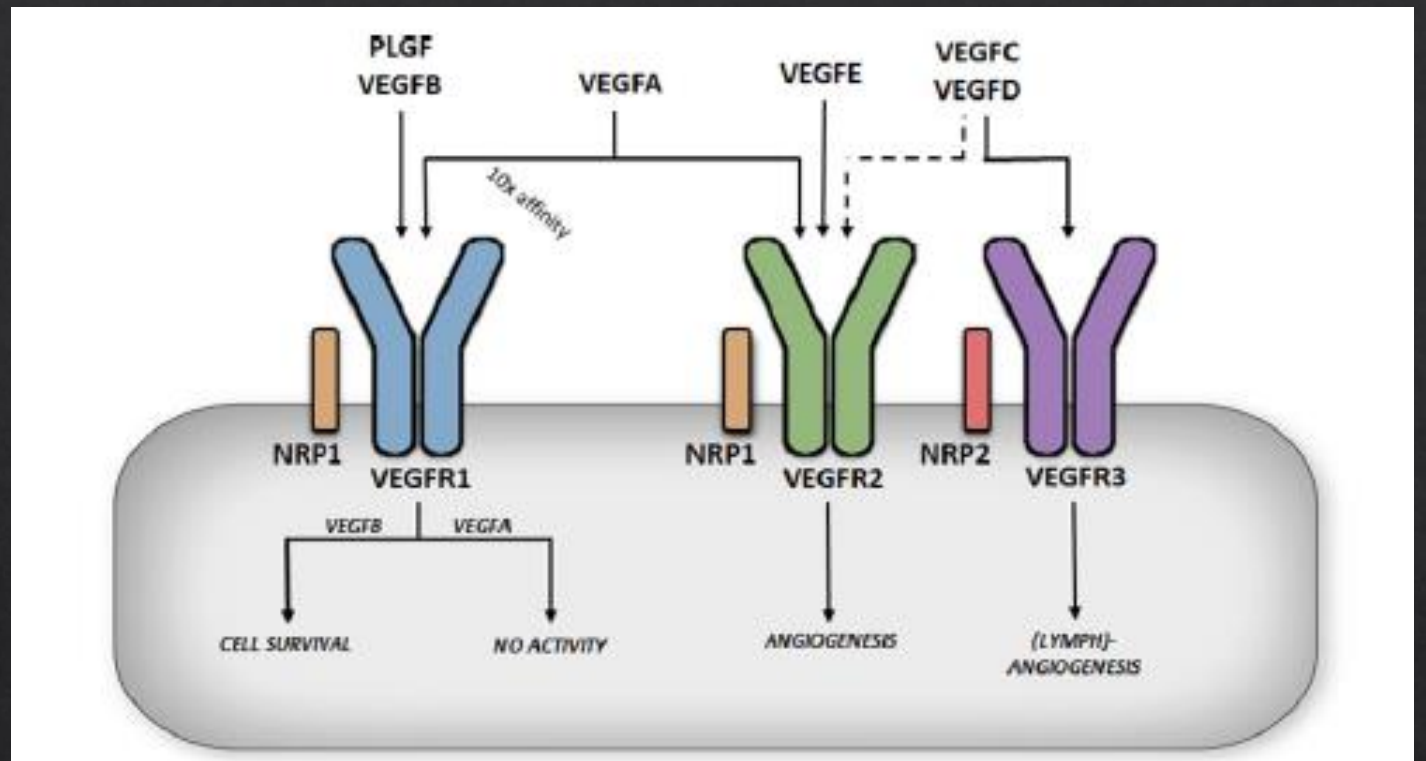
Design, molecular docking, synthesis and biological evaluation of a novel antagonistic peptide of VEGF-A/VEGF-B with domain 2 of vascular endothelial growth factor receptor-1 and domain 2 and 3 of vascular endothelial growth factor-2

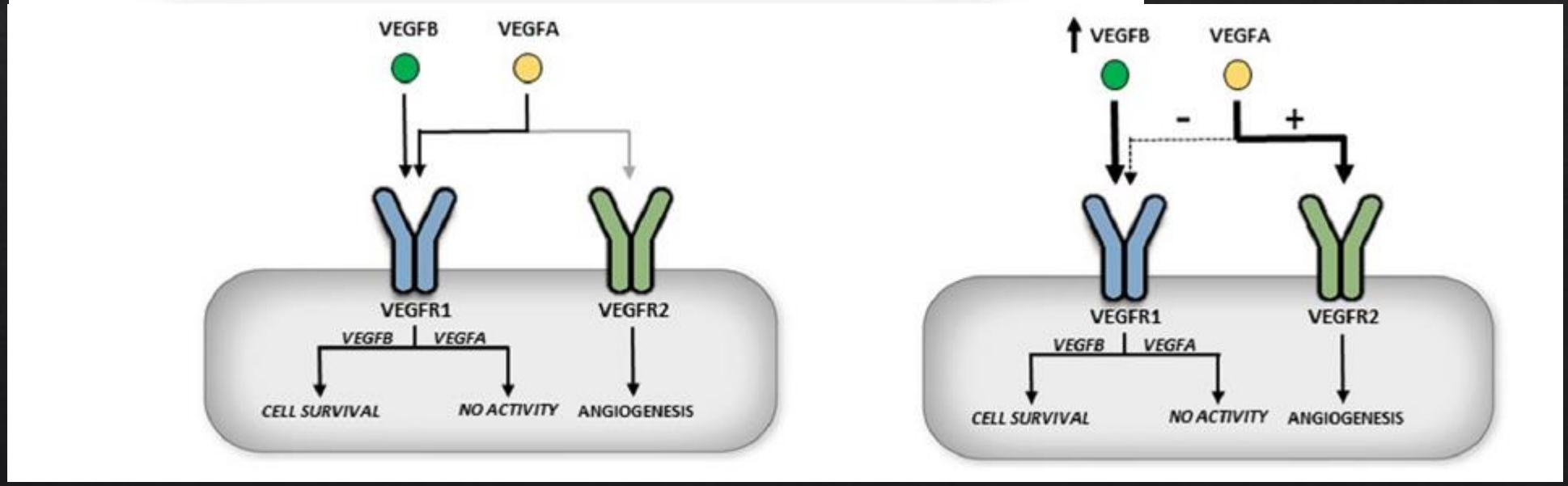


Presenter: Afsaneh Sadremomtaz

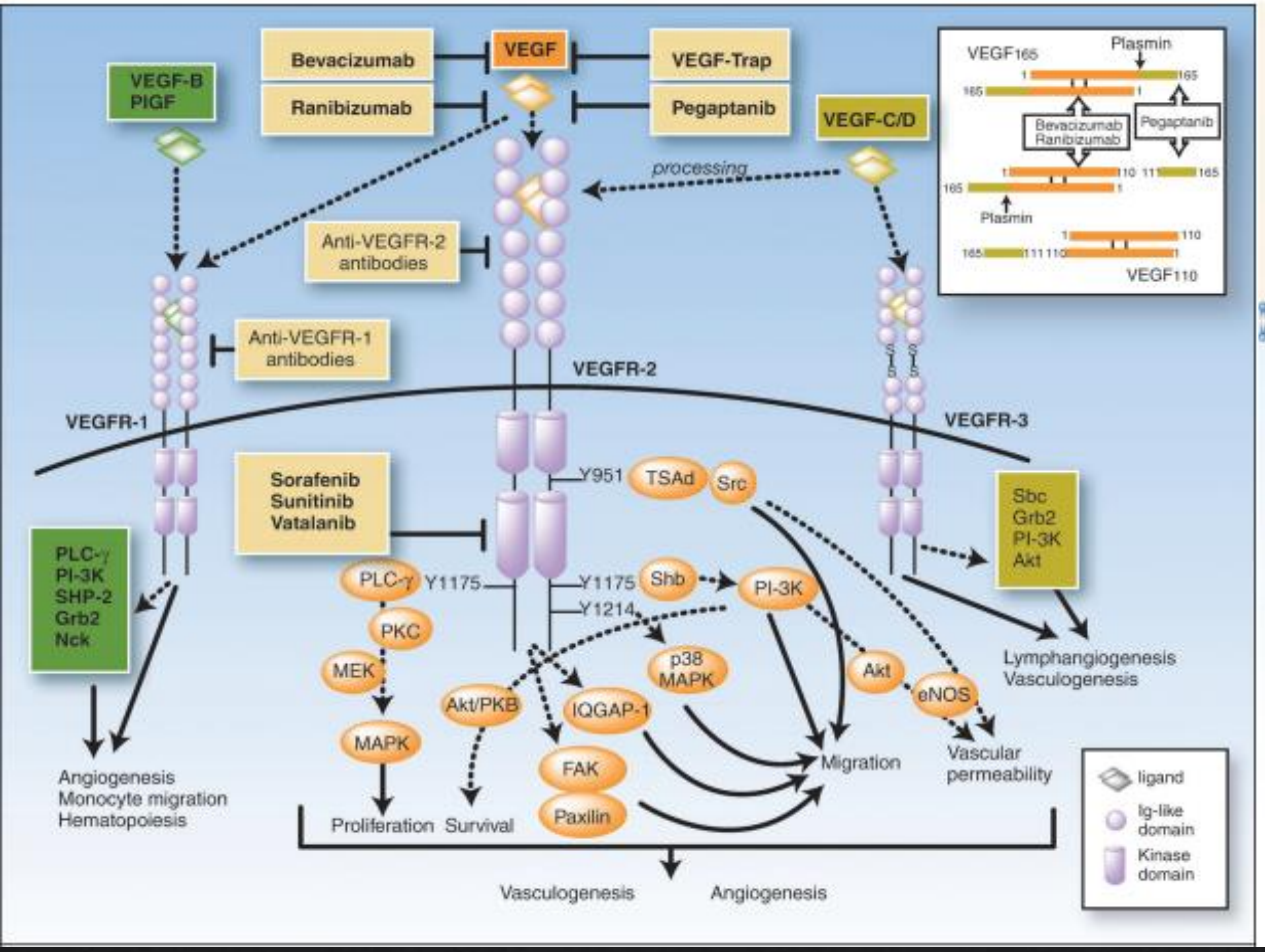
September 2019

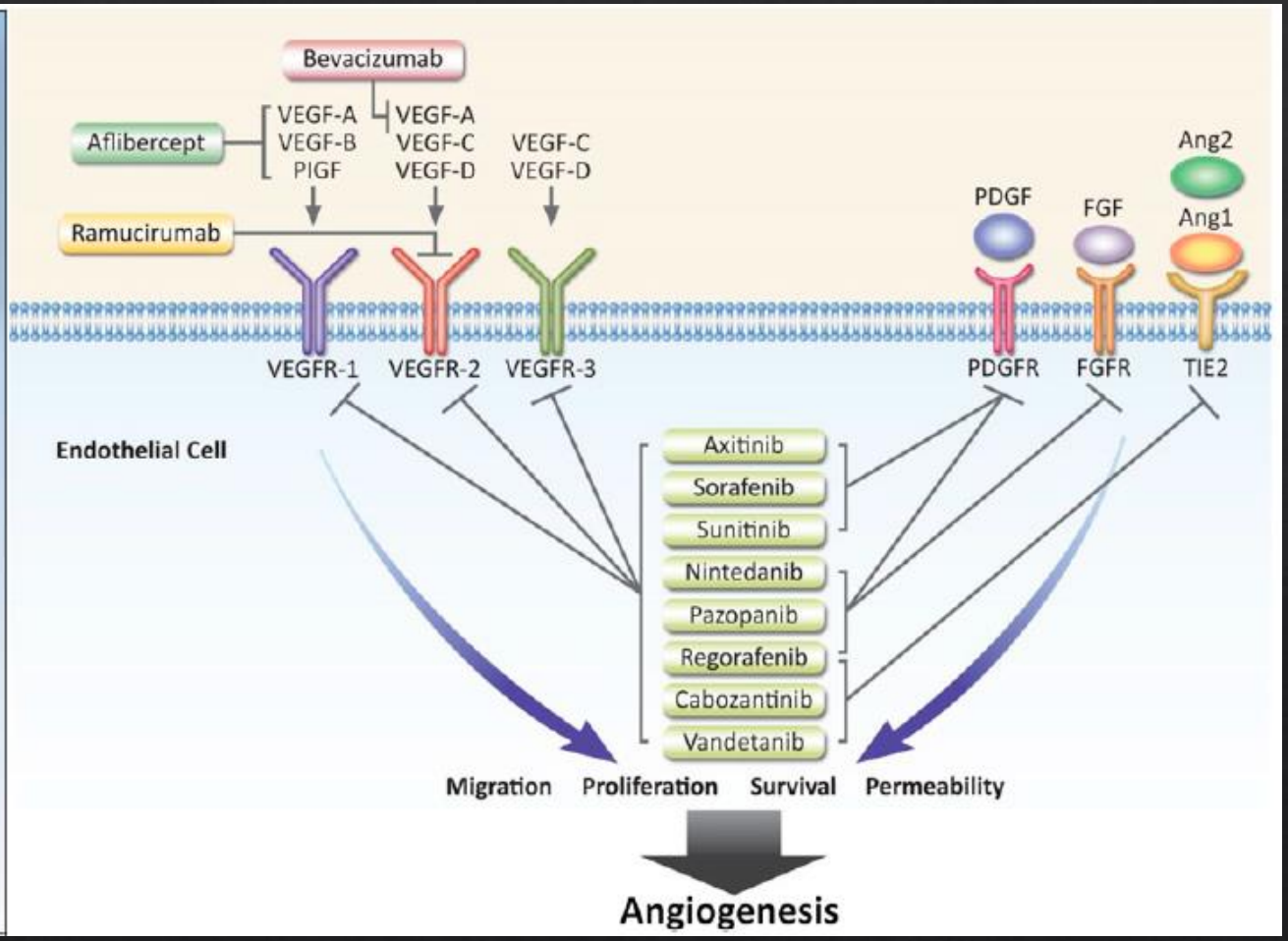
VEGFR Acts as Decoy Receptor in Tumor Angiogenesis





Antiangiogenic compounds





Angiogenesis inhibitors approved by F.D.A.

Drug	Target	Cancer
Avastin (Bevacizumab)	VEGF	mCRC, NSCLC, Advanced breast cancer
Aflibercept	VEGFR1, VEGFR2	mCRC, NSCLC
Lucentis (Ranibizumab)	VEGF	Wet Age-related macular regeneration
Macugen (Pegaptanib)	VEGF	Wet Age-related macular regeneration
Sorafenib (Nexavar)	VEGFR, PDGFR & Raf	Advanced RCC
Sunitinib (Sutent)	VEGFR, PDGFR & c-kit	Advanced RCC & GIST
LY294002	PI3K/AKT/mTOR	NSCLC
Temsirolimus	PI3K/AKT/mTOR	RCC
Wortmannin	PI3K/AKT, MAPK	NSCLC
Rapamycin	MAPK	RCC
Everolimus	**MAPK- Farnesyltransferase Rho and Ras**	Gastric cancer, Hepatocellular carcinoma
Dasatinib (Sprycel)	**Bcr-Abl & Src**	**Gleevec-resistant CML or Ph+ ALL**
Erbitux (Cetuximab)	**EGFR**	**mCRC & Head and Neck cancer**
Lapatinib (Tykerb)	**EGFR & Her2/neu**	**Advanced metastatic Her2+ breast cancer**
Endostar (Endostatin)	**Endogenous angiogenesis inhibitor**	**Lung cancer**

Advantages of peptides in antiangiogenic therapy

- High cost of recombinant proteins
- Small size of peptides
- Ease of synthesis and modification
- Penetrate further into tissues, tumor-penetrating ability, less immunogenic, easy to produce, lower manufacturing costs, greater efficacy, selectivity and specificityand good biocompatibility
- Structure-function relationship and active epitopes of proteins

Aim of study

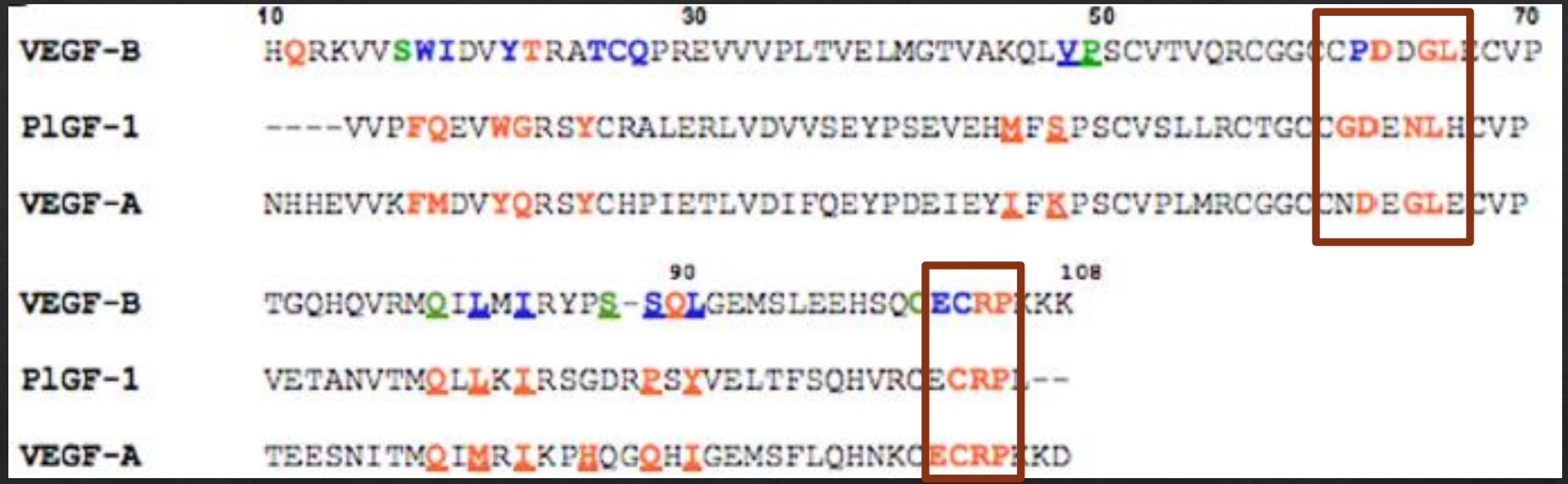
1- Peptide design
2- Peptide synthesis
3- Receptor binding assays
4- *In vitro* studies
5- *In vivo* studies
6- Signal transduction study

Structural biology Study

1- Expression, and Refolding of VEGFR1D2
2- Direct receptor binding assay by Microscale Thermophoresis (MST)

Sequence Alignments of VEGFA& VEGFB and VGB design

3V2A: VEGFA/VEGFR2
1FLT: VEGFA/VEGFR1D2
2XAC: VEGFB/VEGFR1D2



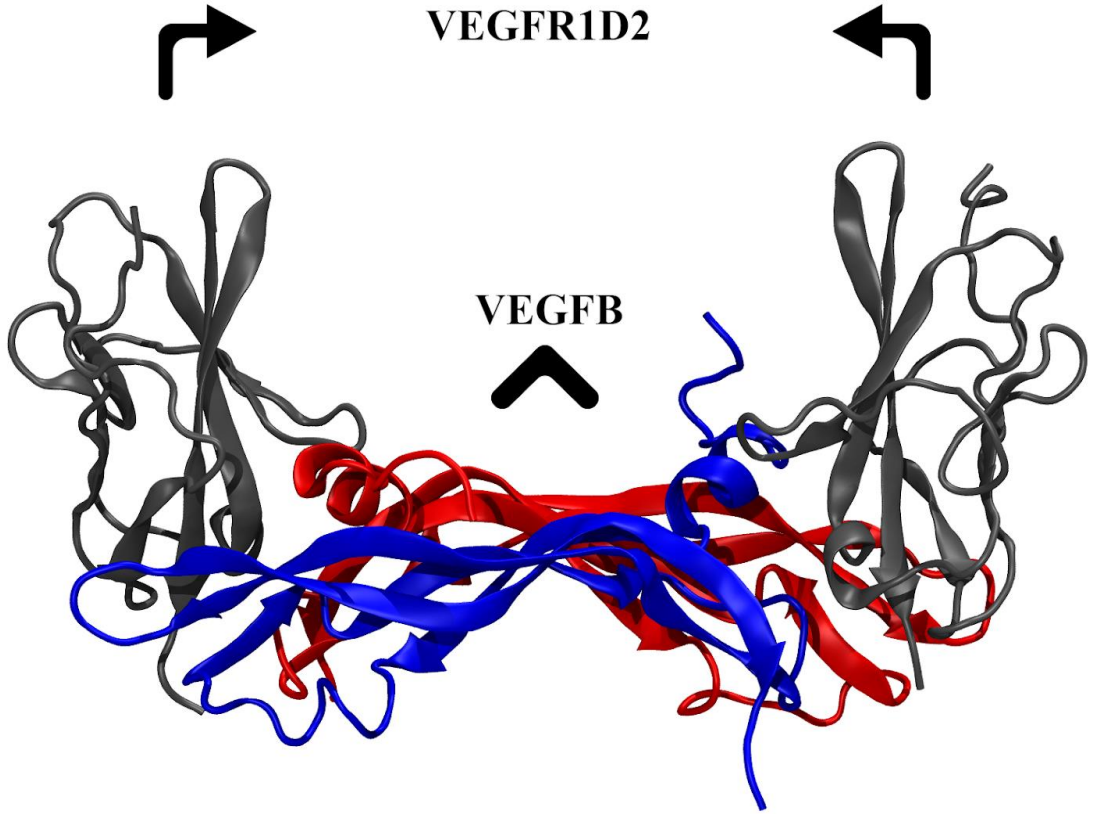
Leppänen VM, Alitalo K. (2009) Proc Natl Acad Sci. 107: 2425–2430.

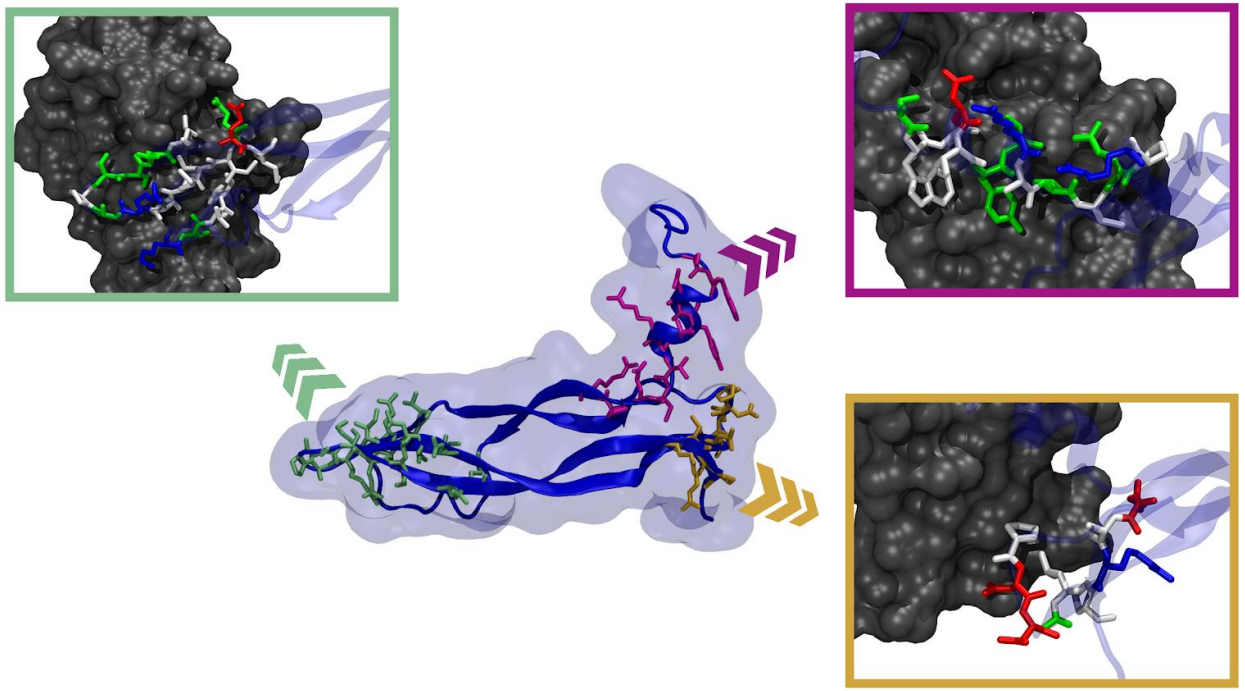
Iyer Sh, Darley PI, Acharya KR. (2010) J. Biol.Chem. 285: 23779–23789.

Brozzo MS, Bjelic´S, Kisko K. (2012) Blood. 119:1781-1788.

Wiesmann Ch, Fuh G, Christinger HW. (1997) Cell. 91:695–704.

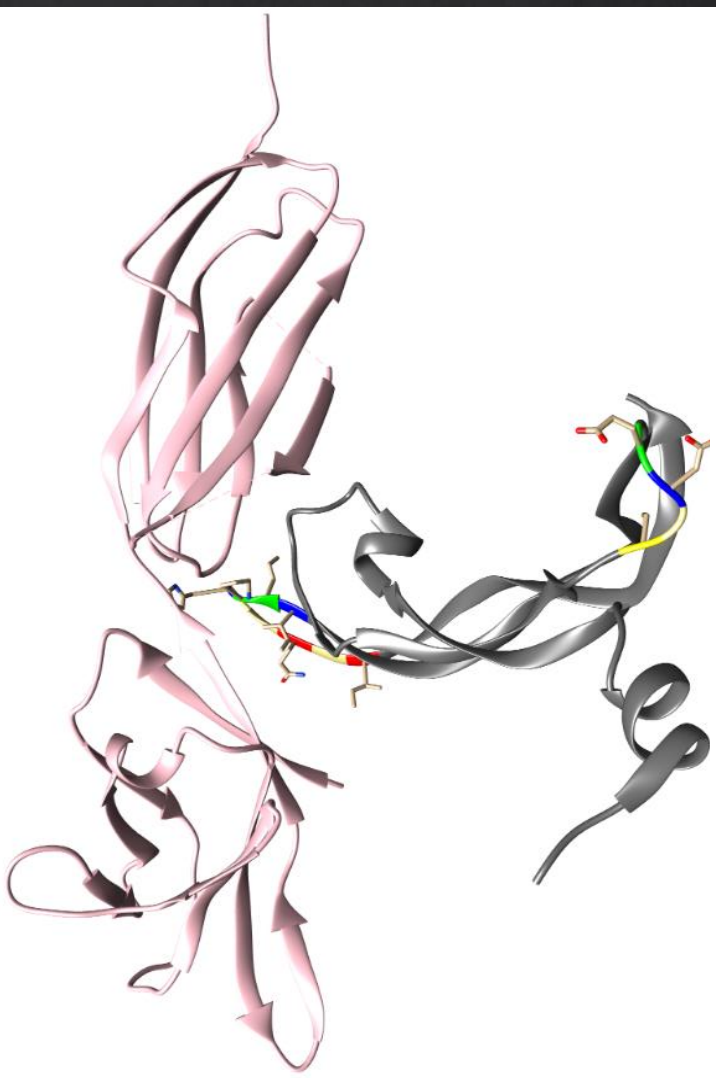
VEGFB/VEGFR1D2: 2XAC

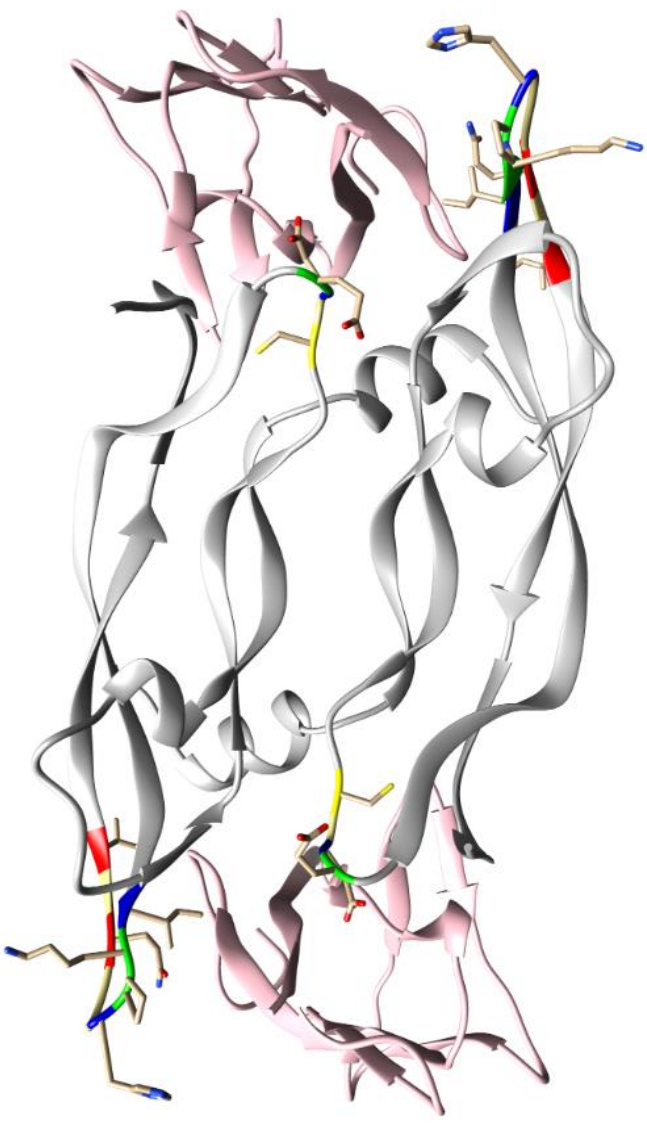


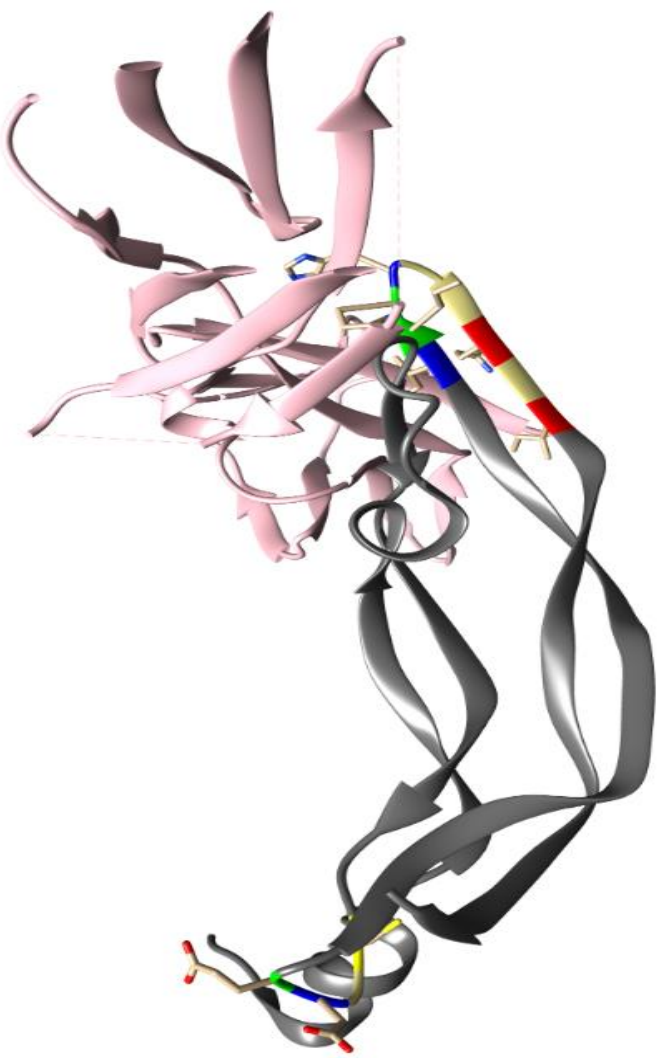


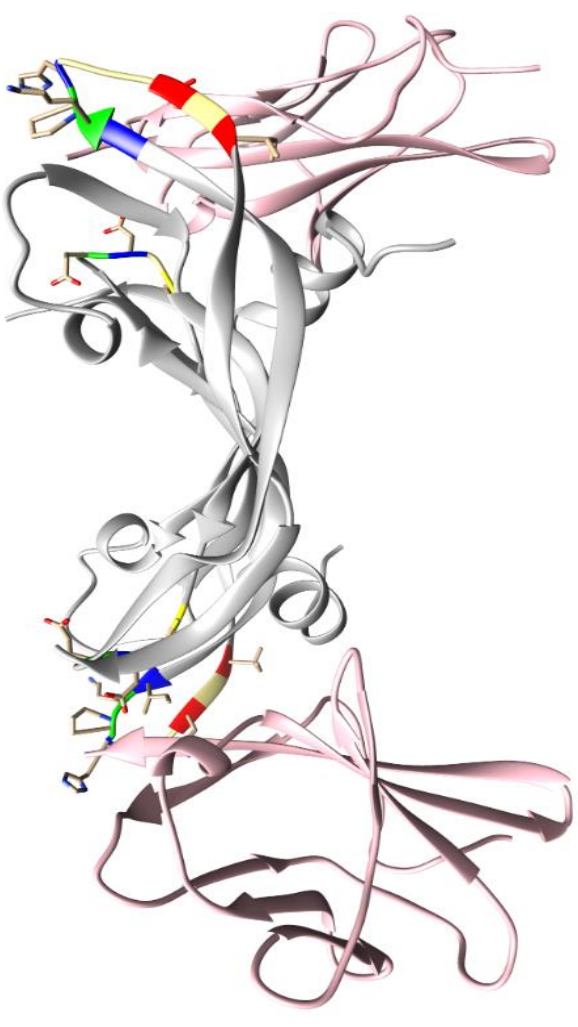
3V2A

1FLT

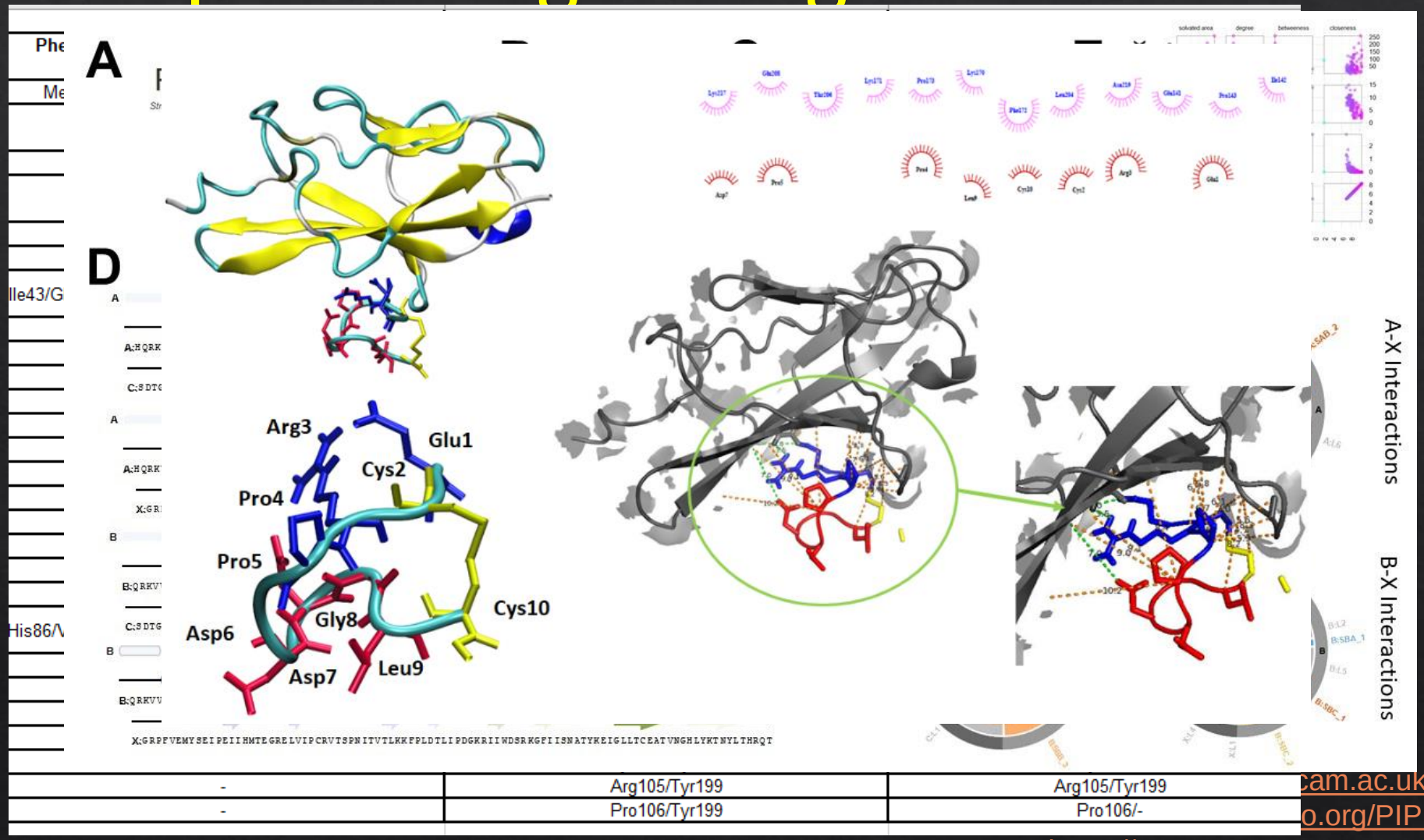








Peptide Design Using Protein Contact Atlas



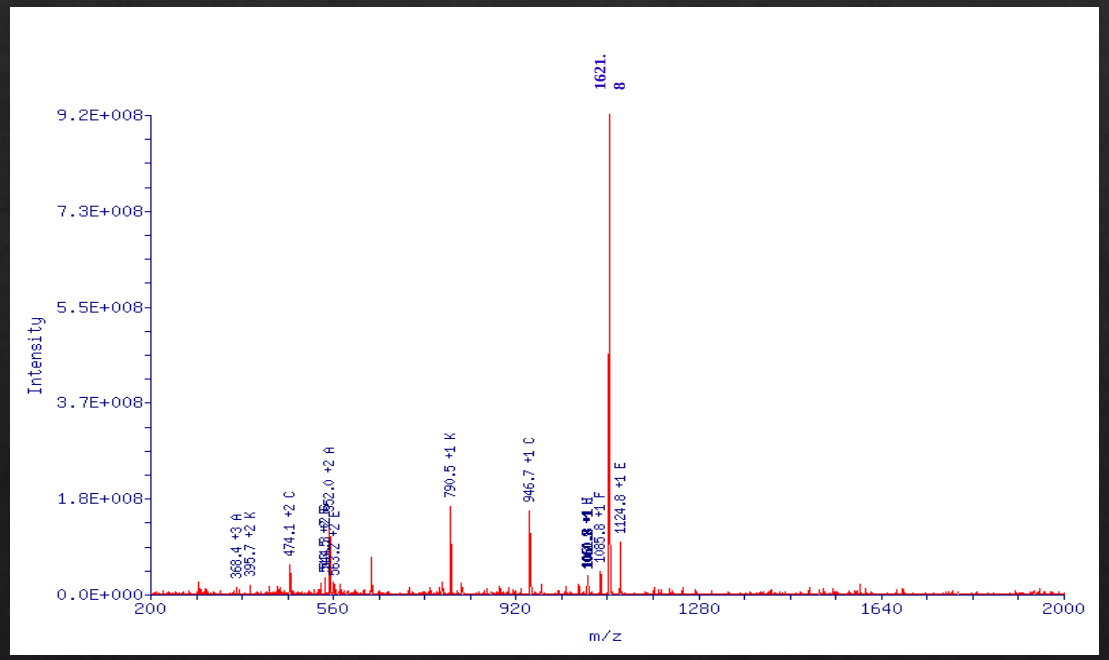
-	Arg105/Tyr199	Arg105/Tyr199
-	Pro106/Tyr199	Pro106/-

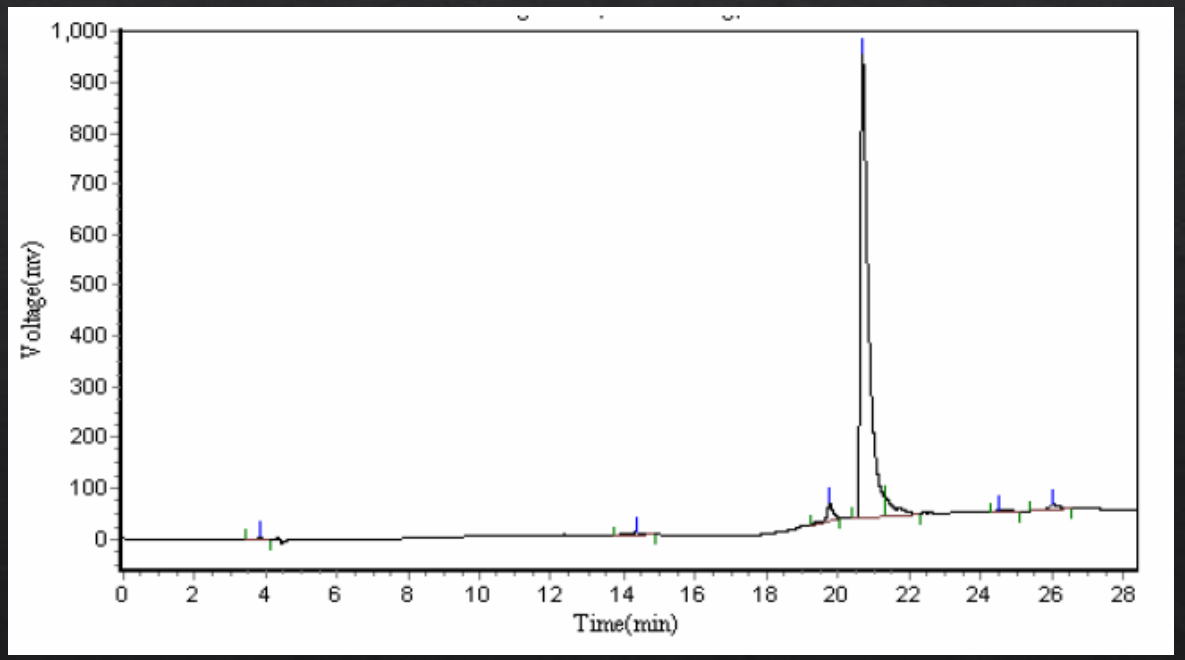
Sadremomtaz A. Groves M, Asghari SM. Signal Transduct Target Ther. 5,76-79 (2020).
Sadremomtaz A. *et al*. Biochim Biophys Acta Gen Subj. 1862, 2688–2700 (2018).
Sadremomtaz A. *et al*. J Recept Signal Transduct Res. 38, 432-441 (2018).

cam.ac.uk/pca/
o.org/PIPRED
https://www.rosettacommons.org/docs/latest/application_documentation/design/design-applications
http://www.yasara.org/

Peptide synthesize

The peptide was synthesized and purified by high-performance liquid chromatography to a purity of 90%, analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDITOF), and confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (Shine Gene Biotechnologies, Shanghai, China). To confirm the presence of disulfide bond in the synthesized peptide, Ellman assay was performed.





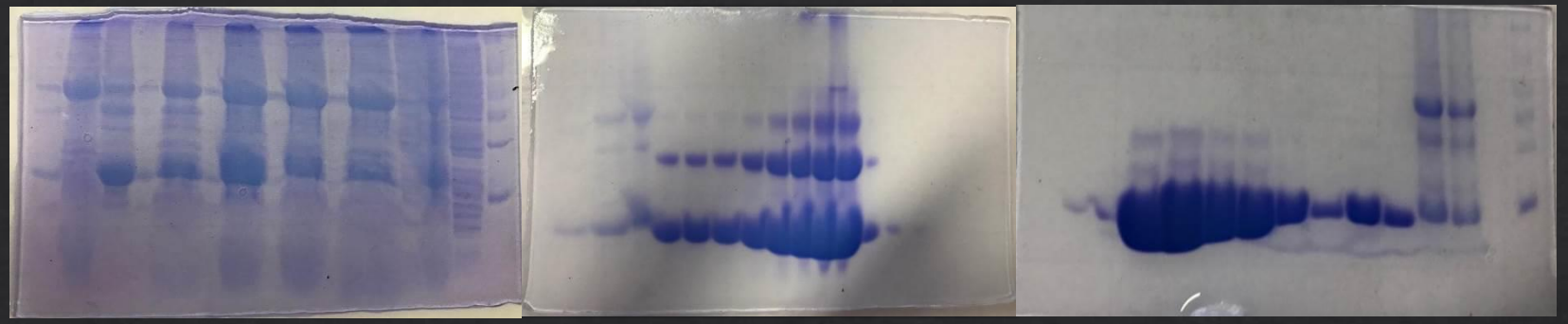
Expression, and Refolding of VEGFR1D2

A significant amount of His-tagged VEGFR1D2 was obtained in the inclusion bodies (Figure 1a). The protein was purified on a Ni^{+2}-NTA agarose resin and refolded in 50 mM Tris-HCl, 10 mM imidazole, 300 mM NaCl, pH 8 with decreasing concentrations of urea (8, 6, 4, 2, 1, 0.5,0.25 and 0 M, Fig 1b,c (with and without BME, respectively). Then, it was eluted increasing imidazole concentration from 10 to 300 mM.

The His-tagged VEGFR1D2 was dialyzed overnight in 50 mM Tris-HCl, pH 7.0, and NaCl 250 mM at 4℃. Glutathione (3 mM reduced/0.3 mM oxidized) was added and incubated for 3h, VEGFR1D2 was purified by size exclusion chromatography by using a S75 column (GE Healthcare) equilibrated in 50 mM Tris-HCl and 250 mM NaCl, pH 7. Finally, it was concentrated until 2mg/ml by the Amicon Ultra system (3000 MWCO, Millipore). (Figure 1d).

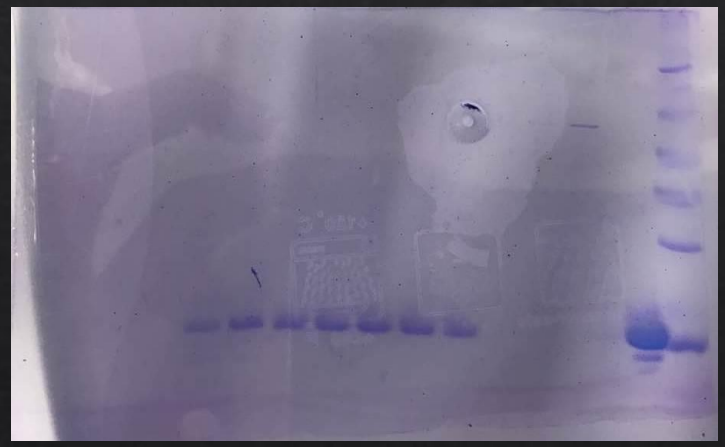
Fig 1. Ni^{+2}-NTA chromatography

a b c



Size Exclusion chromatography

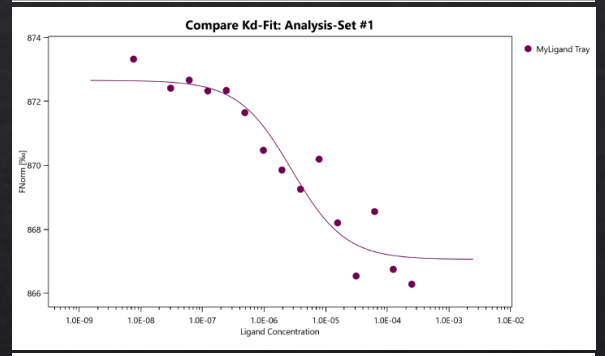
d

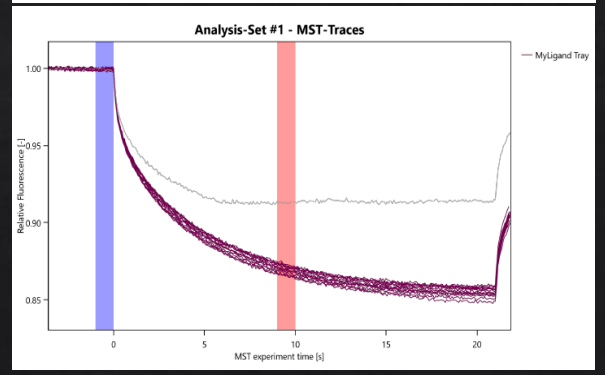


MST

0.25mM-25nM-VGB3-VEGFR1D2

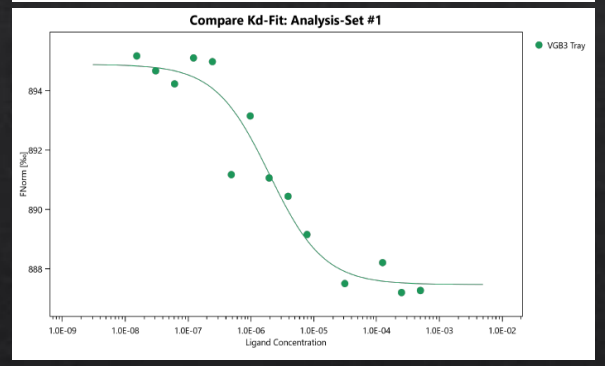
Name:	MyLigand Tray
Graph Color:	
Target Name:	VEGFR
Target Concentration:	25 nM
Ligand Name:	MyLigand
Ligand Concentration:	0.25 mM to 7.63E-06 mM
n:	1
Comments:	
Excitation Power:	80%
MST Power:	40%
Temperature:	22.0°C
Kd:	2.8155E-06
Kd Confidence:	
Response Amplitude:	5.6022328
TargetConc:	1.2533E-12
Unbound:	872.66
Bound:	867.05
Std. Error of Regression:	0.83392148
Reduced x^2:	
Signal to Noise:	7.5788563

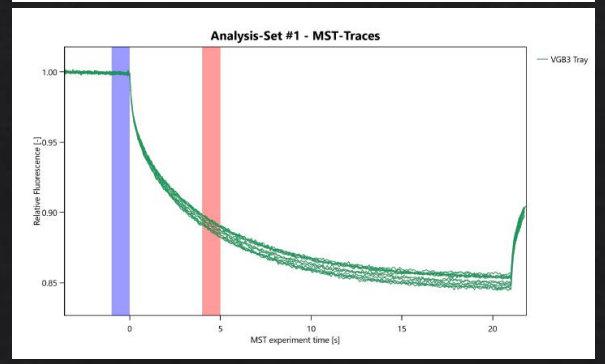




0.5mM-25nM-VGB3-VEGFR1D2

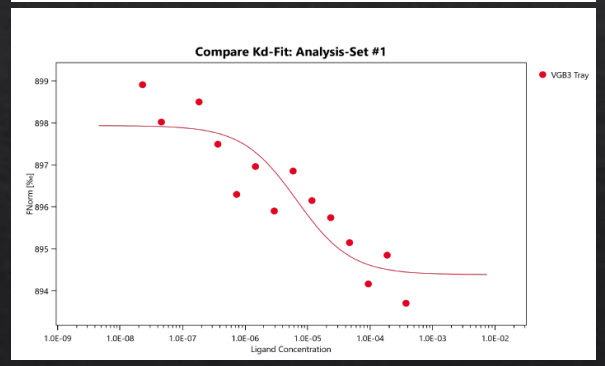
Name:	VGB3 Tray
Graph Color:	
Target Name:	VEGFR
Target Concentration:	25 nM
Ligand Name:	VGB3
Ligand Concentration:	0.5 mM to 1.53E-05 mM
n:	1
Comments:	
Excitation Power:	80%
MST Power:	40%
Temperature:	22.0°C
Kd:	1.9625E-06
Kd Confidence:	± 6.9258E-07
Response Amplitude:	7.4197821
TargetConc:	2.5E-08[Fixed]
Unbound:	894.89
Bound:	887.47
Std. Error of Regression:	0.85933111
Reduced x^2:	
Signal to Noise:	9.3865489

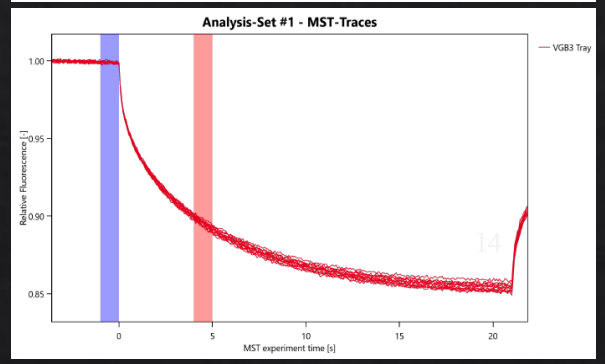




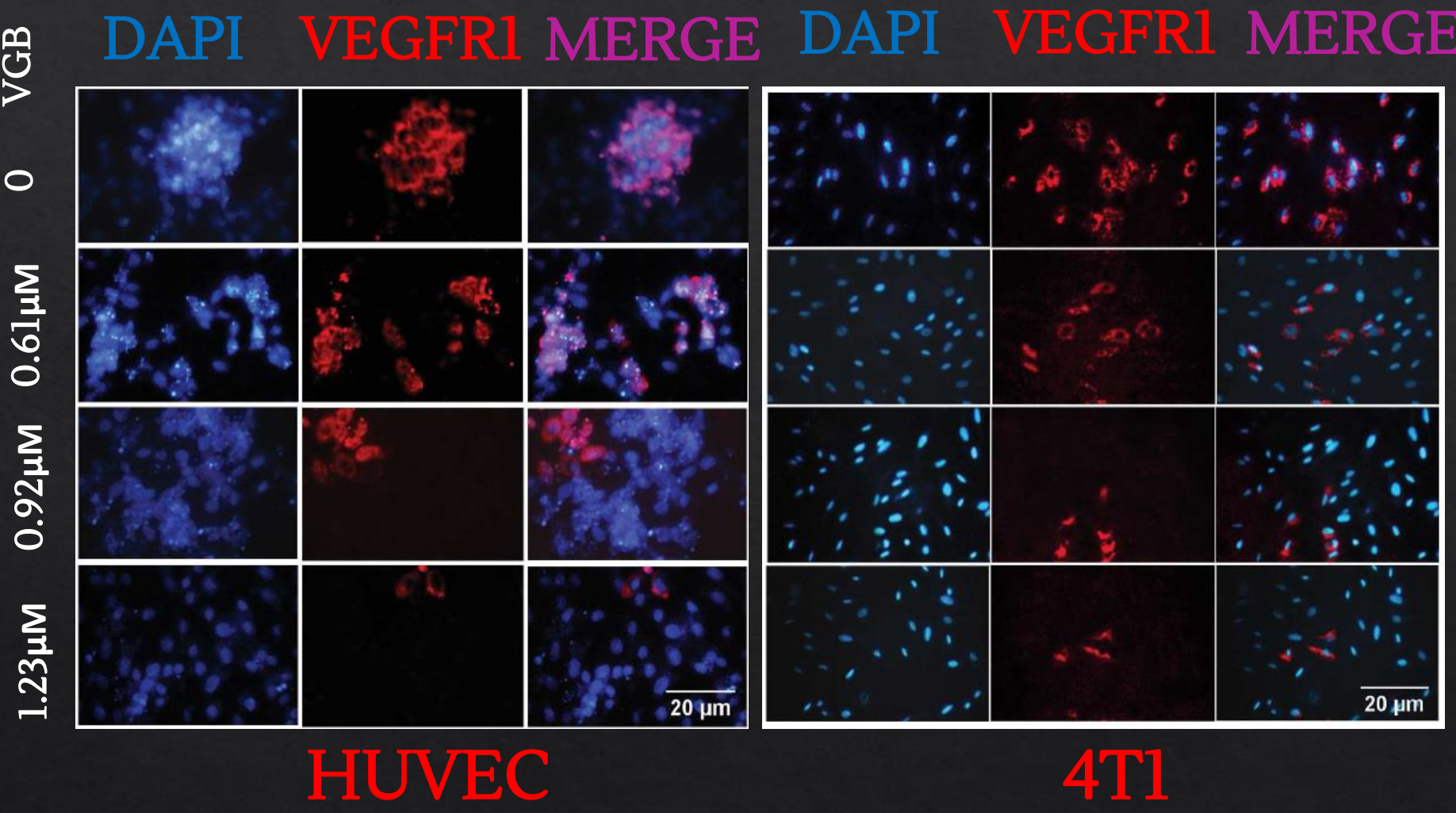
0.75mM-25nM-VGB3-VEGFR1D2

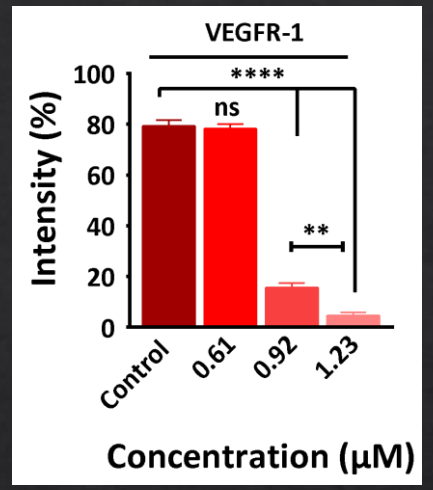
Name:	VGB3 Tray
Graph Color:	
Target Name:	VEGFR
Target Concentration:	25 nM
Ligand Name:	VGB3
Ligand Concentration:	0.375 mM to 2.29E-05 mM
n:	1
Comments:	
Excitation Power:	100%
MST Power:	40%
Temperature:	22.0°C
Kd:	6.6504E-06
Kd Confidence:	± 4.4766E-06
Response Amplitude:	3.5478575
TargetConc:	2.5E-08[Fixed]
Unbound:	897.94
Bound:	894.39
Std. Error of Regression:	0.73994131
Reduced x^2:	
Signal to Noise:	5.2124778



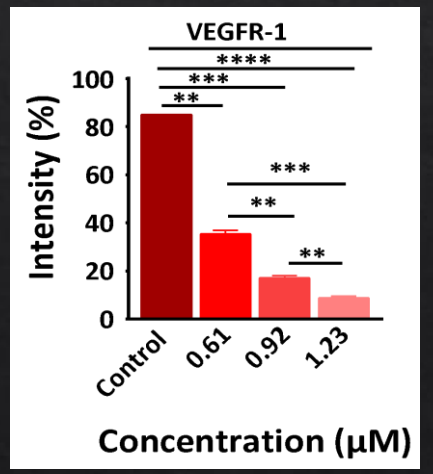


Binding to VEGFR1





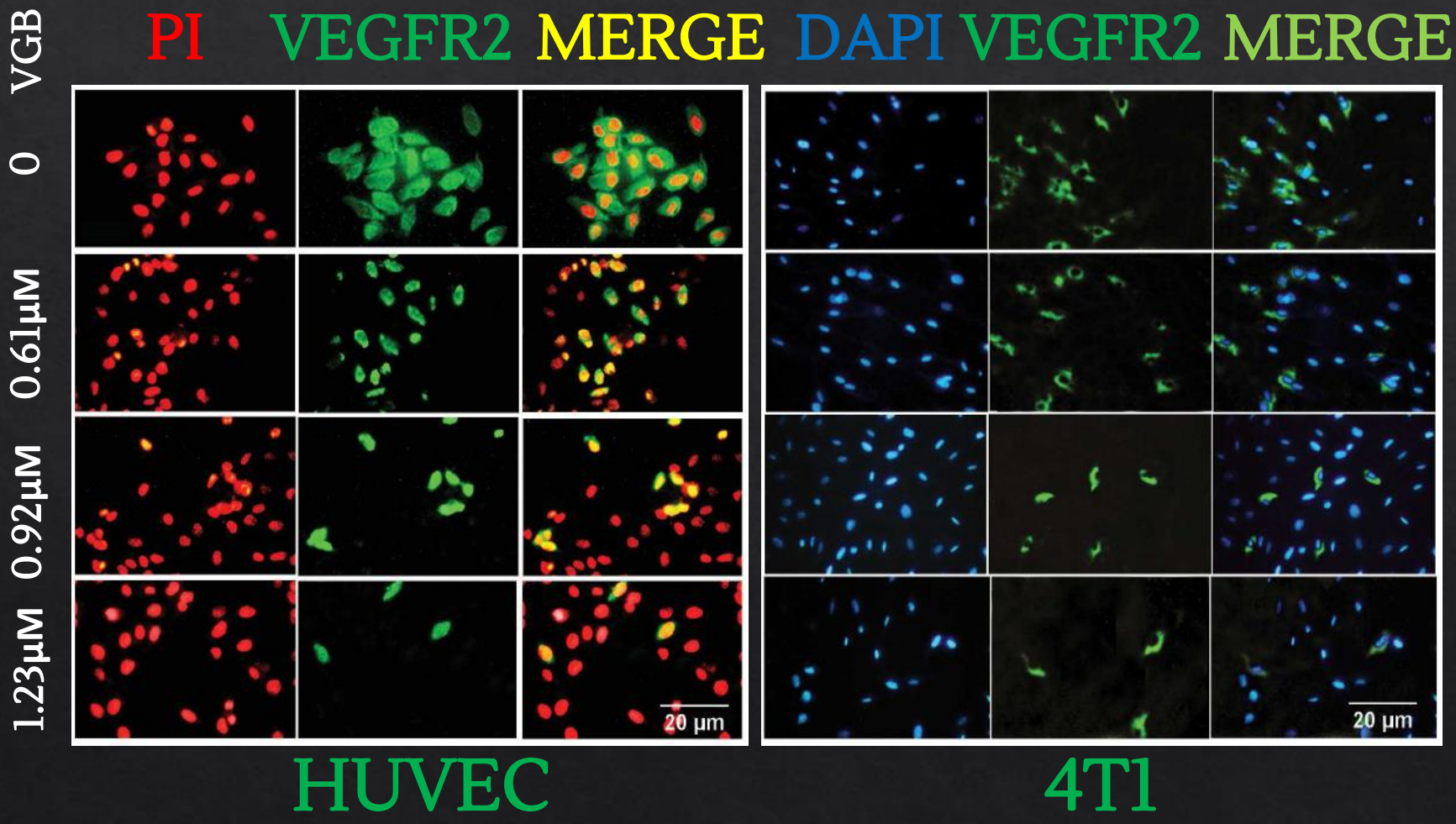
HUVEC

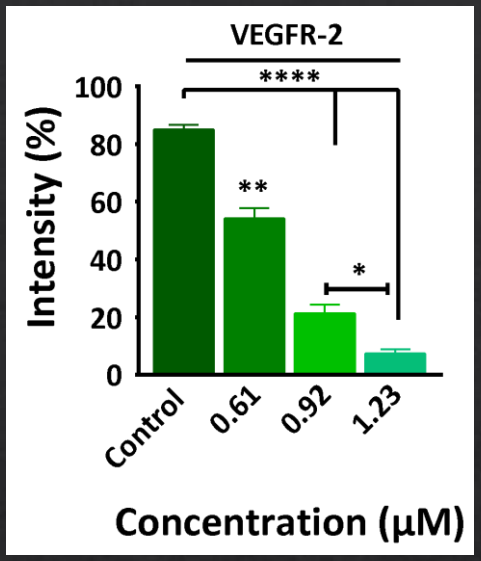


4T1

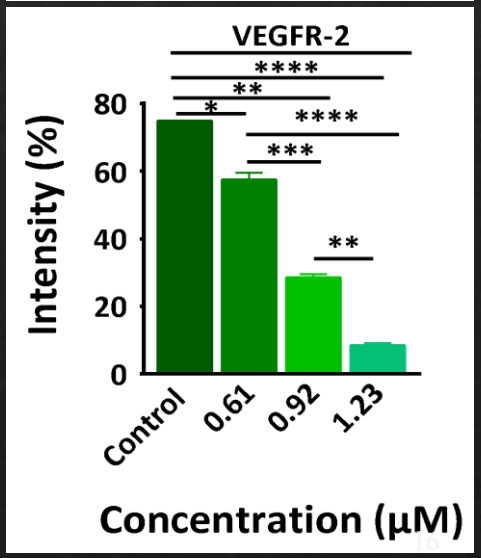
Cell were cultured (5000 cells/well), After 24 h, the media were changed to DMEM without FBS, cell were treated (except the control) with 0.61–1.23 μM VGB, cells were fixed and blocked, followed by adding of PE-conjugated anti-VEGFR1 (5 μg·mL−1) antibody after another round of washes with PBS. Cells were then mounted with DAPI.

Binding to VEGFR2



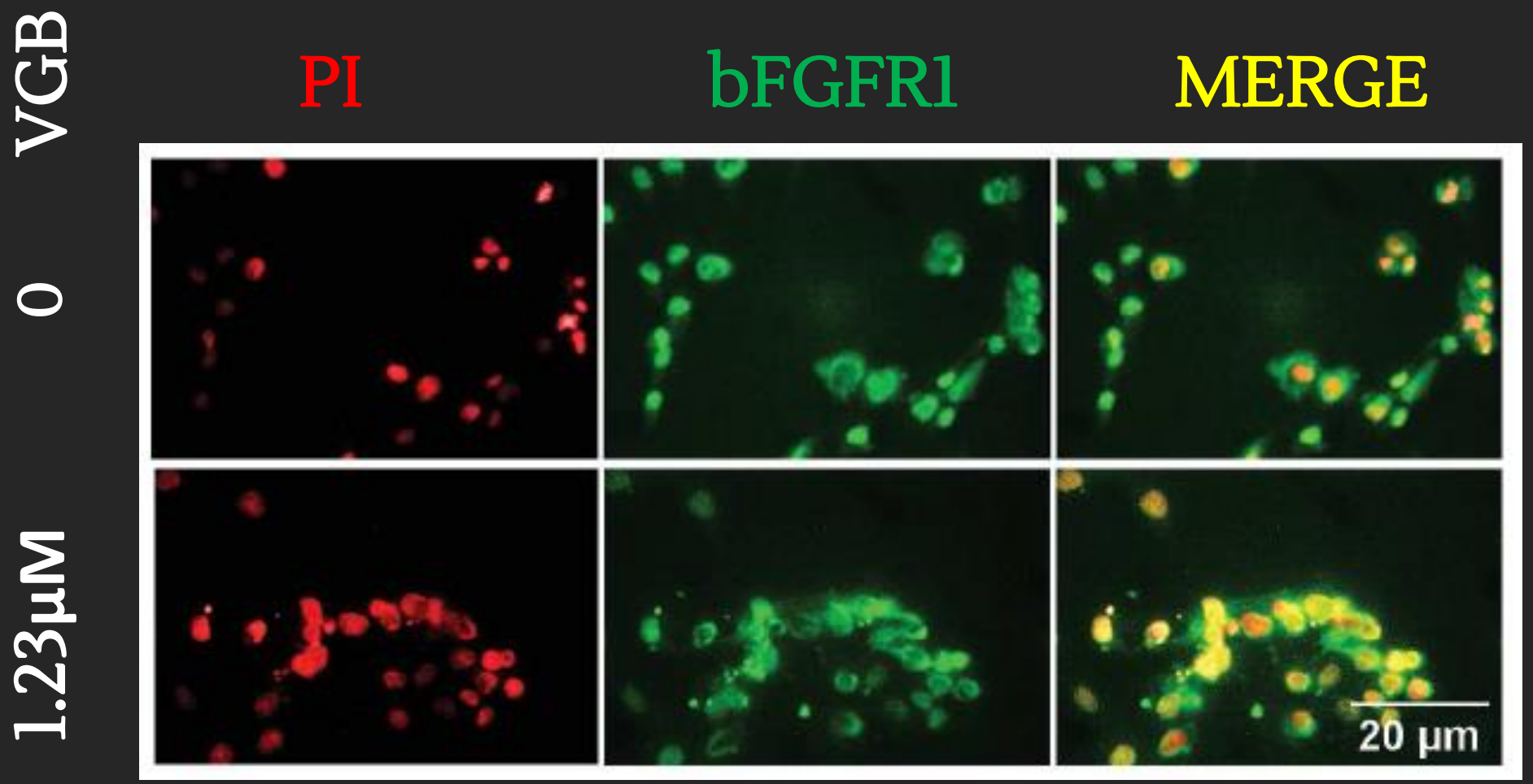


HUVEC

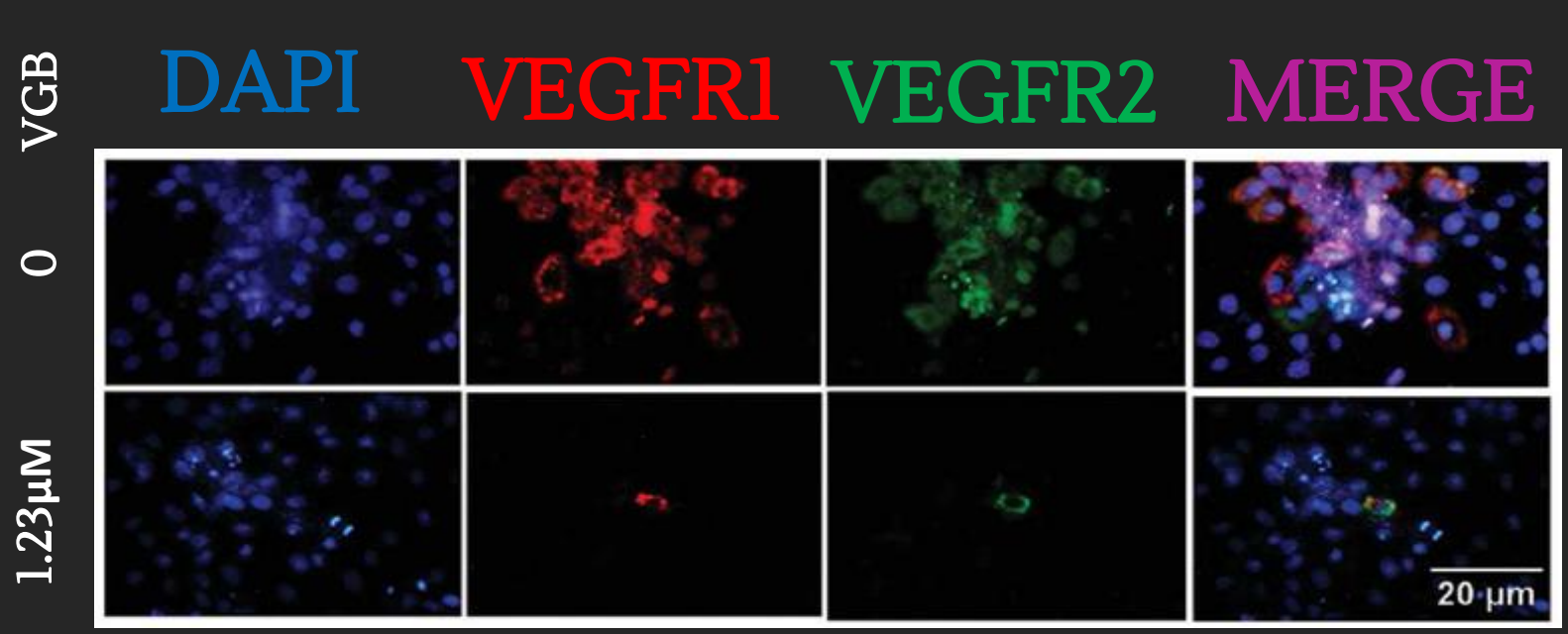


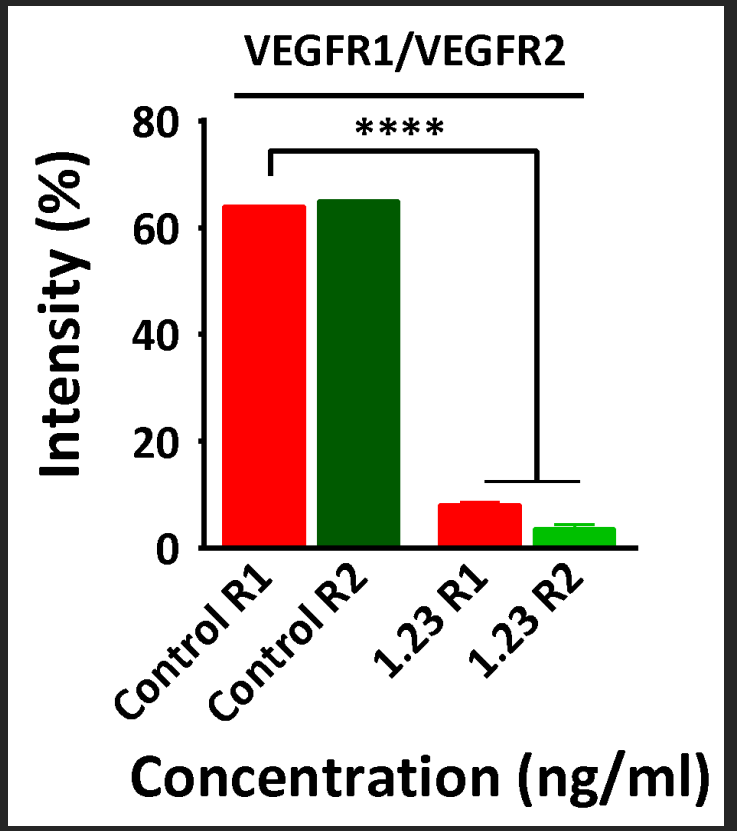
4T1

Receptor binding specificity



Co-localization of VEGFR1/VEGFR2





Studies on difference hallmarks of angiogenesis

Downstream signaling pathways

Antagonistic peptide (VGB) of VEGFA & VEGFB can able to block downstream signaling pathways of VEGFR1 & VEGFR2

Proliferation: Inhibiting activation of MAPK/ERK1/2, PI3K/AKT, NF-kB, c-Myc

Migration: Downregulation on expression of; FAK/Paxilin, PAK/Cofilin, PI3K/AKT, MAPK/ERK1/2, EMT/E-cadherin

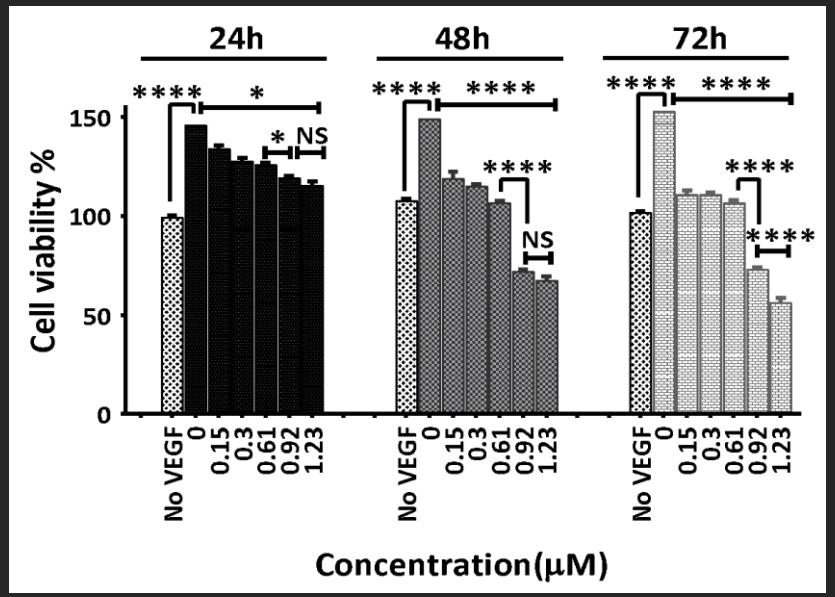
Metastasis: Downregulating on expression of; PI3K/AKT, EMT/E-cadherin, MMP-9, c-Myc, NF-kB

Apoptosis: Downregulation of PI3K/AKT/p53 (proapoptotic), Bcl-2 (antiapoptotic)

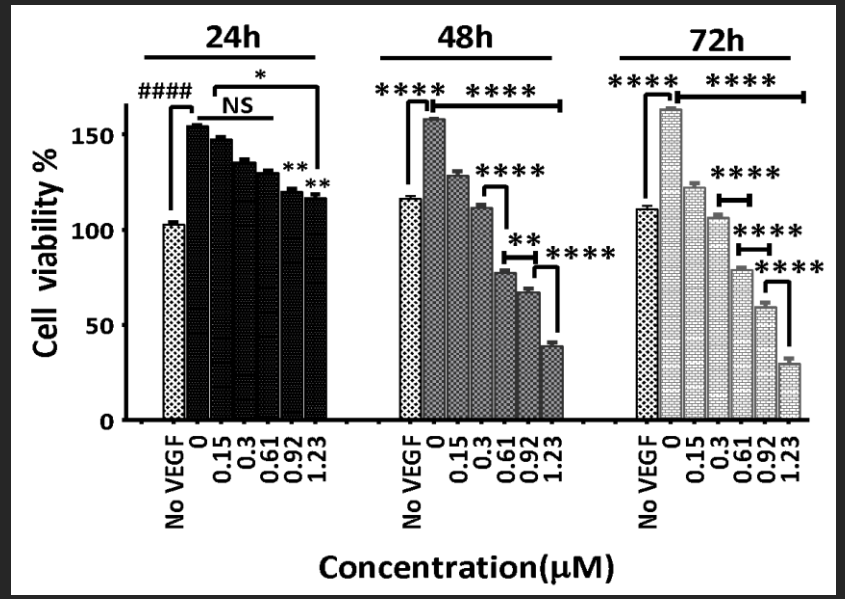
Cell survival: Downregulation of PI3K/AKT/NF-kB

Inhibition of tumor cell proliferation

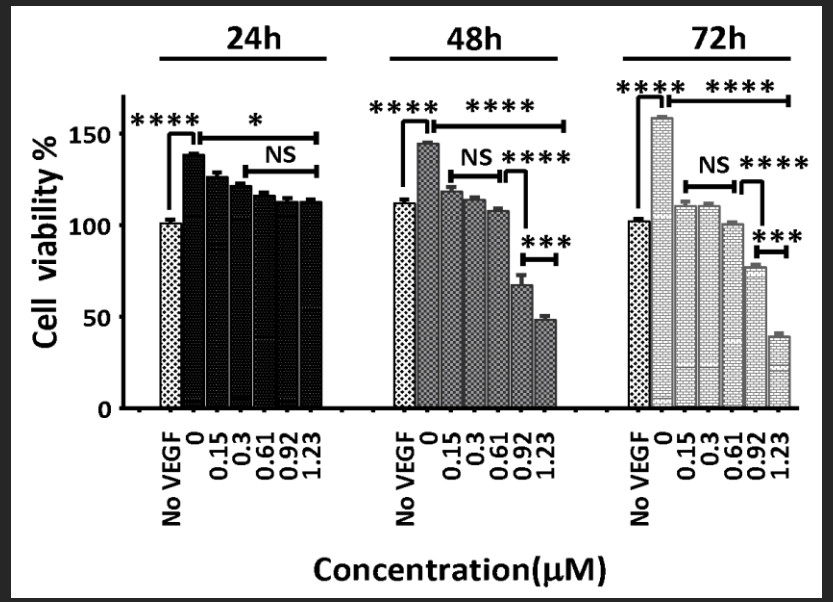
HUVEC



4T1



U87



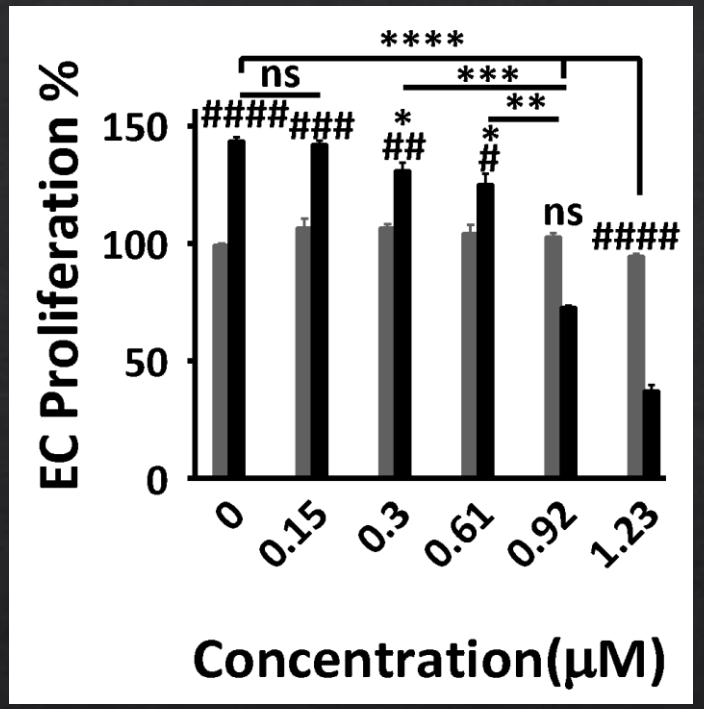
IC50= 0.92μM

IC50= 0.61μM

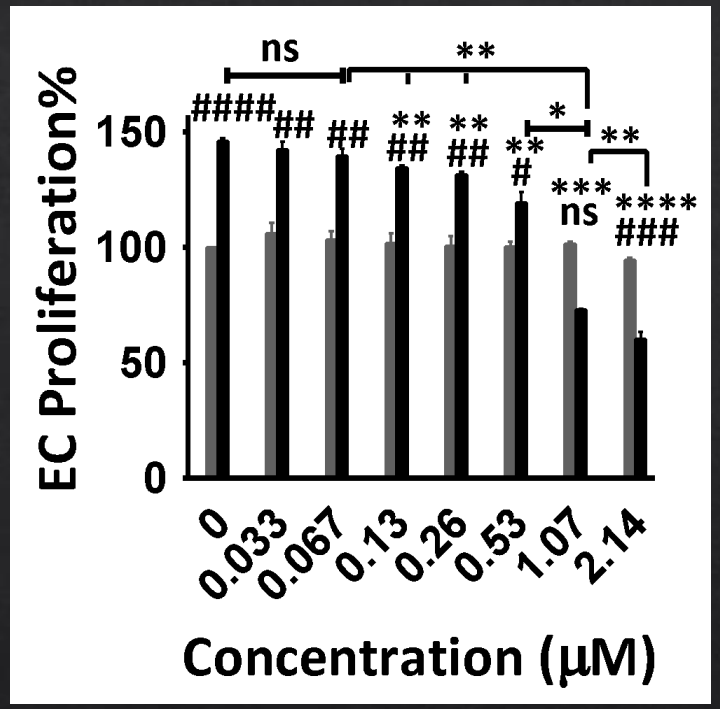
IC50= 0.92μM

The effects of VGB on the proliferation of HUVEC, 4 T1, and U87 cells were quantified after 24, 48, and 72 h by MTT assay. 2×10^3 HUVECs were added to each well of a plate in DMEM media containing 5% FBS and incubated overnight at 37 °C. Cells were then transferred to serum-free medium containing 0.2 $\mu g \cdot mL^{-1}$ VEGF-A at 37 °C, 5%CO_2. cells were treated with varying concentrations of VGB (0.15–1.23 μM) for comparison with untreated control.

VGB *vs* bevacizumab

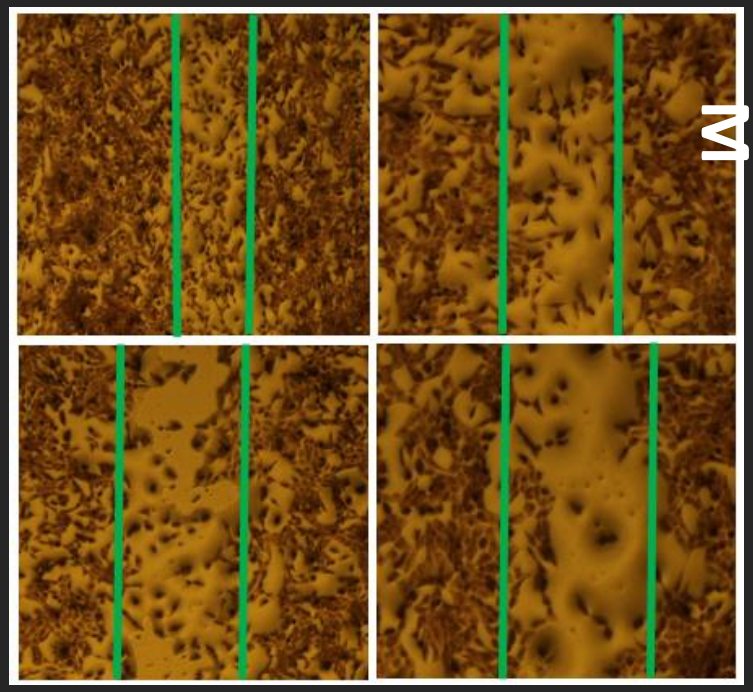


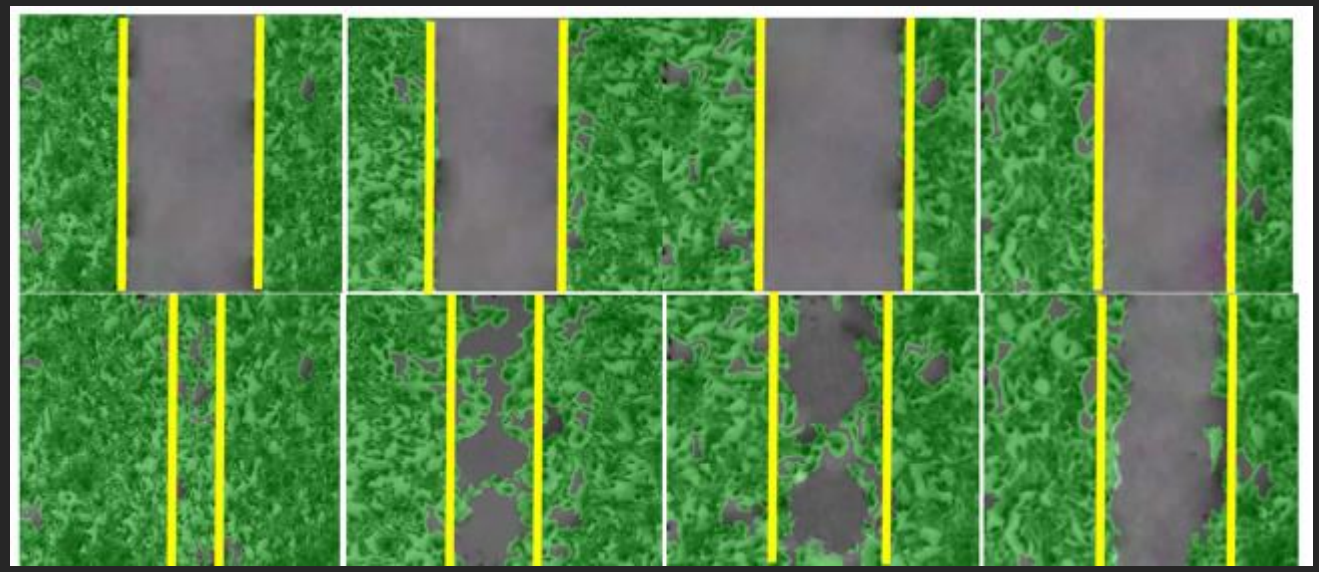
IC50= 0.92μM

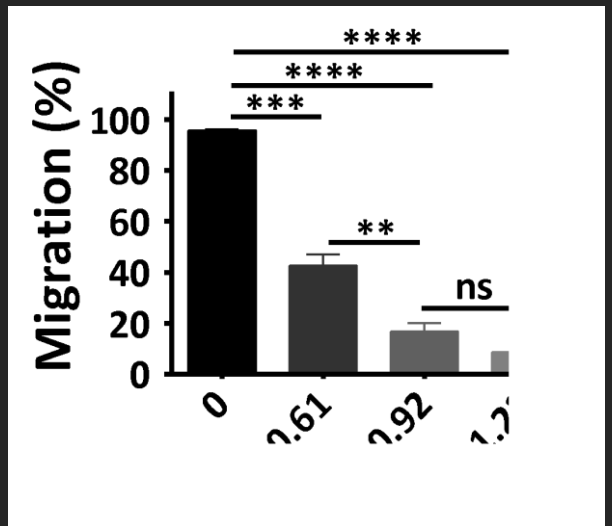


IC50= 1.07μM

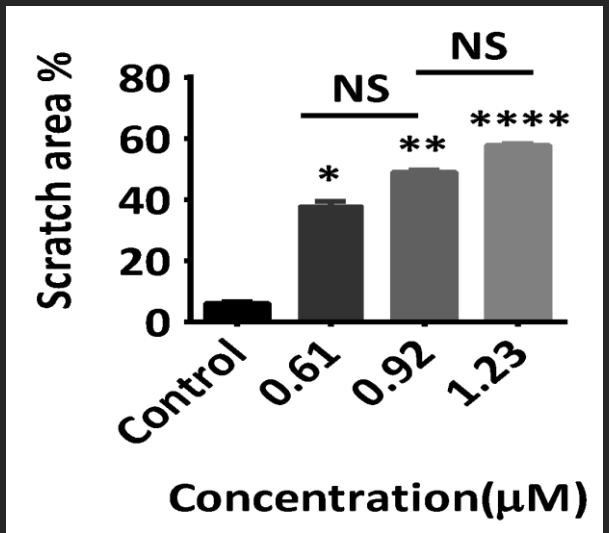
Inhibition of HUVECs migration



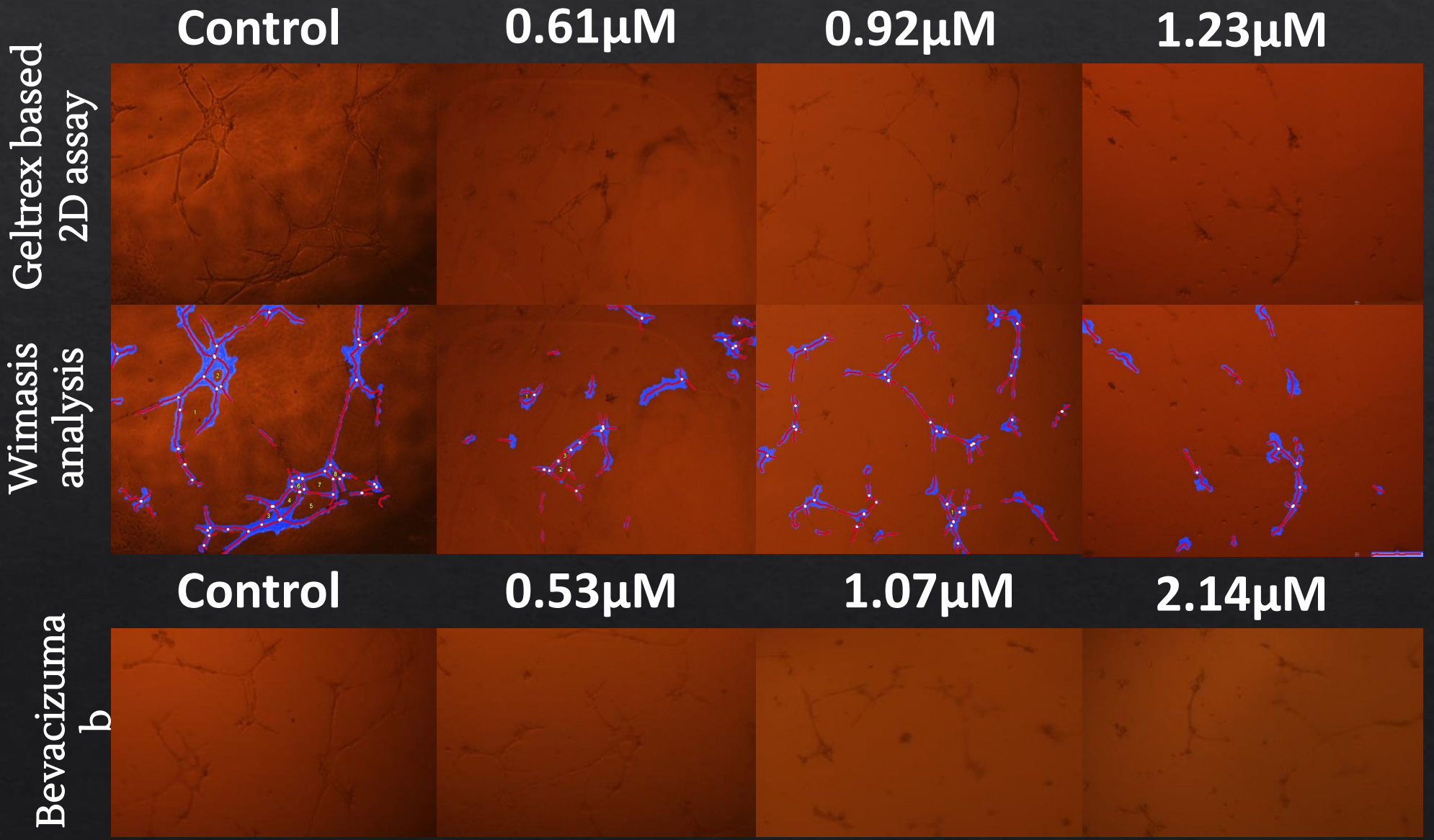




After 24 h, a wound was generated with a 1000 µL pipette tip, and the cells were washed gently with cold PBS 1× and rinsed after washing with serum-free medium twice, after which the medium was changed for one containing 0.2 µg·mL−1 VEGFA. Serially diluted concentrations of the peptide were added to each well for comparison with untreated
control. Wounded area: (1 - (wound area at 24 h / wound area at 0 h))×100.



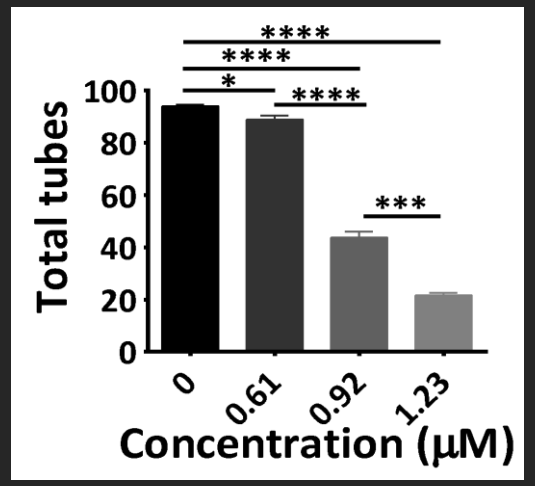
2D angiogenesis assay using Geltrex matrix

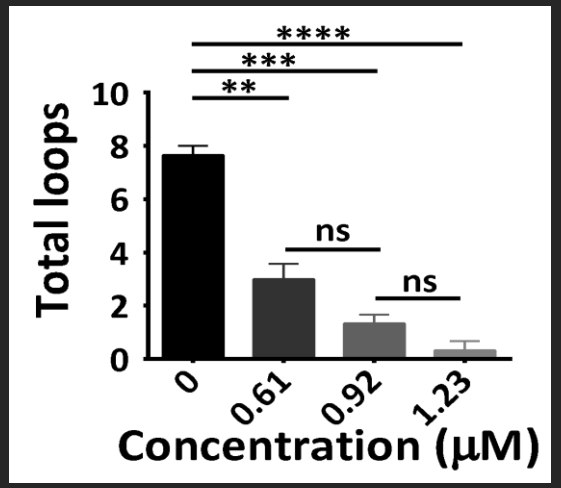


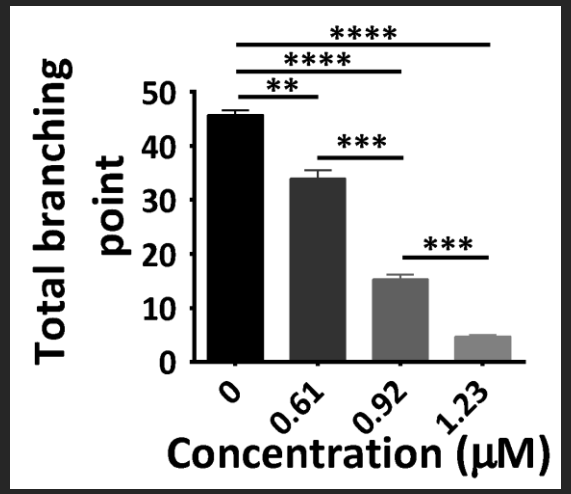
Wells of the tube formation assay were precoated with growth factor-reduced basal membrane extract (Geltrex™). HUVECs were then suspended in M200 or basal media free-serum containing 0.2 µg·mL^{-1} VEGF-A, and seeded at 14×10^3 cells/200 µL per well. Cells were treated with VGB (0.61–1.23 µM) followed by incubation for 14–18 h.

Wimasis analysis

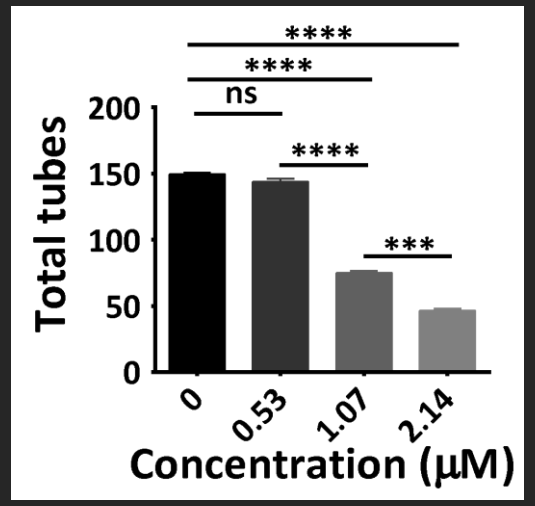
VGB treatment

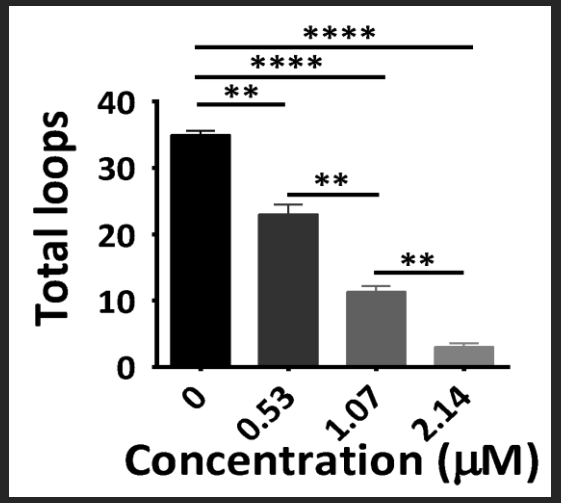


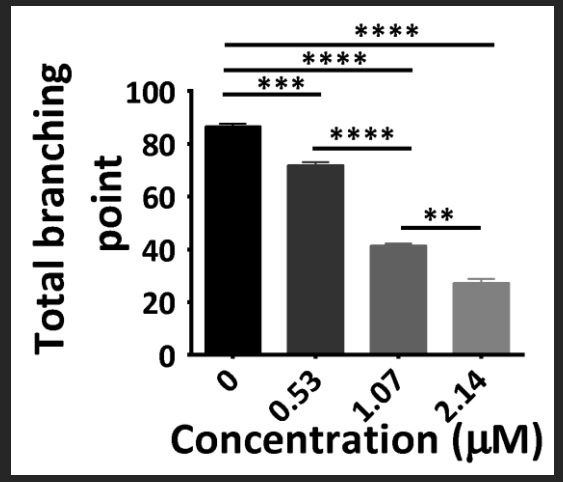




Bevacizumab treatment





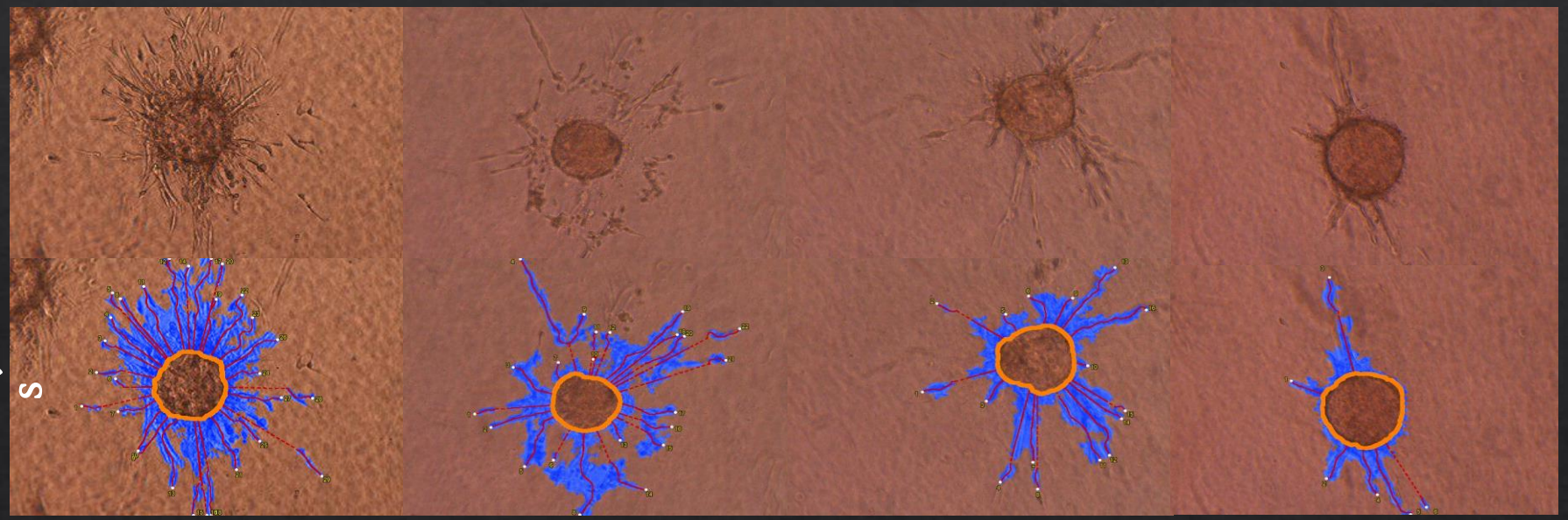


3D angiogenesis assay using Collagen based cytodex

Collagene based cytodex assay

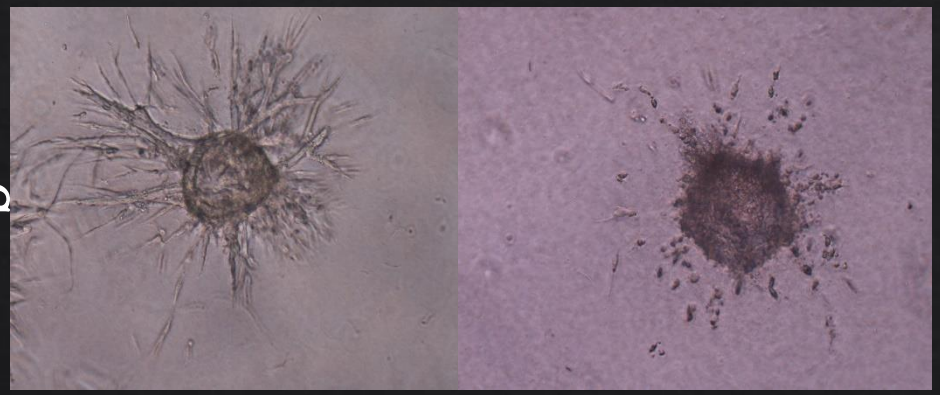
Wimasis analysis

Control | 0.61µM | 0.92µM | 1.23µM



Bevacizumab

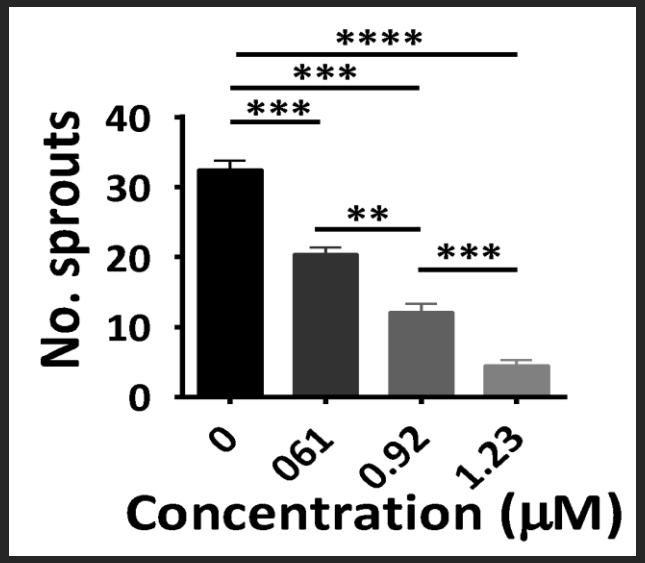
Control | 2.14µM

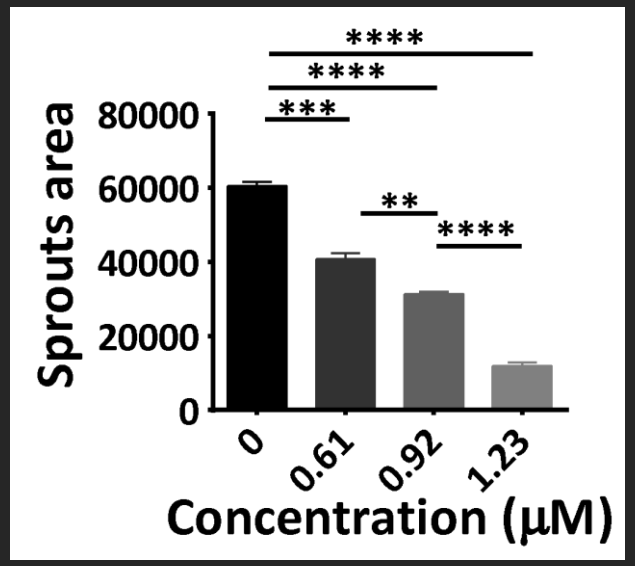


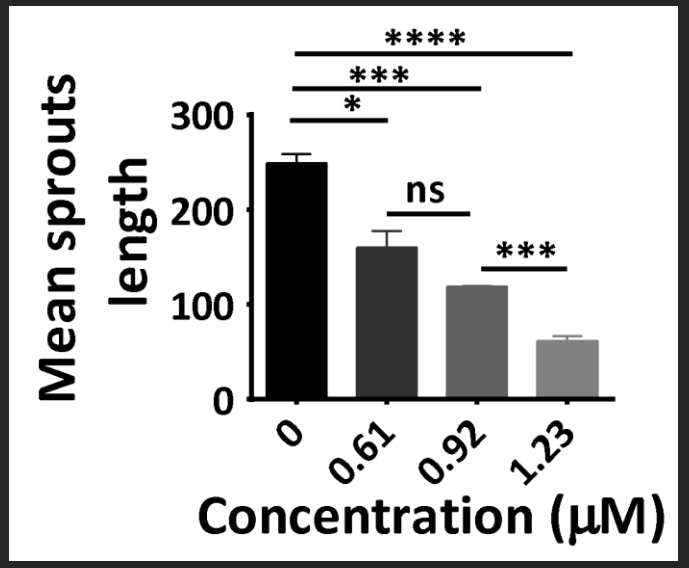
HUVECs were allowed to attach to Cytodex microcarrier beads by incubation in DMEM medium with 10% FBS for 4 h at 37 °C in 5% CO_2 (60 µL of 50 g/mL Cytodex beads were coated with 400 µl of HUVECs). The HUVEC-coated beads were embedded in a collagen matrix under sodium bicarbonate conditions, distributed in 96-well plates (100 µl/well), and placed in a 37 °C, 5% CO_2 incubator for 30 min. The media were renewed by stimulator of angiogenesis VEGF-A (0.2 µg·mL−1) without FBS, after which cells were treated with serially diluted concentrations of VGB (0.61–1.23 µM) and incubated for 72 h at 37 °C, 5% CO_2.

Wimasis analysis

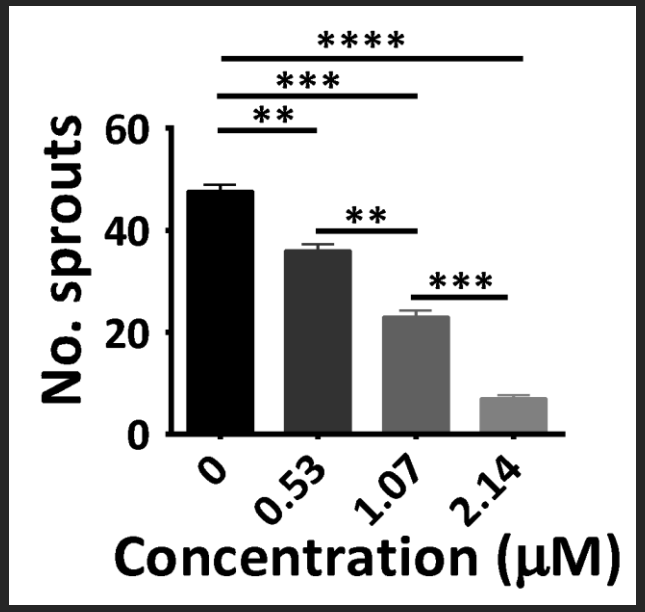
VGB treatment

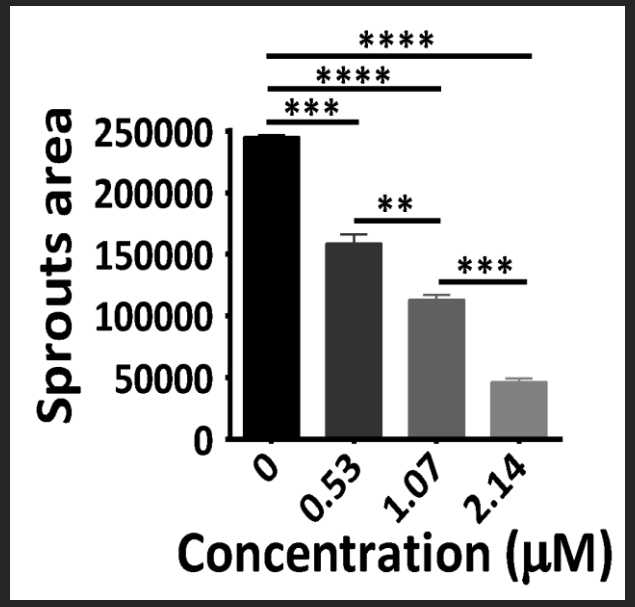


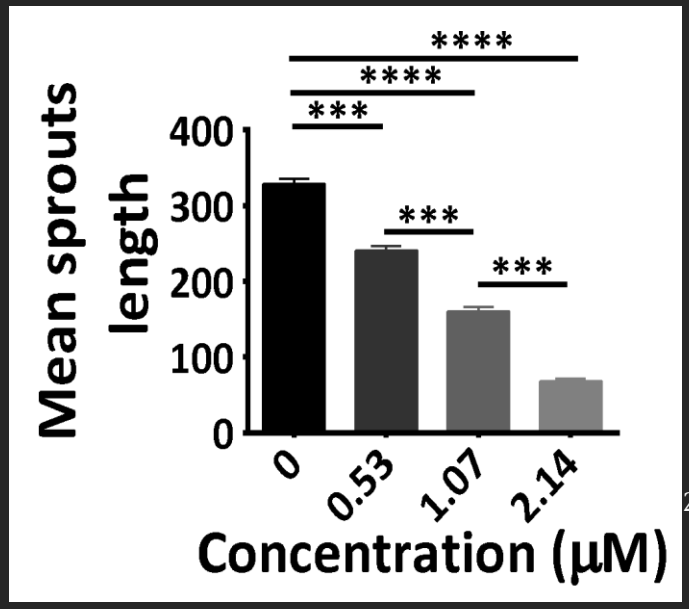




Bevacizumab treatment

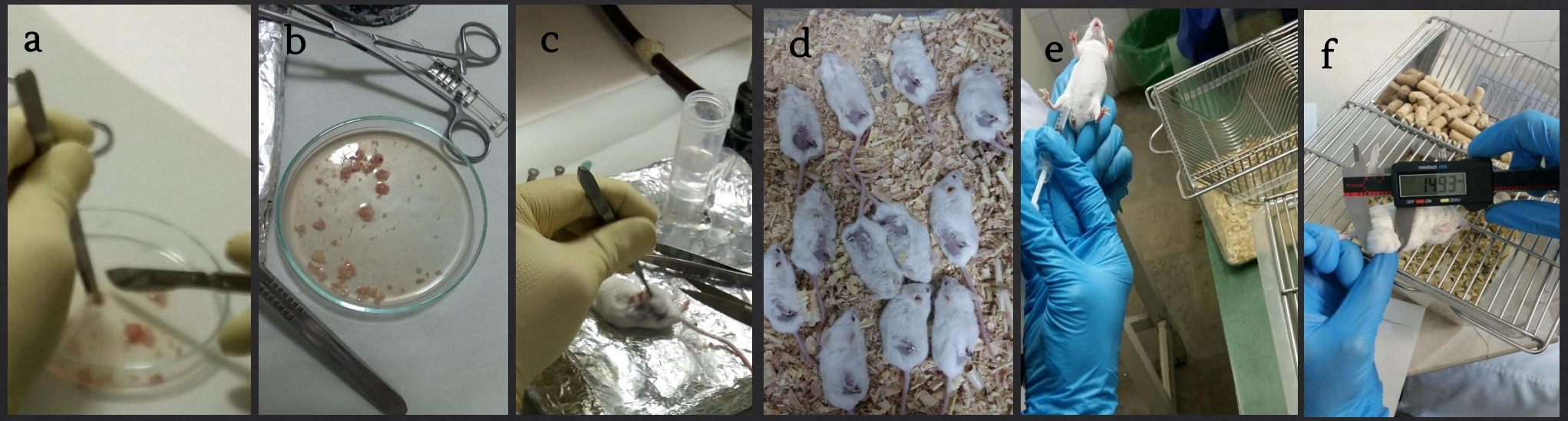






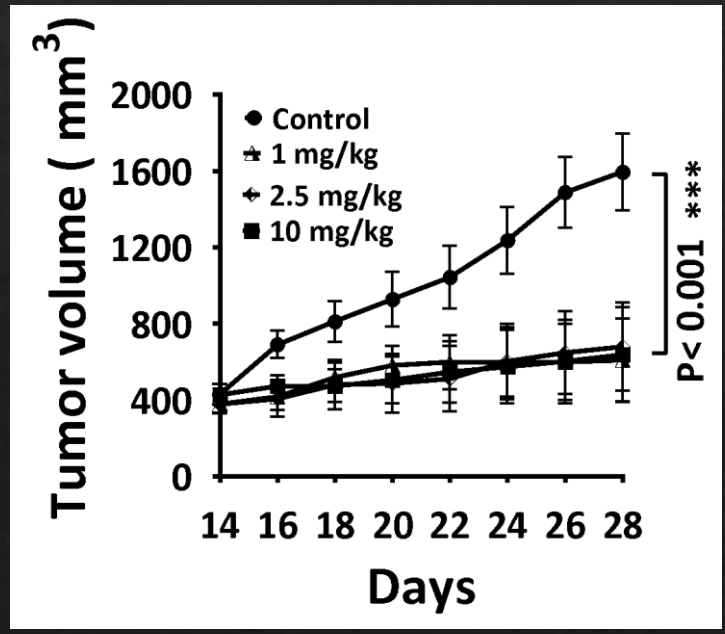
In vivo and Signal transduction studies

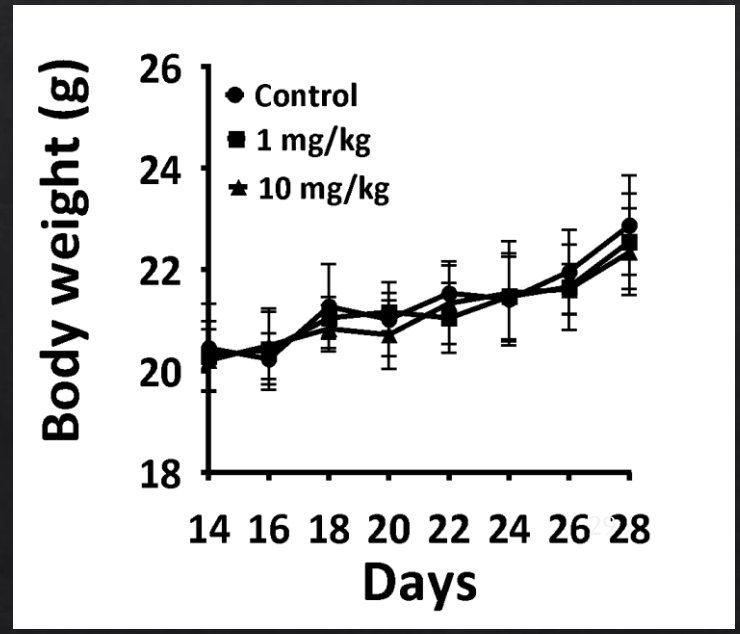
Regression of 4T1 murine mammary carcinoma tumor



Tumor cells (4 T1; 1×10^6 cells/500 µl or 1×10^5 cells/50 µl) were injected subcutaneously into the right flanks of mice (n=3–5). To generate the metastatic model, 4 T1 tumor models were sterilized, excised from the breast cancer bearing BALB/c mice, cut into pieces of<0.3 cm^3, and subcutaneously implanted into the animals' right flanks under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia.

Animals carrying tumors of size ~400mm^3 were randomized to groups (n=6). The treatment groups received 1, 2.5, and 10 mg/kg i.p. of the peptide daily and control group received PBS i.p for two weeks. The tumor volume was measured every two days by a digital Vernier caliper (Mitutoyo, Japan), using the following formula: $v=a^2\times b\times0.52$.

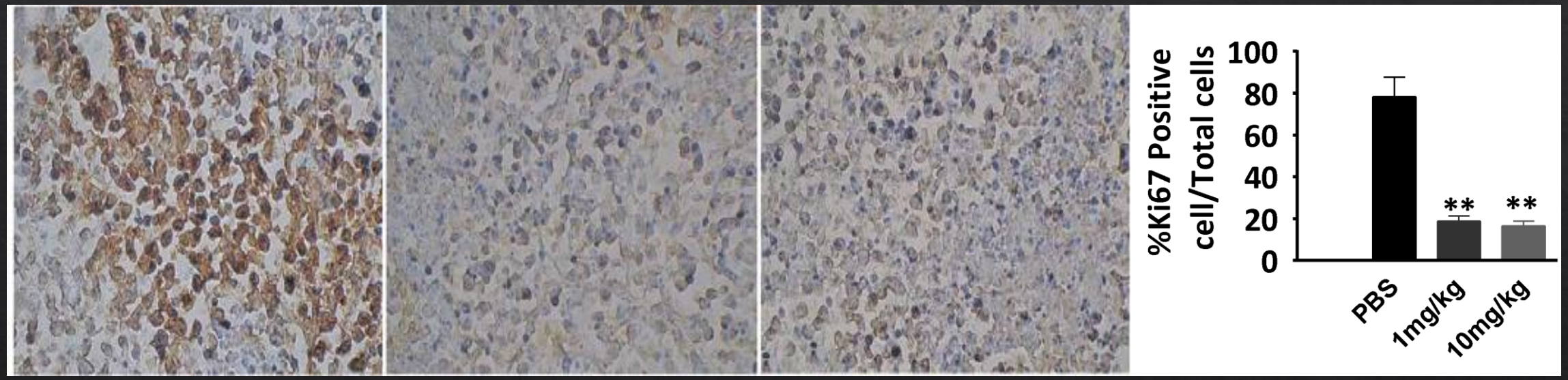




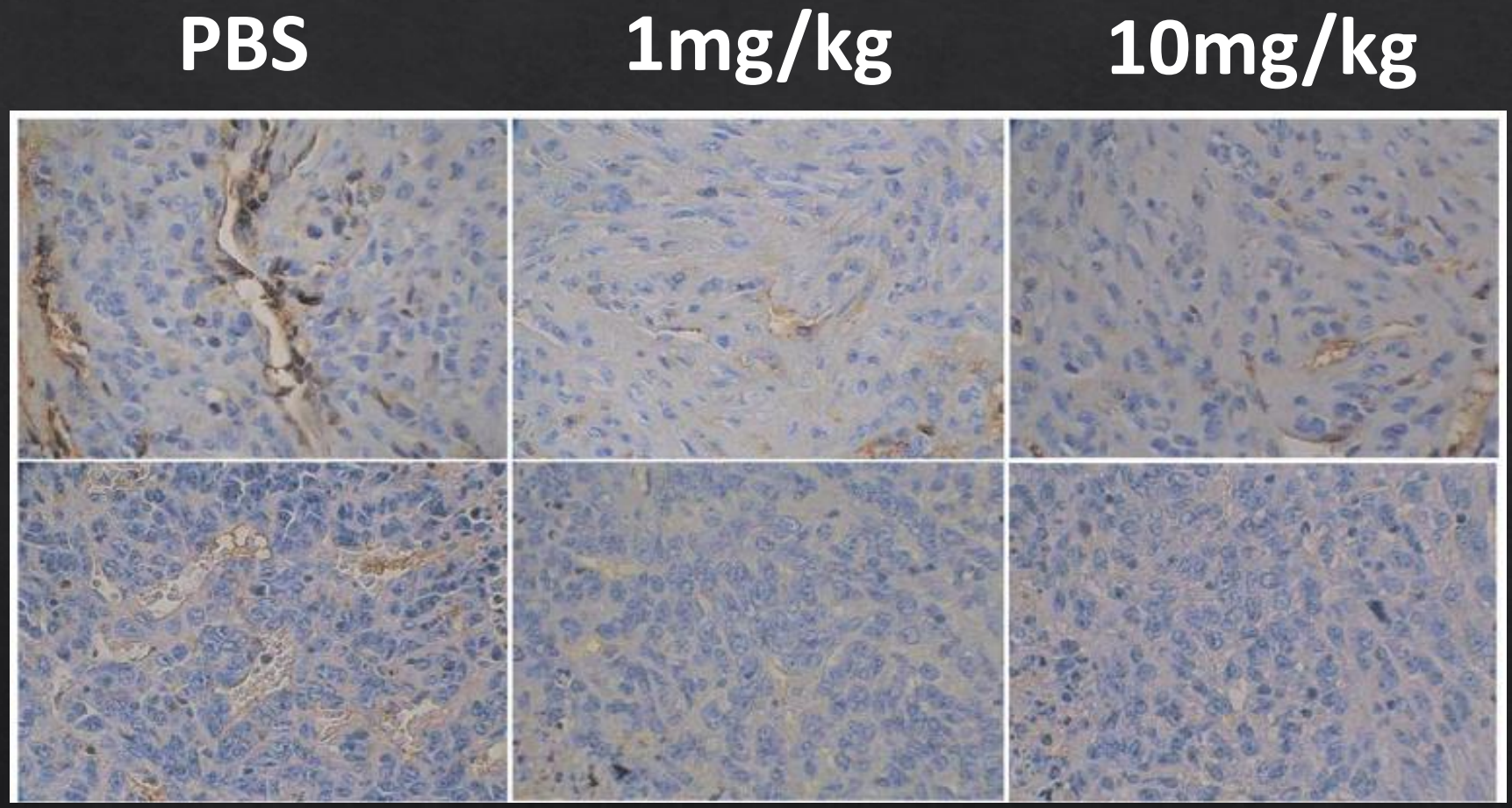
VGB effects on tumor cell proliferation

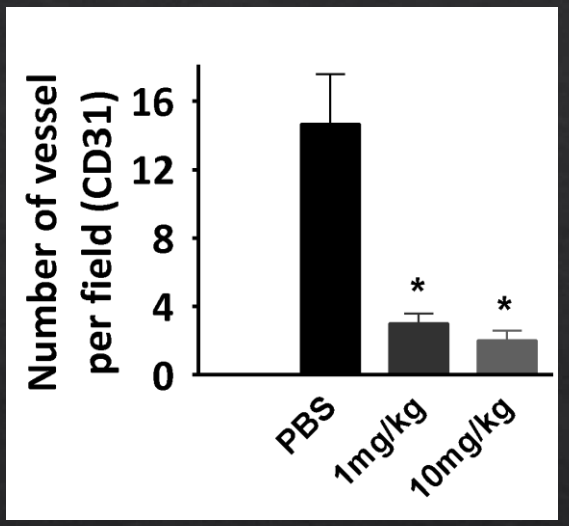
PBS 1mg/kg 10mg/kg

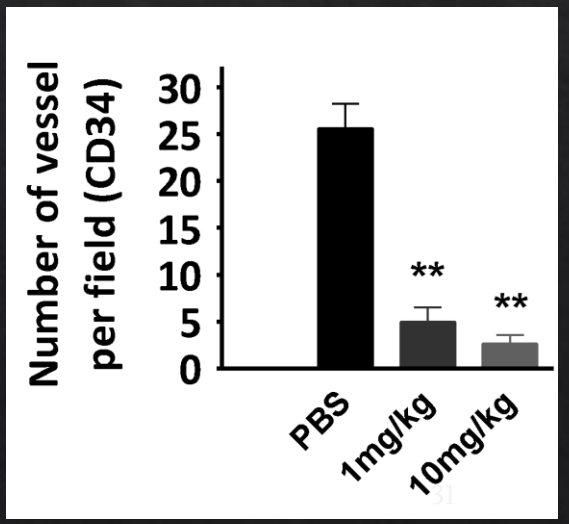
Ki67



VGB effects on tumor microvascular







VGB effects on apoptosis induction

PBS 1mg/kg 10mg/kg

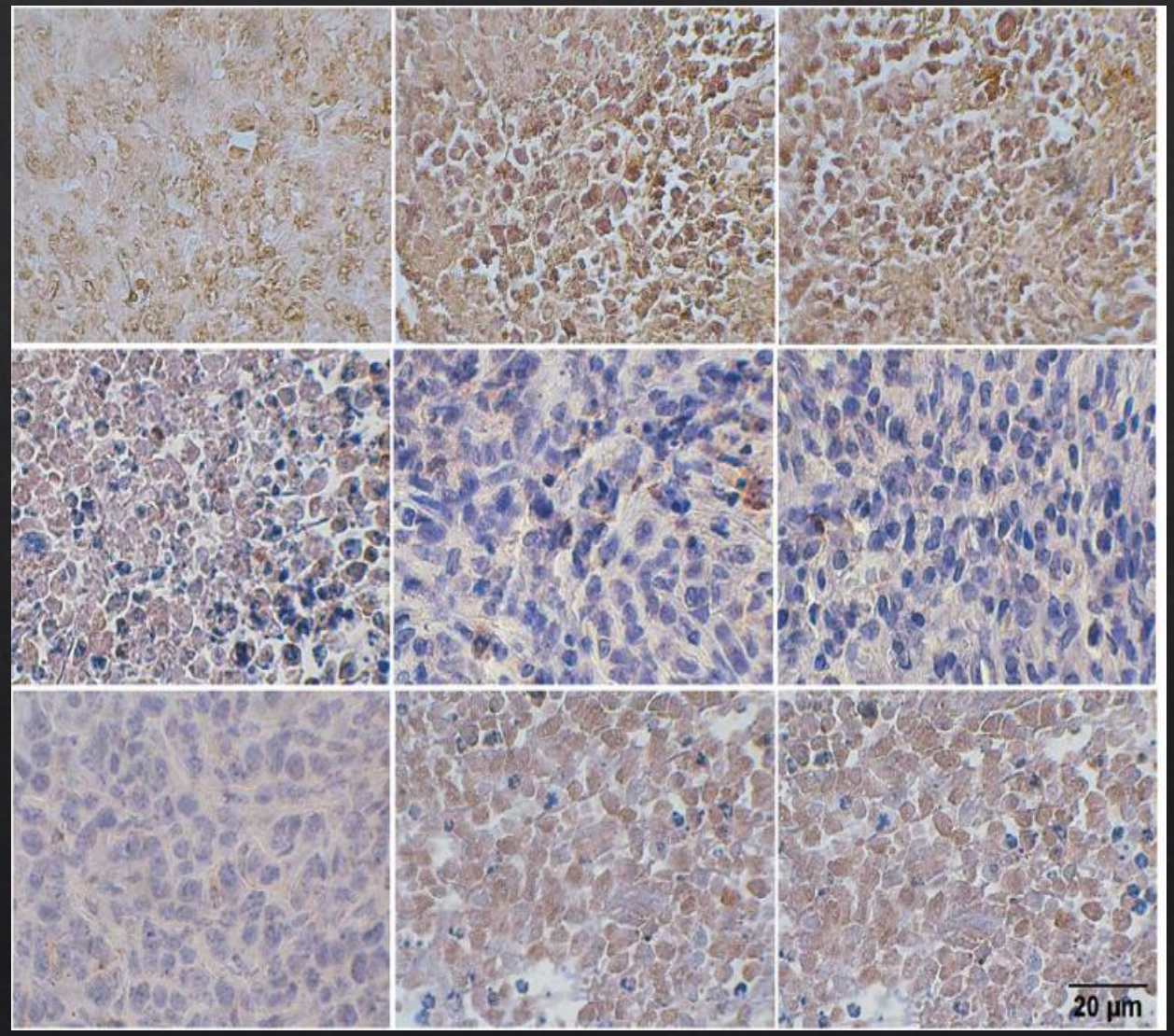
Late apoptosis

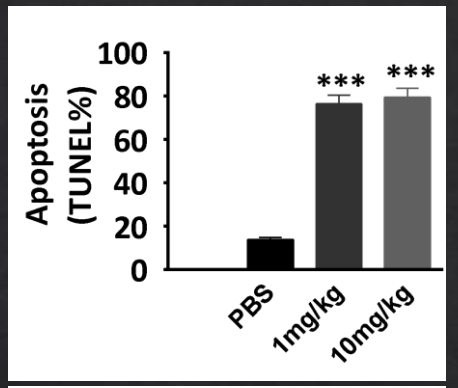
TUNEL

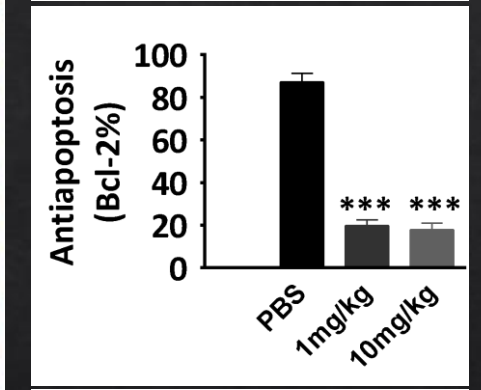
Early apoptosis

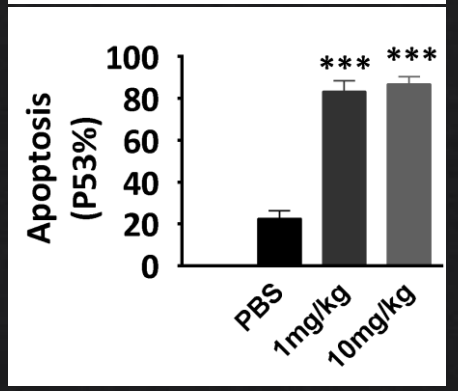
Bcl-2

P53



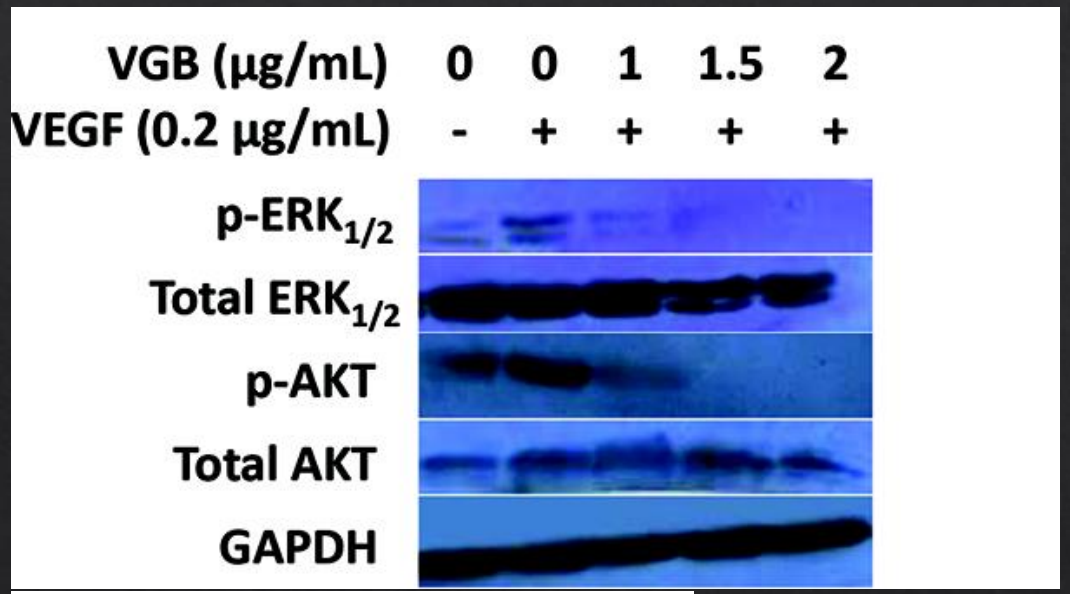


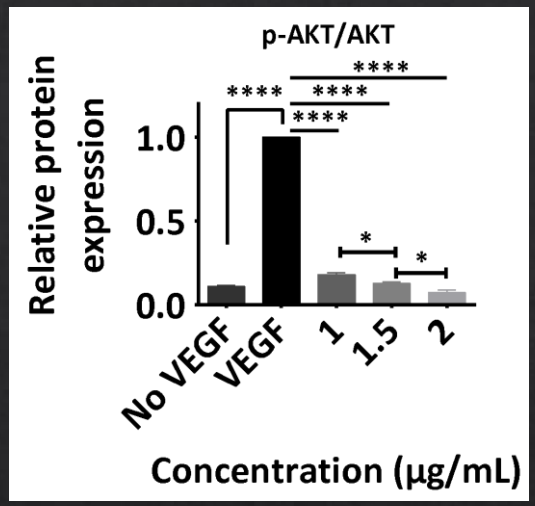


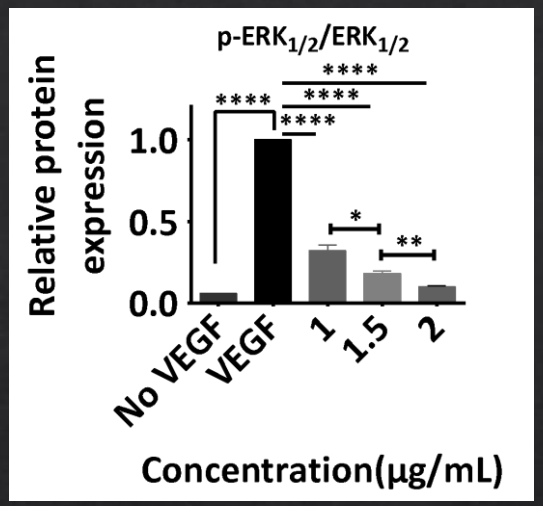


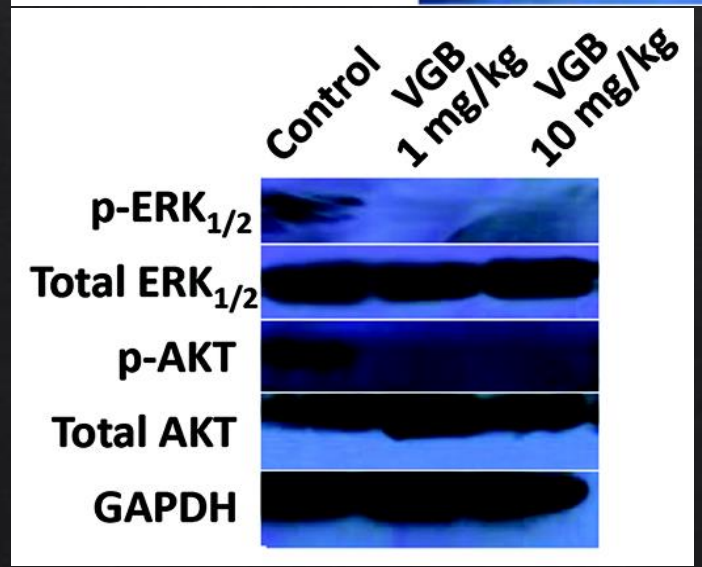
VGB effects on downstream signalling of VEGFR1&VEGFR2: PI3K/AKT and MAPK/ERK1/2

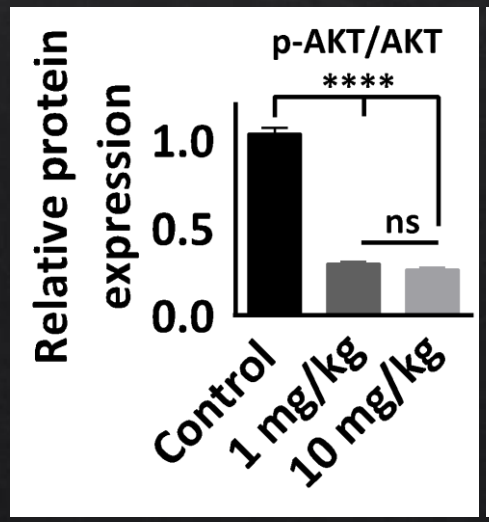
In vivo study *In vitro* study

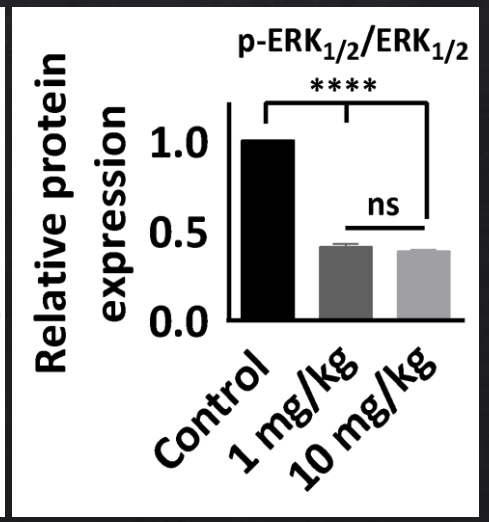




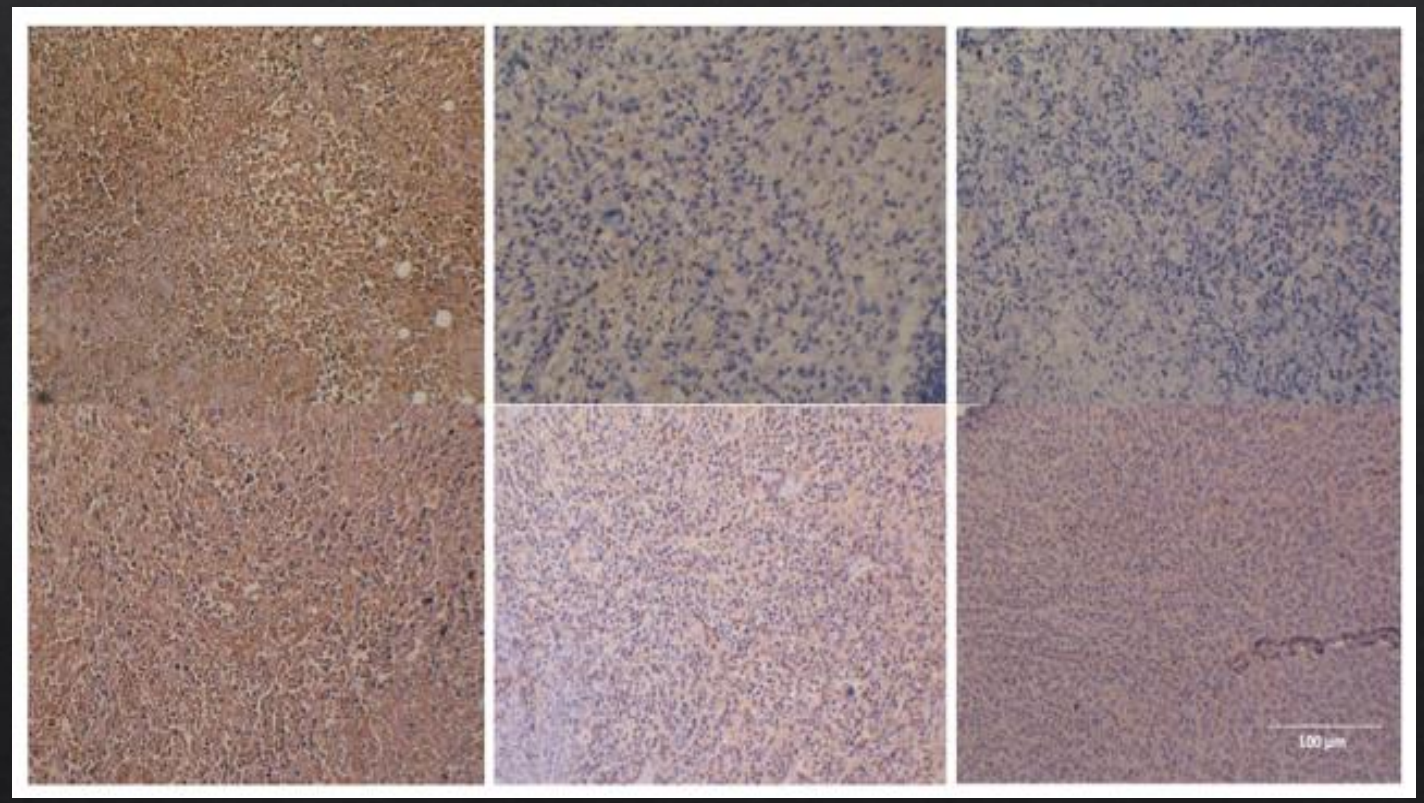


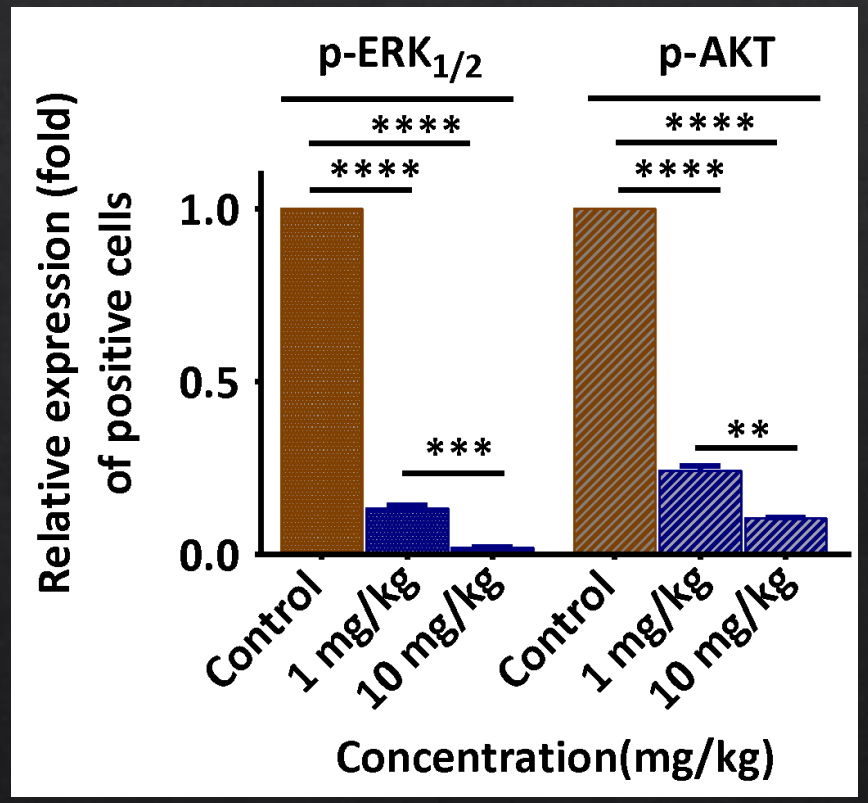




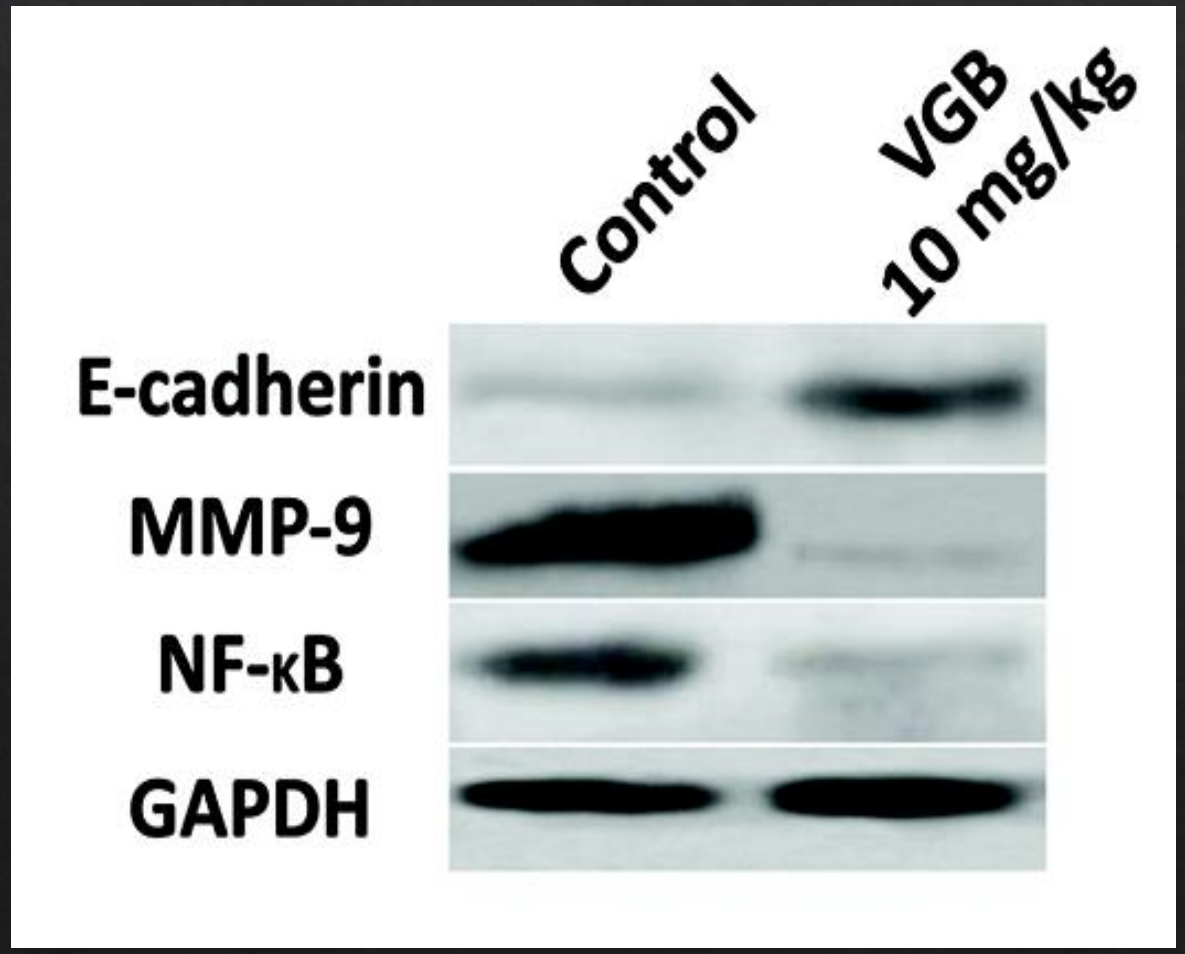


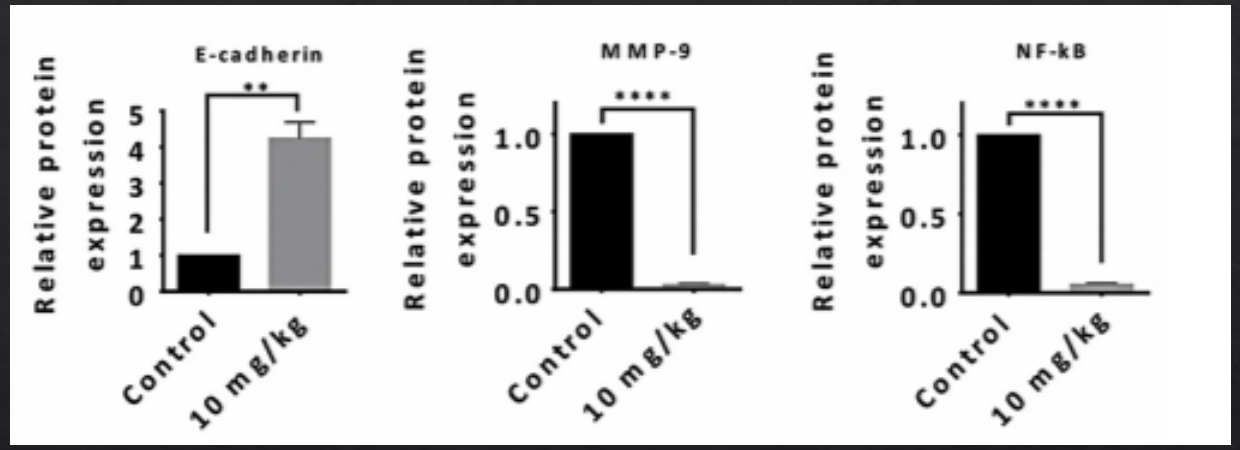
VGB effects on p-AKT and p-ERK1/2 expression





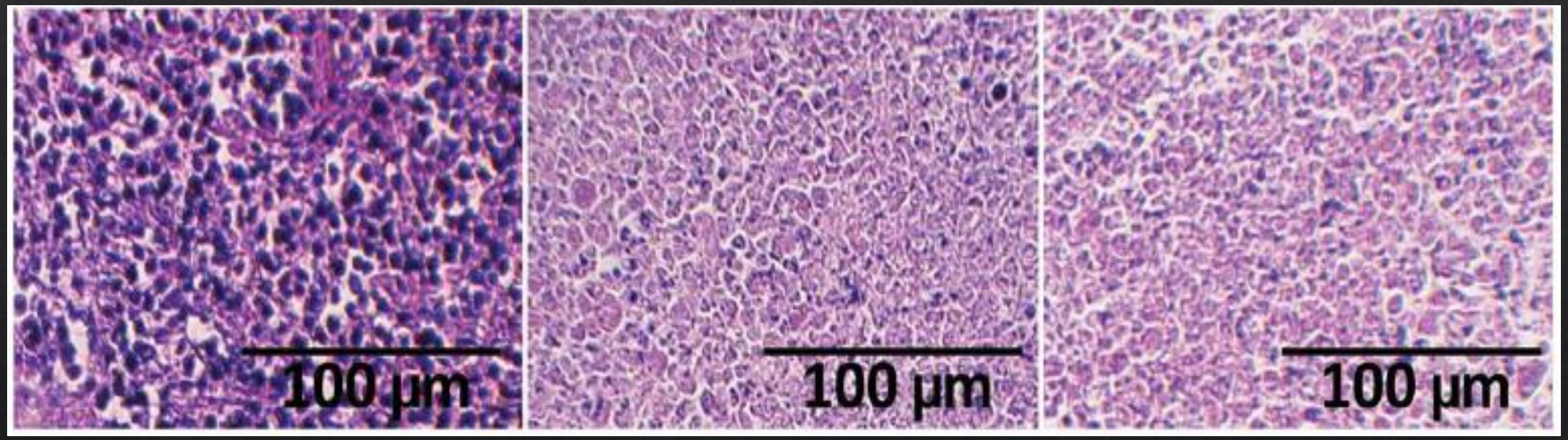
Evaluation of migratory and metastatic pathways





Hemotoxylin & Eosin staining

PBS 1mg/kg 10mg/kg



VEGFR1 blocking peptides

Peptide name	Sequence of peptide	IC50 *in vitro*	Inhibition of tumor growth
- (Giordano et al)	CPQPRPLC	50-100 nM (binding)	-
SPV5.2 (El-Mousawi et al)	NGYEIEWYSWVTHGMY	5 µM (tube formation, 10 nM VEGF)	-
F56 (Song et al)	WHSDMEWWYLLG	10-50 µg/µl (Angiogenesis assay, 2 ng/ml VEGF)	30 µg/2d
- (Goo Bae et al)	GNQWFI	100 µM (MTT assay, 5 ng/ml VEGF)	100 µg/d
BP1 (Taylor et al)	SHRYRLAIQLHASDSSSSCV	2 µM	200 µg/3d/4weeks
Pep.7 (Goncalves et al)	YYDEGLEE	50 µM (Tube formation, 130 pM VEGF)	-
4-23-5 (Ponticelli et al)	Peptoide tetrameric	10 µM (Tube formation, 150 ng/ml VEGF)	-
VG3F (Goncalves et al)	VEGF 3 fragments: 16–26, 60–68 and 102–107 KFMDVYQRSY(Ahx)elGedncs(Ahx)ECRPK-NH2	100 µM (Tube formation, 520 pM)	-
- (Giordano et al)	D(LPR)	2 pM (binding)	20 mg/kg per day
- (García-Aranda et al)	VEGF81–91: (Ac-M-c(CH2-NH-CO-CH2)^{2,10} [GIKPHQGQG]I-NH2)	87.6±5 µM (binding)	-
Pep.16 (García-Aranda et al)	Ac-EVVKFMDVYQRSY-NH2	36 ± 9 µM (binding)	-
HPLC (De Rosa et al)	KQCLWIRSGDRPWYCTS KPDRWSQWRSTYSLSIG	50 ng/mL (MTT assay, 25 ng/ml VEGF)	-
Pep.18 (Wang et al)	LTVELMGTVAKQLVPSC	50 µm (Tube formation, 30 ng/ml VEGF)	-

VEGFR2 blocking peptides

Peptide name	Sequence of peptide	IC50 *in vitro*	Inhibition of tumor growth
V1/A7R (Binetruy-tournaire et al)	ATWLPPR	420 µM (MTT assay, 2 ng/ml VEGF)	-
- (Jia et al)	QKRKRKKSRYKS	20 µM (MTT assay, 10 ng/ml VEGF)	-
K237 (Lei et al)	HTMYYHHYQHHL	100 µM (Angiogenesis assay, 2 ng/ml VEGF)	60 µg/2d
D2 [A] (Shrivastava et al)	P3: Ac-AGPTWCEDDWYYCWLFGTGGGK-NH2 P4: Ac-VCWEDSWGGEVCFRYDPGGGK-NH2	0.65 µm 0.45 µM (Cell migration assay, 100 Nm VEGF)	-
GU40C4 (Udugamasooriya et al)	Peptoide dimeric	1 µM (MTT assay, 1.3 nM VEGF)	800 µg/d
P3 (CYC) (Vicari et al)	Ac-ITMQCGIHQGQHPKICEMSF-NH2	10 µg/Ml (MTT, Tube formation and migration assays, 10 ng/ml VEGF)	500 µg/d
-	RLYE	0.15 nM (MTT, Tube formation and migration assays, 10 ng/ml VEGF)	0.5 and 1.0 mg/kg/d

VEGFR1/R2 blocking peptides

Peptide name	Sequence of peptide	Targeting receptors	IC50 *in vitro*	Inhibition of tumor growth
CBO-P11 (Zilberberg et al)	DFPQIMRIKPHQGQHIGE	VEGFR1 and VEGFR2	5.8 μM (MTT, migration, angiogenesis assays, 10 ng/ml VEGF)	2 mg/kg/d
Pep.1 (Basile et al)	Ac-KLTWMELYQLAYKGI-NH2	VEGFR1 and VEGFR2	12.5 nM (MTT, angiogenesis assayS 25 ng/ml VEGF)	200 nM/d
- (Giordano et al)	PCAIWF WVCSGG	VEGFR1, VEGFR2 and VEGFR3	500μg/Ml (Tube formation, 30 ng/ml VEGF)	-

Conclusion:

1. VGB bound to both VEGFR1 and VEGFR2 and blocked their homo- and heterodimerization in human umbilical vein endothelial cells (HUVECs) as well as 4T1 mammary carcinoma tumor cells.

2. Dual specificity of VGB was confirmed by its dose-dependent inhibitory effect on the VEGF (200 ng/ml)-stimulated proliferation of 4T1 mammary carcinoma tumor cells (that express VEGFR1more than VEGFR-2) and U87 glioblastoma cells (that highly express VEGFR-2). In good agreement with our previous study, MST results reveals that The Kd for the VGB3 (0.5mM)-VEGFR1D2 (25nM) complex (1:1 binding stoichiometry) was 1.96µM.

3. The anti-angiogenic potency of VGB was shown by the observation that, through abrogation of AKT and $ERK_{1/2}$ phosphorylation, VEGFA-stimulated proliferation, migration, and two- and three-dimensional tube formation in HUVECs were inhibited more potently by VGB than by bevacizumab.

4. In a murine 4T1 MCT model, VGB strongly inhibited tumor growth without causing weight loss.

5. Blocking tumor growth and tumor angiogenesis in VGB-treatment against PBS-treated one accompanied by inhibition of AKT and ERK1/2 phosphorylation, a significant decrease in tumor cell proliferation (Ki-67 expression), migration (FAK/Paxilin, PAK2/Cofilin expression), angiogenesis (CD31 and CD34 expression), an increase in apoptosis index (increased TUNEL staining and p53 expression and decreased Bcl-2 expression) and the expression level of a hallmark of EMT axis (E-cadherin expression), and the suppression of systematic spreading of the tumor (reduced NF-κB and MMP-9 and increased E-cadherin expression).

Our results demonstrate the dual specificity of VGB for VEGFR1 and VEGFR2, through which the PI3K/AKT and MAPK/ERK1/2 signaling pathways can be abrogated and, subsequently, angiogenesis, tumor growth, and metastasis are inhibited and apoptosis is induced.

Publications

- 1- **Sadremomtaz A**. *et al*. Dual blockade of VEGFR1 and VEGFR2 by a novel peptide abrogates VEGFdriven angiogenesis, tumor growth, and metastasis through PI3K/AKT and MAPK/ERK1/2 pathway. Biochim Biophys Acta Gen Subj. 1862, 2688–2700 (2018).
- 2- **Sadremomtaz A**. *et al*. Suppression of migratory and metastatic pathways *via* blocking VEGFR1 and VEGFR2. J Recept Signal Transduct Res. 38, 432-441 (2018).
- 3- **Sadremomtaz A**. Groves M, Asghari SM. Molecular docking, synthesis and biological evaluation of Vascular Endothelial Growth Factor (VEGF) B based peptide as anti-angiogenic agent targeting the second domain of the Vascular Endothelial Growth Factor Receptor 1 (VEGFR1D2) for anticancer application. Signal Transduct Target Ther. 5,76-79 (2020); https://doi.org/10.1038/s41392-020-0177-z.


Thank you for your attention