Engineered Conformation-dependent VEGF Peptide Mimics are Effective in Inhibiting VEGF Signaling Pathways.

Daniele Vicari*,†, Kevin C. Foy*,†, Eric M. Liotta§, and Pravin T.P. Kaumaya*,†,††

* Department of Microbiology; † Department of Obstetrics and Gynecology; § School of Biomedical Science; †† Arthur G. James Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

Correspondence to: Pravin T. P. Kaumaya, The Ohio State University, Suite 316 Medical Research Facility, 420 W. 12th Avenue, Columbus, OH 43210. Phone (614) 292-7028; Fax (614) 292-1135; E-mail: Kaumaya.1@osu.edu

Running Title: Engineered VEGF mimicking peptides inhibit VEGF signaling pathways

Keywords List: Peptides; Peptides/Epitopes; Angiogenesis; peptidomimetic, VEGF

Nonstandard Abbreviations Used: VEGF, vascular endothelium growth factor; MVF, measles virus fusion protein; NC, noncyclized; VEGF, vascular endothelial growth factor
ABSTRACT

Angiogenesis, or formation of new blood vessels, is crucial to cancer tumor growth. Tumor growth, progression and metastasis are critically influenced by the production of the pro-angiogenic factor vascular endothelial factor (VEGF). Promising anti-angiogenic drugs are currently available; however, their susceptibility to drug resistance and long-term toxicity are serious impediments to their use, thus requiring the development of new therapeutic approaches for safe and effective angiogenic inhibitors. In this work peptides were designed to mimic the VEGF binding site to its receptor VEGFR-2. The VEGF conformational peptide mimic, VEGF-P3(CYC), included two artificial cysteine residues, which upon cyclization constrained the peptide in a loop native-like conformation in order to better mimic the anti-parallel structure of VEGF. The engineered cyclic VEGF mimic peptide demonstrated the highest affinity to VEGFR-2 by surface plasmon resonance assay. The VEGF peptide mimics were evaluated as inhibitors in several in-vitro assays in which VEGF dependent signaling pathways are observed. All VEGF mimics inhibited VEGFR-2 phosphorylation with VEGF-P3(CYC) showing the highest inhibitory effects when compared to unstructured peptides. Additionally, we show in several angiogenic in vitro assays that all the VEGF mimics inhibited endothelial cell proliferation, migration and network formation with the conformational VEGF-P3 (CYC) being the best. The VEGF-P3(CYC) also caused a significant delay in tumor development in a transgenic model of VEGF^{+/−}Neu2^{+/−}. These results indicate that the structure based design is important to the development of this peptidomimetic and to its anti-angiogenic effects.

INTRODUCTION

The formation of new blood vessels, called angiogenesis, is a process tightly regulated by a balance between pro- and anti-angiogenic factors and, physiologically it is activated in wound healing, ovulation and menstruation. However, it is also stimulated in pathologic conditions such as cancer, macular degeneration in the eyes, psoriasis and diabetes [1-5]. Since most tumors cannot grow beyond a few millimeters in the absence of new blood vessel formation [6], angiogenesis inhibitors have been explored lately as a drug target to be used in combination with several cancer therapies [7-13]. Several studies have explored the use of DNA vaccines, small tyrosine kinase inhibitors, siRNAs, ribozymes, antibodies and receptor blocking agents aimed at better understanding the angiogenic mechanism and development of potential inhibitors [13-15]. Specialized cancer treatments with anti-angiogenic agents approved by the FDA include monoclonal antibody, bevacizumab (Avastin) or small tyrosine kinase inhibitors, SU11248 (sunitinib) and BAY 43-9006 (sorafenib) [7, 16, 17]. Although the clinical application of these drugs in cancer therapy are promising, drug resistance development and long-term side effects like hypertension and endothelium dysfunction remain a concern [14].

The pro-angiogenic factor VEGF is the most studied growth factor in this field, due to its specificity and important role in the activation of all steps of angiogenesis in the endothelium vasculature [18-20]. The splicing variant VEGF_{165} is the predominant form and had been shown to be up-regulated in tumor microenvironment by hypoxia or activation of oncogenes like HER-2 [21-26]. VEGF is a glycoprotein and consists of an anti-parallel homodimer structure containing inter- and intra-disulfide bonds and it has been shown to bind to three receptor types: VEGFR-1 (flt-1), VEGFR-2 (flk-1 or KDR) and neuropilin-1 (NR-1). The VEGF:VEGFR-1 interaction exhibits high affinity, although the role of VEGFR-1 is not fully understood but research suggests its function in activated pathways in macrophages or endothelial progenitor cells (EPC) [27, 28]. In the endothelial cells the majority of angiogenesis signaling (proliferation, migration and survival) proceeds via the interaction between VEGF and VEGFR-2 [18, 29-31].

The binding site of VEGF to its receptors has been characterized by crystal structure analysis as well as alanine scanning and reveals overlapping regions located at the poles in the homodimer [31-36]. VEGF:VEGFR-2 interaction has been explored extensively using antibodies that bind VEGF as well as the extra-cellular domain of VEGFR-2 [13, 37-40], identifying
VEGF epitopes in the binding region that inhibit VEGF dependent pathways [33]. The interaction between VEGF and VEGFR-2 has been identified and comprises residues at a loop region formed by the anti-parallel β-sheets β5-β6 in the VEGF protein [31].

Blockade of receptor-ligand interaction offers a validated and proven approach in drug development because receptor:ligand interaction is usually confined to a defined portion of the ligand and the receptor, and recent technologies have allowed the accurate identification of these binding regions. Peptidomimetics is the approach of reproducing the biological activity or binding properties in a smaller molecule, like peptides or modified peptides which were designed to mimic the desired region. This approach demonstrated successful results in studies with melanocortin MC4, pallet GP IIb/IIIa or CD28 costimulatory receptors [41-46]. Peptides are excellent candidates for drug design because they demonstrate better target specificity and less susceptibility to drug resistance than small molecules. Additionally, peptides demonstrate several advantages like lower developing and manufacturing costs, improved organ or tumor penetration and higher activity per mass when compared to antibodies or large proteins [47, 48].

The main goal of this work is to create molecules that would retain the structural similarity with the binding site region and demonstrate bioactivity “in vivo”. Our hypothesis to a peptide therapeutic approach is that key residues in the binding epitope, in particular side-chain functional groups responsible for a significant portion of the binding affinity to a given ligand, can be transferred to a much smaller fragment while maintaining the contributions to the binding largely intact. The goal is to direct critical amino acids into the same conformational space and orientation as in the bioactive surface yet retain sufficient flexibility to bind cooperatively and with complementarity to a given receptor. The therapeutic targeting of the tumor vasculature has multiple advantages over traditional cancer treatments [49] displaying minimal toxicity. Many new cancer therapies are being directed against VEGF and Flk-1 believed to be the most important for angiogenesis during tumor formation [50-52]. Several antireceptor therapeutic strategies have been pursued. Anti-VEGF strategies include the use of MAbs to VEGF (e.g., bevacizumab) or its receptors, use of ribozymes to decrease receptor expression, small molecule inhibitors of VEGF receptor tyrosine kinase.

Here we report that peptides corresponding to the natural VEGF amino acid sequence 102-122 (76-96) which comprises the important loop-binding residues of VEGF to its receptor were successfully engineered to better mimic the conformational structure of this sequence in the protein. The conformational peptide mimics VEGF-P3(NC) and VEGF-P3 (CYC) sequences demonstrated the highest affinity to VEGFR-2 and were effective as inhibitors of VEGFR-2 phosphorylation and in several angiogenic in vitro assays such endothelial cell proliferation, migration and network formation. The VEGF-P3(CYC) also caused a significant delay in tumor development in a transgenic model of VEGF+/-Neu2-5+/-.[49]. The observed results are consistent with the hypothesis that the structure based design is important to the development of VEGF peptidomimetics and to its anti-angiogenic effectiveness.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized on Milligen/Biosearch 9600 solid-phase peptide synthesizer (Bedford, MA) using Fmoc/t-But chemistry as previously described [53] In case of the synthesis of VEGF102-122 preloaded Fmoc-Phe-CLEAR acid resin (0.41 mmol/gm was used while VEGF-P3 and MVF-VEGF-P3 were synthesized using CLEAR amide resin (0.32 mmol/gm) (Peptides International, Louisville, KY). Peptide P3 were acetylated on resin, using acetyl imidazole reagent as reported earlier [53]. The MVF-GPSL sequence was added on peptide resin of the H2N-VEGF-P3. All peptides were cleaved from the resin using the cleavage reagent B (Trifluoroacetic acid:Phenol:Water:Triisopropyl silane 90:4:4:2) and crude peptides were purified on preparative RP-HPLC (Reverse Phase-High Pressure Liquid Chromatography) using Vydec C-4 column and acetonitrile-water (0.1% TFA) gradient system. All fractions were analyzed on analytical RP-HPLC and characterized by MALDI (Matrix Assisted Laser Desorption Ionization mass spectroscopy) at CCIC (Campus Chemical
Instrumentation Center, The Ohio State University, Columbus, Ohio). RP-HPLC fractions showing same mass spectrum peak were pooled together and lyophilized. RP-HPLC pure peptides MVF-VEGF-P3, VEGF-P3 containing two Cys residues in each peptide were cyclized using acetic acid-iodine method, further purified on RP-HPLC and characterized by mass spectroscopy using established protocol as reported earlier [54]. Amino acid sequences and molecular weight of all peptides and their molecular weights are shown in Table-I.

**Cell lines and Reagents.** All culture media, FBS, and supplements were purchased from Invitrogen Life Technologies (San Diego, CA). HUVEC were purchased from GlycoTech and cultivated in F-12K Nutrient Mixture-Kaighn’s Modification (F-12K) supplemented with 20% FBS, heparin (100 μg/ml) and Endothelial (complement) cell growth factor supplement (ECGS) (50 μg/ml). 293/KDR cells (high expression of VEGFR2) were purchased from SibTech, Inc (Brookfield, CT) and cultivated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and puramycin (0.375 μg/mL). Cells were incubated in a 37 °C humidified 5% CO₂ incubator.

**Surface Plasma Resonance.** Binding assays were performed using a Biacore 3000 instrument (Pharmacia Biosensor, Uppsala, Sweden). The experiments were performed at room temperature using HBS-EP buffer (25 mM HEPES, pH 7.4, 150 nM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20). Peptides were coupled onto CM5 chip surfaces at 10 µL/min using a standard amine coupling protocol with EDC (N-ethyl-N’-dimethylaminopropyl)carbodiimide)/NHS(N-hydroxysuccinimide). KDR-Fc (R&D System) at several concentrations in HBS-EP buffer. Was injected at a flow rate of 10µL/min. Data analysis was performed with BIAsimulation software version 3.1 (Pharmacia Biosensor). For competition assay KDR-Fc and rhVEGF (R&D System) were mixed in HBS-EP and incubated 30 minutes at room temperature and this mixture was injected over the chip where peptides were immobilized. To obtain measurement of anti-peptides binding affinities similar a experiment was carried out, but rhVEGF was immobilized onto CM5 chip surface and anti-peptides were injected at several concentrations.

**Circular Dichroism.** Circular dichroism (CD) measurements were performed on an AVIV model 62A DS instrument. All spectral measurements were obtained at 25°C under continuous nitrogen purging of the sample chamber, using a quartz cuvette of 0.1 cm path length. Spectral measurements of VEGF-P3(NC) and VEGF-P3(CYC) were obtained at a concentration of 100μM in water. Molar ellipticity values were calculated using the formula: \[ \theta = \frac{X \times 100 \times M_r}{n \times c \times l} \]

**Proliferation Assay.** HUVEC (1x10⁴ cells/well) were plated in 96-well flat-bottom plates overnight. Growth medium was replaced with low sera (1% FCS) medium and the cells were incubated overnight. Media were removed from the wells and replaced with low sera medium containing VEGF mimic peptides at concentrations ranging from 50-50,000 ng/ml with or without rhVEGF (10 ng/ml). In the case of antibodies, low sera medium containing purified anti-VEGF peptide mimic antibodies at concentrations ranging from 0.15 to 150 µg/ml with or without rhVEGF (10 ng/ml) was used. Plates were incubated for additional 72 h at 37°C before adding MTT (5 mg/ml) to each well. Plates were incubated 4 h at 37°C, medium was discarded and 100 µl of extraction buffer (20% SDS, 50% dimethylformamide (pH 4.7)) was added to each well. Plates incubated overnight at 37°C and read on an ELISA plate reader at 570 nm with 655 nm background subtraction. Inhibition percentage was calculated as 100% x (VEGF only treated cells – Peptide treated cells)/(VEGF only treated cells).

**Network Formation Assay using Matrigel.** Matrigel (60μl) (B&D Bioscience) was added to 96 well plate and incubated 30 min at 37°C. HUVEC were kept overnight in low sera medium before cells (20,000/well) were seeded with low sera medium F-12K supplemented with 1%FBS and 10ng/ml VEGF (R&D System) with or without inhibitor. The cells were fixed in 4% formaldehyde after overnight incubation at 37°C.
Pictures from magnification 40X from light microscopy were taken and the sprout points counted using the software ImageJ (NIH). Two set of experiments were combined and averaged.

**Scratch Wound Assay.** HUVEC were cultured on 0.1% gelatin coated 24-well plates. Confluent cells were incubated overnight with starving media, then they were scraped using sterilized 200-µl pipette tips and stimulated with 50 ng/ml of rhVEGF with or without VEGF mimic peptides for 16 h at 37°C. Cells were fixed and images were captured immediately at 40X magnification from light microscopy and cells that migrated to the scraped area were counted using ImageJ software.

**Phosphorylation Assay.** HUVEC (5x10^5 cells/well) were grown on 6-well plates in FK-12 endothelial cell growth medium supplemented with ECGS and heparin until 80% confluence. After overnight incubation in starving medium (0.5% FBS) cells were treated with inhibitor (100 µM) for 30 min and then stimulated with 10 ng/ml rhVEGF for 5 min. When using KDR-Fc as inhibitor, it was incubated with rhVEGF for 30 min then added to the cells for 5 min. Cells were washed in cold PBS supplemented with 1mM sodium orthovanadate, harvested into RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated on ice for 30 min and cell lysate was collected after centrifugation at 13000 rpm for 10 min. Cell lysate was kept at -80°C until used for Western blotting detecting phospho-KDR and total KDR as described above.

**Peptide Treatment in Transgenic Mouse.** VEGF^+/−Neu2−5^+/− mice group (n=6) were treated with 500 µg of peptides dissolved in PBS and injected intravenously at the tail weekly from week four to week 10. Mice were euthanized at week 10 and tumors removed. Tumors in each of the ten mammary gland were measured for tumor volume. VEGF^+/−Neu2−5^+/− results were reported as the average of the single largest tumor per mouse. Tumor growth over time was analyzed using Stata’s XTGEE (cross-sectional generalized estimating equations) model which fits general linear models that allow you to specify within animal correlation structure in data involving repeated measurements.

**Immunization of rabbits.** New Zealand White rabbits were immunized with 1 mg of peptide dissolved in ddH₂O emulsified (1:1) in Montanide ISA720 vehicle (Sepsic) with 100 µg of N-acetylglucosamine-3yl-acetyl-l-alanyl-d-isoglutamine (nor-MDP). Rabbits were boosted with the respective doses at 3 week intervals. Rabbit blood was collected via the central auricular artery and sera tested for antibody titers. Anti-peptide antibodies were purified by affinity chromatography using a Protein A/G column (Pierce) from high titer antibody sera.

**ELISA for anti-VEGF antibodies.** Plates were coated overnight at 4°C with 100µl of 2µg/ml
rhVEGF (R&D System), washed four times with 0.1% Tween 20/PBS, and blocked with of 100 μl of 1% BSA/PBS for 2 h at room temperature. Plates were washed four times with 0.1% Tween 20/PBS. Anti-peptide sera were added at several dilutions and incubated 2 hours at room temperature. Plates were washed four times with 0.1% Tween 20/PBS. Anti-peptide sera were added at several dilutions and incubated 2 hours at room temperature. Plates were washed four times with 0.1% Tween 20/PBS, a 1/500 dilution of goat-anti-rabbit IgG HRP was added and incubated 1h. Detection was done using ABTS substrate and absorbance reading at 415nm. Ab titers were determined as previously described [56] and defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting background.

Direct Peptide-cell Binding Assay. The peptide VEGF-P3-CYC was biotinylated at the N-terminus during synthesis. Peptide binding to the VEGFR2 was evaluated using both HUVECS and 293-KDR cells. 1 X 10⁶ cells were incubated with the biotinylated peptide in 100μl of 2%FCS in PBS for 2h at 4°C. Unbound peptides were removed by washing 3 times with PBS and the cells incubated with Alexa Fluor 594-Streptavidine (Molecular Probe) for 1h. Cells were then washed with PBS three times and fixed with 1% formaldehyde before being analyzed by phase contrast, fluorescence and confocal microscopy.

Flow Cytometry. Binding of the VEGF P3 (CYC) peptide antibodies to KDR cells was evaluated using flow cytometry. 1X10⁶ KDR cells were incubated with 100μl PBS+ 5% BSA+ 0.02% sodium azide for 30min at 4°C in order to block non specific binding. 50μg of anti-peptide abs purified from rabbits were then added and incubated for 1hour. The cells were then washed by adding 2mls of PBS+ 0.05% BSA+ 0.02% sodium azide and centrifuged for 5min at 12000rpm. Cells were then suspended in 100μl of PBS+ 0.05% BSA+ 0.02% sodium azide before adding 1μl of of Alexa Fluor 488 goat anti-rabbit IgG antibody at 1:100 dilution, mixed and incubated for 30min at 4°C. The cells were then washed again as before then fixed with 2% formaldehyde before being analyzed by Coulter ELITE flow cytometer. A total of 10.000 cells were analyzed by light scatter assessment before single parameter histograms were drawn. Controls included cells alone and cells plus secondary antibody alone.

RESULTS
Selection and Design of VEGF Peptide Mimics.

The crystal structure of the complex between VEGF and the Fab fragment of a humanized antibody [33], and analysis of the contact residues on both sides of the interface was published by Muller et al., [35, 57]. Zilberberg et al., also identified that the sequence 79-93 of VEGF is involved in the interaction with VEGF receptor-2 [58]. Although the VEGF residues critical for antibody binding are distinct from those important for high-affinity receptor binding, they occupy a common region on VEGF demonstrating that the neutralizing effect of antibody binding results from steric blocking of VEGF-receptor interactions. Thus, it appears that only a small number of the residues buried in the VEGF–Fab interface are critical for high-affinity binding and are concentrated in one continuous segment of polypeptide loop between β5-β6 (Fig. 1A). Several residues are important for VEGF receptor binding, including Met 81, Ile 83, Lys 84, Pro 85, Gln 89, and Gly92 [31, 59]. We have selected to use a peptide encompassing residues 102-122 (numbered as 76-96 in the crystal structure) which mimics the overlapping VEGF binding sites to VEGFR-2 and Avastin.

The strategy to create a conformational epitope consisting of an anti-parallel β-sheet as shown schematically in Fig.1B requires two artificial Cysteine to be introduced between Gln79 & Gly92, and between Ile80 & Glu93 for disulfide pairing to enable the formation of the twisted anti-parallel β-sheet. It also required two artificial cysteines to be introduced between Gln79 & Gly92, and between Ile80 & Glu93. After synthesis and purification of VEGF-P3 (NC) (non-cyclized) peptide, the disulfide bond was formed by oxidation reaction enabling the formation of the
twisted anti-parallel β-sheet structure in the VEGF-P3 (CYC) (cyclized).

**MS Analysis Peptides.** All pure peptides showed uniform peaks on analytical HPLC (purity >95%) and were further characterized using MALDI mass spectroscopy analysis to confirm the calculated and observed (Cal/Obs) molecular weight. In brief VEGF102-122 (M+H⁺) Cal/Obs 2482.24/2482.32; VEGF-P3(NC) (M+H⁺) Cal/Obs 2727.27/2727.61; VEGF-P3(CYC)(M+H⁺)Cal/Obs 2725.27/2725.43 MVF-VEGF-P3(NC)(M+H⁺)Cal/Obs 5023.67/5023.82; and MVF-VEGF-P3(CYC) (M+H⁺) Cal/Obs 5021.62/5021.19.

**Structural Characterization.** To verify the secondary structure of VEGF peptide mimics circular dichroism experiments were carried out. CD analyses of VEGF peptide mimics demonstrated a shift in the minimum of the non-cyclized peptide (197nm) spectrum to minima in the cyclic peptide (203, 205 and 210nm) (data not shown). The shift in the CD spectrum is characteristic of an assumed β-turn II conformational structure [60, 61], indicating that the cyclic peptide may adopt a configuration more similar of the anti-parallel β-sheet structure present in the loop of VEGF protein. This similarity in the binding region is expected to confer more binding ability to the receptor.

**Biotinylated Conformational VEGF Peptide Mimic binds to cells that express VEGFR2.** To determine whether the conformational peptide mimic VEGF-P3(CYC) has the ability to recognize and bind cells that express VEGFR2, we biotinylated the peptide. The biotinylated VEGF-P3-CYC peptide was shown to specifically bind to VEGF2 expressing cells as detected by streptavidin Texas red after incubation with the peptide (Fig. 3A). Results indicate binding and internalization in HUVEC and in a tumor cell line expressing only this receptor (293-KDR). Most interestingly, the binding of the peptide was seen unevenly distributed over HUVEC (Fig. 3B, D), where the receptors are known to be expressed in clusters and reside in an endosomal population close to the plasma membrane [62]. In the case of the 293-KDR cells, the binding could be seen all over the expressing cells (Fig. 3C) because expression is uniform throughout the cell surface. This explains the increased accumulation in some cells (293-KDR) and the clustering in others (HUVECS). These results clearly illustrates that the VEGF-P3-CYC peptide is specific to the VEGFR2 and recognize cells that are known to express the receptor, in a pattern which is consistent with its known distribution.

**Conformational VEGF Peptide Mimic Prevents VEGFR-2 phosphorylation.** VEGFR-2 (also known as flt-1 or KDR) has been characterized as a tyrosine receptor type III. VEGF-2 activation
is promoted by dimerization upon VEGF binding. VEGFR-2 contains several tyrosine residues that can be phosphorylated, triggering several pathways such as proliferation, migration and survival in the endothelial cells [18, 63, 64]. Phosphorylation assay with HUVEC (cells that expresses physiological levels of VEGFR-2) was used to explore the ability of VEGF mimic peptides to block VEGF-VEGFR-2 interaction and consequently phosphorylation. All three VEGF mimic peptides were able to decrease the level of receptor phosphorylation (Fig. 4); however, the inhibitory effect was unable to be quantified, due to the limited detection of VEGFR-2 in the Western blotting. To gauge the effect of VEGF mimic peptides on VEGFR-2 phosphorylation, we used 293/KDR cells, which have been demonstrated to be an excellent model for VEGFR-2 phosphorylation since they over-express VEGFR-2 (2.5x10⁶ receptors per cell) [65]. As seen in Fig. 5A, the degree of VEGFR-2 phosphorylation is notably increased in the presence of exogenous VEGF (10 ng/ml) and decreased when an exogenous receptor (KDR-Fc at 100 ng/ml) was used as a competitor. The level of inhibition in VEGFR-2 phosphorylation was similar with the VEGF natural sequence VEGF 102-122 or irrelevant control. When engineered peptides VEGF-P3(NC and CYC) were used as inhibitors, the level of VEGFR-2 phosphorylation was diminished, with the VEGF-P3(CYC) being the most potent inhibitor. These results were confirmed with the quantification of VEGFR-2 phosphorylation using the Human Phospho-VEGFR R2/KDR DuoSet IC kit (Fig. 5B). The highest inhibition was observed with the VEGF-P3(CYC) (25%) followed by VEGF-P3(NC) (12%) while no inhibition was observed with irrelevant peptide (-2%) and a low level of inhibition with the natural sequence peptide VEGF 102-122 (4%). The percentage of inhibition was calculated assuming the phosphorylation level of control (only rhVEGF) was 100% and the results are represented in Fig. 5B.

Activation of VEGFR-2 also triggers the MAPK (mitogen-activated protein kinase) pathway as one of the downstream signaling in the endothelial cells [66-68]. The level of phosphorylation of MAPK p44ERK1 and p42ERK2 was observed using western blotting and antibodies against phospho-p44/42 (Fig. 4B). Decrease of phosphorylation level was greater when the VEGF-P3(CYC) was used as inhibitor followed by the non-cyclic peptide VEGF-P3(NC) and VEGF 102-122. The small tyrosine kinase SU1498, used as one of positive controls, has been shown to accumulate phosphorylated MAP kinases in endothelial cells because it interacts with other kinases such as ERK 1/2, affecting other pathways [69]. Tyrosine kinase inhibitors usually act by binding the kinase active site blocking ATP binding; consequently the phosphate is not transferred to the tyrosine residue. This mechanism of action has two major drawbacks as tyrosine kinase inhibitors: low specificity and high susceptibility to resistance (enzymes often mutate themselves to recover activity [70-72]. VEGF peptide mimics demonstrated the same pattern of inhibition in VEGFR-2 and MAPK phosphorylation, indicating that downstream MAPK signaling of VEGFR-2 is being inhibited by decreased VEGFR-2 activation.

**Conformational Peptide Inhibits HUVEC proliferation.** Endothelial cell proliferation is VEGF dependent, and mostly activated by VEGFR-2 activation [30]. Thus, angiogenesis inhibitors should inhibit HUVEC proliferation. This assay was carried out in the presence of several concentrations of VEGF mimic peptide to verify their ability to inhibit VEGF dependent proliferation. Fig. 6A shows that all VEGF mimics can inhibit HUVEC proliferation in a dose dependent way and that the conformational peptide VEGF-P3(CYC) demonstrated the highest inhibitory effect. The toxicity of the VEGF mimic peptides was verified using HUVEC proliferation assay in the absence of VEGF where no significant differences between peptide treated and untreated cells were observed (data not shown).

**Conformational Peptide Decreases HUVEC Network Formation in Matrigel Assay.** Activation of VEGFR-2 also triggers the MAP Kinase pathway that leads to the formation of cell cords and tubes by the endothelial cells [73]. The in vitro Matrigel assay is an appropriate model for assessing network formation as it takes advantage of the capacity of cell cord formation by HUVECs growing in an extracellular matrix (Matrigel). Network formation is clearly VEGF dependent as
can be seen in Fig. 6B, where a cell network with several sprout points is more evident in the VEGF treated HUVEC than the non-VEGF treated HUVEC. Decrease in the network branching and tube formation was observed in VEGF treated HUVEC in the presence of VEGF mimic peptides and no significant effect was seen with the irrelevant control (Fig. 6C). The best inhibitory effect was demonstrated by engineered mimic peptides VEGF-P3 (NC and CYC). These results are in agreement with VEGFR-2 phosphorylation and HUVEC proliferation assay, indicating that VEGF mimic peptides can block VEGF and VEGFR-2 interaction.

**VEGF Peptide Mimics Inhibit Cell Migration in a Scratch Wound Assay.** New blood vessel formation requires that the endothelial cells migrate towards the sources of growth factor. This process has similar characteristics with wound healing in which VEGF has been shown to play an important role throughout VEGFR-2 activation [74]. We used the scratch wound assay with HUVEC to observe the ability of the VEGF mimic peptides in inhibiting endothelial cell migration. As can be seen in Fig. 7A, cells were able to migrate towards the scratched area in higher number when exogenous rhVEGF was added compared to the absence VEGF. Growth medium was supplemented with 20% FBS and endothelial cell growth supplements. Fig 7B shows a slight increase in percentage of migrated cells, probably due to the complexity provided by the supplements. Irrelevant peptide control had a comparable number of migrated cells when compared to rhVEGF control, indicating no inhibition. All three VEGF mimic peptides demonstrated ability of inhibiting HUVEC migration at similar levels (approximately 50%) of the small VEGFR-2 tyrosine kinase inhibitor (SU1498) at a standard concentration (Fig. 7B) indicating that VEGF mimic peptides are capable of blocking the VEGF dependent migration in endothelial cells.

**VEGF Peptide Mimics as treatment in a VEGF over-expressing mouse model.** We have extensively studied conformational peptides of HER-2 as peptide based immunotherapy. We used the double transgenic mice VEGF+/−Neu2-5+/− in our in vivo experiments taking into consideration that our ultimate goal is to combine HER-2 immunotherapy with anti-angiogenic therapy. The transgenic Neu2-5+/− mouse develops spontaneous mammary tumors at age of 111 days due to a mutation in the neu gene (mouse homologue of HER-2). When they are crossed with the MMTVVEGF-164, which over express VEGF under a MMTV promoter, the offspring resulting is the double transgenic VEGF+/−Neu2-5+/− in which spontaneous tumors developed much earlier, around 51 days, due to excessive activation of angiogenesis by increased expression of VEGF. VEGF+/−Neu2-5+/− mice treated with VEGF peptide mimics developed tumors after 57 days -old while non-treated mice had tumors around 51 days old. However, using the statistical model XTgee only the group treated with VEGF-P3(CYC) demonstrated statistical significance (p=0.0074) in delaying tumor burden (Fig 8). The tumor doubling time in the VEGF-P3(CYC) treated group was 1\( \frac{1}{2} \) times longer than the control group (3.6 vs 2.3 days). Design of constrained VEGF-P3(CYC) in order to mimic VEGF binding sites seems to be important to produce the best inhibitor in vitro and in vivo.

**Anti-VEGF Peptide Antibodies Validate Peptidomimetic Approach.** In order to develop therapeutic approaches to inhibit angiogenesis by using peptide mimics, we wanted to explore the ability of the VEGF peptide mimics to generate native-like antibodies. Such anti VEGF antibodies could be used to further demonstrate the validity of the peptidomimetic approach and could themselves be used as inhibitors of angiogenesis. We have developed strategies for using epitope-based peptides to generate antibodies with better affinity and specificity. Similarly, we have successfully predicted oncogenic peptide epitopes to develop conformational peptides for cancer vaccine approaches [54, 56, 75-78]. VEGF sequence 102-122 (76-96) comprises the region containing several residues important for antibody neutralization of VEGF. We hypothesized that antibodies elicited against the engineered peptide mimics VEGF-P3(NC and CYC) would retain or enhance the specificity for VEGF protein. A “promiscuous” T cell epitope MVF 288–302, which has been demonstrated to enhance immune response [79] was incorporated into VEGF mimic...
peptides and used for raising antibodies in rabbits. All three constructs of VEGF peptide mimics demonstrated high immunogenicity (data not shown) and were able to recognize the rhVEGF in an ELISA assay (Fig.9).

**Specificity of Anti VEGF Peptide Mimic Antibodies.** To confirm their specificity, we carried out competitive ELISA assay using rhVEGF on the plate with antibodies to the various mimics and VEGF mimic peptides as inhibitors. Fig. 10 shows the results for anti-MVF-VEGF-P3(CYC). Engineered VEGF-P3 peptides, in the cyclic form, were able to compete for the binding site in the anti-VEGF-P3(CYC) but not to the antibodies generated against the natural sequence. This indicates that the engineered peptides did not generate antibodies against the linear sequence of VEGF but most importantly that they mimic the conformational epitope in the VEGF protein.

**Antibodies to Conformational VEGF Peptide Mimic VEGF P3(CYC) have high affinity for rhVEGF.** Kinetic parameters of antibodies raised against VEGF mimic peptides were obtained by surface plasma resonance using direct binding assay in BIAcore 3000. Anti-peptide antibodies were injected as ligands over rhVEGF immobilized onto CM5 chip. The binding affinity to the whole protein was higher for the antibody raised against the conformational epitope, anti-VEGF-P3(CYC) (KD=146nM), followed by the anti-VEGF-P3(NC) (KD=251nM) and the antibody raised against the natural sequence anti-VEGF 102-122 (KD=552nM). As can be seen in Table III, the Ka for the anti-VEGF mimic peptides demonstrated only 10 fold decrease in association rate constant, Ka and comparable dissociation constant rate Kd when compared to a commercially available monoclonal antibody against VEGF.

**Reactivity of Peptide Antibodies to Conformational VEGF P3(CYC) with VEGFR2 expressing cells (KDR).** Binding of peptide Abs to intact VEGFR2 cells was evaluated by immunofluorescence staining of single cell suspension of 293-KDR cells. The peptide antibodies were able to specifically bind the cells (Fig.11). The binding was also dose dependent with increase binding when higher concentrations of the Abs were used (results not shown). These results demonstrate that the peptide vaccine is able to prevent VEGF binding to it receptor VEGFR2. The peptide when added before the anti-peptide abs was also able to prevent the binding of the abs illustrating its specificity for the VEGFR2 (results not shown).

**Anti-VEGF Peptide Antibodies Inhibits HUVEC proliferation.** VEGF neutralizing antibodies blocks the interaction of VEGF and VEGF receptors by binding to and occluding VEGF binding sites. Since our anti-VEGF mimic peptide antibodies were able to bind VEGF, we tested their ability of inhibiting VEGF dependent HUVEC proliferation assay. All three anti-VEGF peptide antibodies were able to inhibit HUVEC proliferation in a dose dependent way when compared to the preimmune-serum control. Anti-VEGF-P3(CYC) demonstrated the highest inhibition and anti-VEGF-P3(NC) seems to be slightly more efficient than the natural sequence (Fig. 12). In this model the proliferation inhibition is believed to be due to blockage of interaction between VEGF and VEGFR-2, indicating that the engineered VEGP-P3(CYC) which contains twisted ends and the disulfide bond to mimic the binding region of VEGF can generate antibodies against the conformational epitope which resulted in the highest neutralization effects.

**DISCUSSION**

Protein-protein interactions trigger a wide variety of cellular pathways, representing a target for drug development. The active or passive binding sites of a protein are confined to a small set of amino acids; therefore smaller sequence like peptides can be designed to simulate these regions, potentially acting as an agonist or antagonist. Synthesis of peptides is easier and cheaper than proteins and recent approaches have brought many new improvements to the delivery and stability of peptide in vivo. Recently, a 17 amino acid cyclic VEGF peptide (79-93) has been shown to VEGF receptor-2 and block angiogenesis [58] and a peptide inhibitor of VEGF receptor KDR/Fk-1 was identified by phage display [80]. Several peptides have been identified that block VEGF/VEGFR interactions and may be potent inhibitors of tumor angiogenesis and metastasis[40]. Peptides which mimic the VEGFR-2 binding site of VEGF were designed to block VEGF:VEGFR-2 interaction,
which has been characterized as the most important for angiogenesis activation.

Here we report that peptides corresponding to the natural VEGF amino acid sequence 102-122 (76-96) (Fig. 1A) which comprises the loop region with the important binding residues of VEGF to its receptor was engineered to better mimic the conformational structure of this sequence in the protein. The two conformational peptide mimics VEGF-P3(NC) and VEGF-P3 (CYC) sequence were specially designed such that the ends were twisted with cysteines artificially inserted to enable cyclization (Fig. 1B). CD analysis confirmed that the VEGF-P3(CYC) assumes characteristics of β-turn II and surface plasmon resonance analysis demonstrate that VEGFR-2 had a higher binding affinity for this cyclic peptide VEGF-P3(CYC) than to the non-cyclized version and/or the natural sequence (VEGF 102-122) indicating the importance of the constrained structure for enhancing binding activity. Competition assay showed that VEGF peptide mimics and VEGF are binding to VEGFR-2 in the same region indicating the peptide mimics could act as antagonist to VEGF. The conformational peptide VEGF-P3(CYC) also demonstrated to bind cells expressing VEGFR2. Antibodies raised in rabbits against VEGF mimic peptides showed to be specific for each peptide and also recognize the native protein rhVEGF. Anti-VEGF-MVF-P3(CYC) demonstrated better affinity for rhVEGF in SPR experiments, indicating that the conformational peptide construction is mimicking better the portion comprising the loop in VEGF.

Next we evaluated whether inhibition of VEGFR-2 cascade signaling would be translated in inhibition of activation of endothelial cell network formation, migration and proliferation. In order to determine these effects we tested VEGF peptide mimics in several in vitro angiogenesis assays. Several in vitro assays have been established to explore VEGF-dependent angiogenesis [81, 82] and we carried out them to test whether the antagonist effect of VEGF peptide mimics could block VEGF action in these assays. Upon VEGF binding, VEGFR-2 dimerizes leading to phosphorylation of tyrosines in the kinase domain which triggers several pathways including endothelial cell proliferation, migration and survival. The inhibitory effects of VEGF peptide mimics on VEGFR-2 phosphorylation were evaluated indicating that they were able to inhibit VEGFR-2 phosphorylation in a cell line (HUVEC) physiologically expressing VEGFR-2, as well as in the over-expressing cell line (293/KDR). We also observed a decrease in p44/42 MAPK phosphorylation which is one of the downstream signaling resulting from VEGFR-2 activation. The designed peptide VEGF-P3(CYC) displayed the best inhibitory effect on phosphorylation assay following the pattern observed with surface plasmon resonance (SPR) experiment, indicating that the design to better mimic conformational structure of VEGF binding site confers better inhibitory effects on VEGF activated signaling. The biotinylated VEGF-P3-CYC peptide was also shown to specifically bind to cells that have different expression of the VEGFR2 and the pattern of binding was coherent with the receptor expression.

To confirm the effect of the VEGF peptide mimics as angiogenesis inhibitors, we used several in vitro angiogenic assays: scratch wound (migration), Matrigel (network formation) and HUVEC proliferation assay (proliferation). All three VEGF peptide mimics were able to inhibit cell migration in the presence of exogenous rhVEGF in the wound assay, indicating that the peptides were effective inhibitors of VEGF:VEGFR-2 interaction. Our results of whether all three VEGF peptide mimics could inhibit the network formation in Matrigel show that the engineered peptide mimic VEGF-P3(CYC) had the largest inhibition compared to the natural sequence and/or the uncyclized peptide. Proliferation of endothelial cells is essential to formation of the new wall vessels and inhibition of HUVEC proliferation was observed in a dose dependent manner with VEGF-P3(CYC) as the most potent inhibitor. The conformational peptide VEGF-P3(CYC) demonstrated the best inhibitory effects, the highest binding affinity, and is most likely due to the loop stabilization by the disulfide bond between the two cysteines. Our biochemical and in vitro experiment results were in agreement and established that VEGF-P3(CYC) had the best potential of inhibiting angiogenesis further emphasizing that receptor:ligand interaction is exquisitely dependent on the conformation of the peptide structure.
Peptides can be used as antigen to generate high affinity antibodies specific for an entire protein. These peptides must include the antigenic determinant residues which usually are hydrophilic and are exposed in the protein [83]. These can be achieved by rational design of peptides that may include few modifications in order to obtain similar conformational structure of the protein. Our primary goal was to evaluate VEGF peptide mimic as angiogenesis inhibitors. However, the VEGF peptide mimic was designed to mimic the binding region of VEGF to VGFR-2 which overlap with a B-cell predicted epitope. We also tested if synthetic VEGF peptide mimics could be used to generated antibodies against native VEGF protein. Since combining the B-cell and the T-cell epitope have allowed us to increase the immunogenicity of peptides, we linked VEGF peptides to a promiscuous T-cell epitope from MVF. These peptides were highly immunogenic in outbred rabbits and purified antibodies against all three VEGF peptide mimics recognized rhVEGF. We quantified the binding affinity of these antibodies by using the SPR experiments. Among anti-VEGF peptide mimics antibodies, anti-MVF-VEGF-P3(CYC) has the highest binding affinity, suggesting that the structural arrangement of VEGF-P3(CYC) were able to generate antibody that can bind tighter to the VEGF. Competitive ELISA results showed that the epitope recognized in VEGF by anti-VEGF peptide mimics are not the same, indicating that the anti-MVF-VEGF-P3 (NC) and (CYC) bind to VEGF by recognition of conformational instead of the linear epitopes.

VEGF neutralizing monoclonal antibodies, such as Avastin®, binds to VEGF preventing VEGF-VGFR-2 interaction and as consequence inhibits angiogenesis. Anti-peptide generated against VEGF peptide mimics were able to specifically recognize the native protein and anti-MVF-VEGF-P3(CYC) demonstrated better affinity to rhVEGF. We further evaluated if these anti-peptide antibodies would block VEGF-VGFR-2 interaction and as expected, the inhibitory effect on HUVEC proliferation of anti-MVF-VEGF-P3(CYC) was slightly better than the other anti-VEGF peptide mimic antibodies.

The design of the peptide, VEGF-P3(CYC) that would mimic a structural binding site of VEGF to its receptor was shown to be important in obtaining a better inhibitory molecule in several in vitro assays as well as in the transgenic mouse model of VEGF+/Neu2.5+/. These findings motivate the development and potential of using VEGF-P3(CYC) as an alternative of peptide therapeutic drug to inhibit angiogenesis. Still, future analysis involving animal models of angiogenesis-dependent tumor formation will give insight into the efficacy of these peptides in inhibiting angiogenesis given the complexity of the tumor microenvironment. In the tumor vicinity stromal cells are involved in angiogenesis and they also can activated other processes like neovascularization in which endothelial progenitor cells (EPC) can initiate the formation of completely new blood vessels [84].

It also will be interesting to observe whether this peptide would have an effect on other important aspects of VEGF signaling via VEGFR-1 in other cells like macrophage or EPC [85, 86]. VEGF-P3(CYC) is not expected to interact with VEGFR-1 once it does not include the VEGF residues responsible for binding to VEGFR-1. However, VEGF-P3(CYC) may interfere with signaling activated by the heterodimer VEGFR-1/VEGFR-2 which can also activate angiogenesis[87, 88]. VEGF-P3(CYC) may also be relevant in inhibiting autocrine activation in cancer cells once cells lines derived from breast cancer had been shown to overexpress VEGFR-2 that can be activated in an autocrine loop via upregulation of VEGF[89].

In conclusion, we showed that VEGF receptor-specific peptides can interfere with the interaction between VEGF and VEGFR-2 inhibiting several VEGF dependent pathways and indicating that VEGF mimic peptide have the a clear potential as candidate in preclinical studies using animal models as alternatives to the development of new anti-angiogenesis therapeutic approaches.
ACKNOWLEDGEMENTS.

The authors are grateful to Mirela Anghelina, MD for skillful technical assistance and Dr Nikanor Moldovan for useful discussions.

REFERENCES:

Table I - Amino acid sequences and molecular weight of VEGF mimic peptides

Table II: Kinetic parameters of VEGF peptide mimics binding to VEGFR-2. Results were obtained from surface plasmon resonance assay where binding of peptide mimics to immobilized KDR was observed at different concentrations.

Table III: Kinetic parameters of anti-VEGF peptide antibodies binding to rhVEGF. Results were obtained from surface plasmon resonance assay where binding of anti-peptide antibodies to immobilized rhVEGF on the chip was observed at different concentrations.

Figure 1A: Representation of the region selected for VEGF peptide mimic. VEGF Crystal structure (pdb 2VPF) with the sequence 102-122 (76-96) shown in detail. VEGF fragment sequence 76-96 is a loop between anti-parallel β-sheets 5 and 6 and contains the residues involved in VEGF-VEGFR-2 interaction.

Figure 1B: Schematic representation of the VEGF peptide mimic design. VEGF sequence 102-122 (76-96) from crystal structure (pdb: 2VPF) with labeled residues. Peptide mimic VEGF-P3(CYC). In black is shown the VEGF-P3(CYC) peptide sequence with labeled residues; arrows show anti-parallel β-sheet orientation based in the crystal structure as represented in (A).

Figure 2: Surface Plasmon Resonance Assay. A-C Sensograms of VEGFR-2 binding to VEGF mimic peptides. VEGF 102-122(A), VEGF-P3(NC)(B) and VEGF-P3(CYC)(C) were immobilized and KDR-Fc at shown concentrations were injected at 10μl/min. D. Competition assay in which KDR-Fc at 500nM were incubated with indicated rhVEGF concentration for 30 min, prior to injection over peptide immobilized onto chip. Percentage was calculated using the equation:100% x (RU_max_sample/ RU_max_control) where control was the KDR-Fc at 500nM. Error bars represent standard deviations of the mean.

Figure 3: Binding of biotinylated VEGF-P3(CYC) peptide to cells expressing VEGFR2. A. HUVEC incubated with streptavidin-Texas Red reagent only (negative control). B. HUVEC incubated with peptide (100 µg) for 2 h, followed by streptavidin for one hour. Accumulation of the fluorescent conjugate on plasma membrane patches and/or in endocytosis vesicles is apparent. C. Staining of cells overexpressing the VEGFR2/KDR receptor. Note increased accumulation in some cells (arrows), while non-expressors remained negative (arrowhead). D. Optical sectioning of the peptide-labeled plasma membrane of a HUVEC (arrow). A, B. Combined phase contrast and fluorescence microscopy. C, D, confocal microscopy. In blue, nuclear staining with DAPI

Figure 4: Inhibition of phosphorylation in HUVEC. Cells were grown in 6 well plates and incubated with inhibitor as indicated for 30 minutes prior to stimulation with rhVEGF (10ng/ml). Representative Western blots of cell lysates that were dissolved in SDS-PAGE, transferred to PVDF membranes and detected phosphorylated KDR (A) and MAPK p44<sup>ERK1</sup> and p42<sup>ERK2</sup> (B),
using specific anti-phospho and anti-total KDR, and anti-p44/42 MAPK and anti-CD31, respectively.

**Figure 5:** Inhibition of KDR phosphorylation in 293/KDR cell line. Cells were grown in 6 well plates and incubated with inhibitor as indicated 30 minutes prior to stimulation with rhVEGF (10 ng/ml). (A) Representative Western blot using 293/KDR cell lysates that were solved in SDS-PAGE, transferred to PVDF membranes and probed using specific anti-phospho and anti-total KDR. (B) Percentage of VEGFR-2 phosphorylation calculated using human Phospho-VEGF R2/KDR DuoSet IC kit (R&D Systems).

**Figure 6:** Inhibitory effects of peptides on (A) HUVEC proliferation and (B) Network formation in Matrigel. The results represent an average from two set of experiments, each one done in triplicate, and expressed as inhibition percent relative to the control (VEGF stimulated cells without inhibitors). Pictures at magnification 40X from light microscopy are shown for the matrigel assay (B) and average sprout points counted using the software imageJ (NIH) are shown in (C). Inhibitors were used at indicated concentration. KDR-Fc is an extracellular domain from VEGFR2 and SU1498 (CalBiochem) is the small molecule tyrosine kinase inhibitor. ECGS represent a positive control where growth condition was used, i.e. medium supplemented with 20%FBS and ECGS (endothelial cell growth supplement). Irrelevant peptide represents a scrambled sequence with similar molecular weight. * Represent p<0.05 using T-test, indicating that only cells treated with irrelevant peptide were not significantly different from the control. Error bars represents SEM

**Figure 7:** HUVEC migration in scratch wound assay using peptides as inhibitors. A. Pictures taken at magnification 40X in light microscopy. B. Average percentage of migrated cells, counted using the software imageJ (NIH) assuming rhVEGF control as 100%. Inhibitors were used at indicated concentration. α-vegf represents a monoclonal antibody demonstrated to block VEGF-dependent pathways. Results shown represent an average of two different experiments each performed in duplicates and error bars represent SEM.

**Figure 8:** Passive treatment using peptides as inhibitors of mammary tumor development in mouse VEGF-+/+Neu2-5-+/. Groups of 6 mice were treated once a week with I.V. injection of 500µg of peptide (in PBS) from 4 to 10 weeks-old. Control represents no treatment. The data are presented as the average tumor size per group and are reported as mm3. Average of largest tumor volume calculated as (long measurement x short measurement2)/2. Statistical analysis demonstrates significance in the growth tumor rate in mice treated with VEGF-P3(CYC) (*p value=0.0074) Error bars represent SEM
**Figure 9: Detection of anti-peptide antibodies by ELISA**. Plate was coated with rhVEGF (1μg/ml) overnight and ELISA was done as usual. Purified anti-peptide antibodies were used as primary antibodies. The control represents a commercially available polyclonal antibody to detect VEGF(R&D Research). Goat anti-rabbit antibody conjugated to HRP was used as secondary antibody at 1:500 dilution factor. Pre represents blood drawn before immunization. Results shown represent an average of three different ELISAs each performed in duplicates and error bars represents SEM.

**Figure 10: Competitive ELISA**. Plates were coated with peptide as indicated. Competitive assay was carried out using constant amount of anti-peptide and several concentrations of peptides as competitors. Results represents average of two different experiments each done in duplicates.

**Figure 11. Cross-reactivity of antipeptide Abs to VEGFR2.** Flow cytometry was used to assess the binding capabilities of the peptide Abs (I) to KDR cells. 50ug of purified Abs from immunized rabbits were used before adding secondary Abs (KDR, I, II). The binding results was compared to cells alone (KDR) and cells with secondary Abs alone (KDR, II). Ab binding was detected by goat anti-rabbit Alexa Flour 488 secondary Abs (II). The x-axis represents fluorescent intensity and the y-axis represents relative cell number.

**Figure 12: Proliferation assay using antibodies as inhibitors.** Anti-VEGF 102-122, P3 NC and P3 CYC correspond to polyclonal antibodies against peptides MVF-VEGF 102-122, MVF-VEGF-P3(NC) and MVF-VEGF-P3(CYC) raised in our laboratory. Antibodies were purified from bleed 3Y+2 or 3Y+3. Rabbit IgG: Rabbit total IgG. MAb anti-VEGF: monoclonal antibody against human VEGF protein (R&D System) shown to block proliferation. The results represent average from two set of experiments, each one done in triplicate and is expressed as inhibition percentage relative to the control (VEGF stimulated cells without inhibitors). Error bars represent SEM.
Table I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>#AA</th>
<th>Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF 102-122</td>
<td>(102)-76-ITMQ IMRIKPHQGQHI8 EMSF-96-(122)</td>
<td>21</td>
<td>2481</td>
</tr>
<tr>
<td>VEGF-P3(NC)</td>
<td>76–ITMQ-79-C-92-GIHQGQHPKIRMI-80-CEMSF-96</td>
<td>23</td>
<td>2726</td>
</tr>
<tr>
<td>VEGF-P3(CYC)</td>
<td>76–ITMQ-79-C-92-GIHQGQHPKIRMI-80-C-EMSF-96</td>
<td>23</td>
<td>2724</td>
</tr>
</tbody>
</table>

Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>ka (1/Ms) x10^4</th>
<th>kd (1/s) x 10^-4</th>
<th>KD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF 102-122</td>
<td>1.4</td>
<td>6.3</td>
<td>45</td>
</tr>
<tr>
<td>VEGF-P3(NC)</td>
<td>1.6</td>
<td>7.5</td>
<td>49</td>
</tr>
<tr>
<td>VEGF-P3(CYC)</td>
<td>1.9</td>
<td>2.0</td>
<td>11</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ka (1/Ms)x10^3</th>
<th>kd (1/s) x10^-3</th>
<th>KD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti VEGF 102-122</td>
<td>6.2</td>
<td>3.6</td>
<td>552</td>
</tr>
<tr>
<td>Anti VEGF-P3(NC)</td>
<td>6.7</td>
<td>1.4</td>
<td>251</td>
</tr>
<tr>
<td>Anti VEGF-P3(CYC)</td>
<td>8.6</td>
<td>1.3</td>
<td>146</td>
</tr>
<tr>
<td>Anti-mAb VEGF</td>
<td>86.1</td>
<td>2.7</td>
<td>29</td>
</tr>
</tbody>
</table>
Figure 2

(A) VEGF 102-122

(B) VEGF-P3(NC)

(C) VEGF-P3(CYC)

(D) rhVEGF concentration

% VEGF-2 bound

VEGF 102-122 | VEGF-P3(NC) | VEGF-P3(CYC)
Figure 3
Figure 4

A

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>100 M</th>
<th>IRRELEVANT</th>
<th>VEGF-P3 (NC)</th>
<th>VEGF-P3 (CYC)</th>
<th>SU1498</th>
<th>VEGF 102-122</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhVEGF</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Anti-pY KDR

Anti-total KDR

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>No VEGF</th>
<th>Only VEGF</th>
<th>VEGF-P3 (NC)</th>
<th>VEGF-P3 (CYC)</th>
<th>Irrelevant</th>
<th>Su1498</th>
<th>KDR-Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>pY42/44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5

A

Inhibitor
100 nM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phosphorylation Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>5%</td>
</tr>
<tr>
<td>+ none</td>
<td>6%</td>
</tr>
<tr>
<td>+ VEGF 102-122</td>
<td>12%</td>
</tr>
<tr>
<td>+ VEGF P3(NC)</td>
<td>12%</td>
</tr>
<tr>
<td>+ VEGF P3(CYC)</td>
<td>12%</td>
</tr>
<tr>
<td>+ Irrelevant</td>
<td>12%</td>
</tr>
<tr>
<td>+ KDR-Fc 100ng/ml</td>
<td>12%</td>
</tr>
<tr>
<td>+ SU1498</td>
<td>12%</td>
</tr>
</tbody>
</table>

B

Antibody

Anti-VEGF

Phosphorylation inhibition

12% 25% 51% 45% 46% -2% 4% None
Figure 6

A

B

C

No VEGF 10
only rhVEGF 39
irrelevant 31
VEGF 102-122 19
VEGF-P3(NC) 9
VEGF-P3(CYC) 7
SU1498 14
KDR-Fc 27
ECGS 91
Figure 7

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Migrated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>44%</td>
</tr>
<tr>
<td>Only rhVEGF</td>
<td>100%</td>
</tr>
<tr>
<td>IRR</td>
<td>91%</td>
</tr>
<tr>
<td>VEGF 102-122</td>
<td>52%</td>
</tr>
<tr>
<td>VEGF-P3(NC)</td>
<td>54%</td>
</tr>
<tr>
<td>VEGF-P3(CY)</td>
<td>55%</td>
</tr>
<tr>
<td>SU1498 mAb</td>
<td>50%</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>79%</td>
</tr>
<tr>
<td>ECGS</td>
<td>128%</td>
</tr>
</tbody>
</table>

B

Wound assay

% migrated cells

- None: 44%
- Only rhVEGF: 100%
- IRR: 91%
- VEGF 102-122: 52%
- VEGF-P3(NC): 54%
- VEGF-P3(CY): 55%
- SU1498 mAb: 50%
- Anti-VEGF: 79%
- ECGS: 128%
Figure 8

The figure shows a line graph that compares the tumor volume (average in mm³) over age (in days) for different groups: VEGF 102-122, VEGF-P3(CYC), VEGF-P3(NC), and Controls. The graph indicates a significant increase in tumor volume over time for all groups, with VEGF-P3(CYC) showing the most rapid increase, followed by VEGF-P3(NC), VEGF 102-122, and Controls. The asterisk (*) indicates a statistically significant difference.
Figure 10

Competitive Elisa - Plate coated with rhVEGF
Anti-VEGF-P3(CYC) dilution - 1/4000

[Graph showing absorbance at 415 nm vs. [inhibitor] nMolar levels for VEGF 102-122, VEGF-P3(NC), VEGF-P3(CYC), and Irrelevant]
Figure 11

<table>
<thead>
<tr>
<th>Key</th>
<th>Name</th>
<th>Parameter</th>
<th>Gate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KDR .001</td>
<td>FL1-H</td>
<td>No Gate</td>
</tr>
<tr>
<td></td>
<td>KDR II 1:200.002</td>
<td>FL1-H</td>
<td>No Gate</td>
</tr>
<tr>
<td></td>
<td>KDR I II 1:200.003</td>
<td>FL1-H</td>
<td>No Gate</td>
</tr>
</tbody>
</table>
Engineered conformation-dependent VEGF peptide mimics are effective in inhibiting VEGF signaling pathways
Daniele Vicari, Kevin C. Foy, Eric M. Liotta and Pravin T. P. Kaumaya

J. Biol. Chem. published online February 14, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.216812

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts