RESEARCH HIGHLIGHT

Inhibition of VEGF/VEGFR1 interaction by a series of C-terminal modified cyclic peptides

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Inhibition of the interaction between vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) is a validated therapeutic strategy of anti-cancer treatment. This approach consists in indirect blockage of the kinase activity on VEGFR with inhibitors of protein-protein interactions, which showed great interests in oncology. The FDA approved anti-cancer agents bevacizumab (Avastin®) and ziv-aflibercept (Zaltrap®) bind specifically to VEGF are from anti-VEGF strategy. The very recently approved agent ramucirumab (Cyramza®), a recombinant humanized monoclonal antibody that specifically binds to VEGFR2 is from anti-VEGFR strategy. Based on a cyclic peptide antagonist of VEGFR1 designed from VEGF fragments, we developed, by a new synthesis process, a series of C-terminal modified cyclic peptides to improve their receptor binding ability. Three of such peptides with aromatic groups showed greatly increased VEGFR1 binding affinity in a competition ELISA-based test. This research highlight discusses the processing and findings of the recent study.

Keywords: VEGF; VEGFR; angiogenesis; cyclic peptides

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Cancer is characterized by uncontrolled cell proliferation with an ability to spread from one organ or part to another, leading to the formation of tumor mass and metastasis. Cancer is one of the most dangerous threats to human health. At the same time, anti-cancer research has continuously improved, evolving during the past decades from the use of nonspecific cytotoxic agents to the development of selective, mechanism-based targeted therapeutics [1]. Since tumor growth and metastasis require new blood vessels formation (angiogenesis) for the supply of nutrients and oxygen and the removal of waste products, angiogenesis constitutes a new target in anti-cancer research [2].

Angiogenesis, involving the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels, plays an important role in cancer progression and has been recognized as one of the six hallmarks of cancer [3]. It is regulated tightly by pro- and anti-angiogenic factors. The pro-angiogenic factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placenta growth factor (PlGF), angiogenin, transforming growth factor (TGF)-α, TGF-β and tumor necrosis factor (TNF)-α [4]. The binding of these factors to their receptors induce receptor dimerization, resulting in the tyrosine kinase domain activation and downstream signalization to eventually cell progression [5]. Among these pro-angiogenic factors, the VEGF and their receptors (VEGFR1 and VEGFR2) play a critical role in the

field of neoplastic vascularization [6].

Anti-angiogenic therapy interferes with various steps in this process. For example, at the level of VEGF, bevacizumab (Avastin®) is a humanized monoclonal antibody that specifically recognizes and binds to VEGF [7]. Similary, ziv-aflibercept (Zaltrap®) is a recombinant fusion protein consisting of the extracellular domains of human VEGFR1 and VEGFR2, which binds VEGF and PlGF [8]. When VEGF or PlGF is attached to these inhibitors, it is unable to activate the VEGF receptors and their downstream signaling pathways. At the level of receptors, ramucirumab (Cyramza®) approved by FDA in 2014 is a recombinant humanized monoclonal antibody that specifically binds to VEGFR2 and blocks the receptor to interact with VEGF [9]. Other angiogenesis inhibitors, including sorafenib (Nexavar®), sunitinib (Sutent®) and pazopanib (Votrient®) inhibit VEGFR1 and VEGFR2 and also some other receptors tyrosine kinase activity [10, 11]. In the clinic, anti-angiogenic therapy reduces tumor progression and metastasis in various types of cancers. However, antibodies or tyrosine kinase inhibitors have their proper drawbacks either with a high pharmacokinetic variability (antibodies) or a low specificity (tyrosine kinase inhibitors) [12]. Angiogenesis inhibition therapy does not necessarily kill tumor cells but instead may prevent tumors from growing, and most frequently, it is combined with additional therapies, especially chemotherapy [13, 14].

The development of small antagonists blocking protein-protein interaction thus draws more and more attention [15]. Such antagonists include rationally designed peptides [16] and library screened peptides or non-peptidic chemical molecules [17]. Peptides resembling more the natural protein would have higher specificity for the target protein and lower cellular toxicity. Driven by the progress of protein-protein interaction structure determination, computer homology modeling and also the chemical synthesis technology, many anti-angiogenic peptides have been developed [18]. Several peptides have shown activity in pre-clinical models of cancer or have been tested in clinical trials. Based on structural data of the complex of VEGF binding on the second domain (D2) of VEGFR1 [19], which is the main VEGF binding site, rational design [20-22] and library screening [23-25] have been pursued to develop antagonists of VEGF/VEGFR. The molecules optimized from the hits thus designed or identified have shown effective anti-angiogenic and anti-cancer activities.

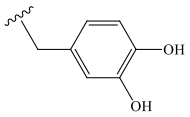
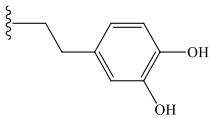
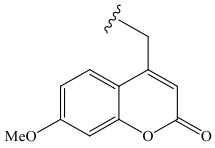
In our laboratory, we particularly focused on α1 helix and β3-β4 loop of VEGF implicated in VEGFR1 binding [20]. Our previous work has led to a cyclic peptide antagonist c[YYDEGLEE]-NH_2, combining the residues of β3-β4 loop and two important tyrosine residues of the α1 helix of VEGF. This cyclic peptide has been shown capable of antagonizing VEGF binding to VEGFR1 and inhibit VEGF induced receptors phosphorylation, endothelial cell signalization pathway, cell migration and capillary-like tube formation [20, 21]. Starting from this cyclic peptide, we described in our recent study entitled "Design and Synthesis of C-Terminal Modified Cyclic Peptides as VEGFR1 Antagonists" the optimization of synthesis process, the design of new peptides and the evaluation of peptides VEGFR1 binding ability and the study of the structure-activity relation [26].

Based on the cyclic peptide c[YYDEGLEE]-NH_2 binding with VEGFR1 D2 domain model after manual docking and energy minimization [20] and the mutation of the second Tyr by a Lys residue [21], we designed and synthesized a series of peptides c[YKDEGLEE]-NH-R with modifications on the C-terminal end by aliphatic and aromatic groups (R). The C-terminal elongation is expected to create new interactions with the D2 domain of VEGFR1. A new synthetic pathway combining solid phase peptide synthesis and solution phase cyclization was described. The synthesis began with the first Gly residue loaded on an acid labile 2-chlorotrityl resin. The second glutamic acid derivative (Fmoc-Glu-NH-R) prepared in solution and the following residues were coupled to the resin following microwave-assisted solid phase peptide synthesis procedure. Then, the peptides were cleaved by 2% TFA in dichloromethane, cyclized in solution, and fully side chain deprotected at the final step. This synthetic process eliminated guanidine formation during on-resin cyclization [21] and avoided the use metal catalyst (Pd^0) required in the previous synthesis pathway [20, 21]. We have recently shown that trace of metal ions would greatly influence biochemical and biological assay results [27].

About two dozen peptides with additional aliphatic or aromatic groups at the C-terminal position of the parent peptide have been obtained. A competition ELISA type test was applied to determine the peptide VEGFR1 binding ability [28]. The assay permitted peptides and biotinylated VEGF to bind competitively with recombinant human VEGFR1 extracellular domains, which were adsorbed beforehand on the surface of a 96-well microplate. Then the percentages of biotinylated VEGF displaced by peptides were calculated according to chemiluminescence detected of the remaining btVEGF *via* an HRP-conjugated streptavidin. Among these modified peptides, three peptides showed greatly increased binding affinity: two peptides with C-terminal substitution of benzyl groups linked through one (Peptide **1**) or two methylene (Peptide **2**) and the third peptide with C-terminal substitution of a coumarinyl group linked by a methylene (Peptide **3**). All three peptides have shown dose dependent inhibition of VEGF/VEGFR1 binding

Table 1. Dose dependent inhibition of selected peptides on VEGFR1 binding measured by ELISA. The values are the average of at least 3 tests each in triplicate. NA means no activity.

Tyr-Lys-Asp-Glu-Gly-Leu-Glu-Glu-NH-**R**

Peptide	R	Displacement (%)		
		100 µM	50 µM	30 µM
Ref	H	12	7	NA
1		58	37	14
2		53	31	NA
3		68	41	11

(Table 1). Structure-activity relationship was discussed in our published paper [26].

The structure of peptide **2** (Table 1) alone in solution has been studied recently by NMR, and the determination of the structure of its complex with the D2 domain of VEGFR1 is now in progress. Compared with the previously published docking complex of the parent peptide c[YYDEGLEE]-NH_2 with D2-VEGFR1, this new peptide (peptide **2**) mimics better the β3-β4 loop of VEGF in the new docking model, and interestingly, it creates one more hydrogen bond with D2 VEGFR1 than the parent peptide. Furthermore, the peptide **3** featuring a fluorescent coumarinyl group at the C-terminal end will be a useful tool in co-crystallization and biological imaging studies. These peptides have also been shown recently able to inhibit VEGF-induced endothelial cell tube formation. Further anti-angiogenic and anti-tumor cellular assays are now undergoing. The structure and biological results will be published later.

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Conflict of Interests

The authors declare no conflict of interests.

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