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# Recent developments in the inhibition of angiogenesis: examples from studies on platelet factor-4 and the VEGF/VEGFR system

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**Abstract**

Inhibition of angiogenesis is an important strategy to block tumor growth and invasion. We discuss herein results from our ongoing investigations on platelet factor-4 (PF-4) and the VEGF/VEGFR system. Platelet factor-4 (PF-4) is an anti-angiogenic ELR-negative chemokine. PF-4 inhibits endothelial cell proliferation and migration, and angiogenesis in vitro and in vivo. We have studied the structure and anti-angiogenic activities of a C-terminal fragment of PF-4 named PF-4 CTF. This molecule retains anti-angiogenic activity, blocks the interaction of angiogenesis factors with their receptors and may also be improved by mutation or domain-swapping. It seems, therefore, to be a good candidate for further development. Furthermore, we have developed a cyclic vascular endothelial growth inhibitor (Cyclo VEGI) from the structure of VEGF-A. In aqueous solution, cyclo-VEGI adopts an $\alpha$ helix conformation. Cyclo-VEGI inhibits binding of iodinated $VEGF_{165}$ to endothelial cells and angiogenesis. Furthermore, cyclo-VEGI significantly blocks the growth of established intracranial glioma in nude and syngeneic mice and improves survival.


*Keywords:* Angiogenesis inhibitors; Cancer; Cardiovascular disease

## 1. Introduction

Vascular development is regulated by soluble factors, proteases, cell adhesion molecules and integrins [1]. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) and are amongst the principal soluble regulators [1–3]. VEGF and FGF-2 bind to specific cell surface receptors and induce endothelial cell proliferation, migration and differentiation. Heparan sulfate proteoglycans (HSPGs) modulate the binding of VEGF and FGFs with their respective receptors.

HSPGs also stabilize the FGF-2/FGF receptor complex, protect FGF-2 from degradation or facilitates FGF-2 dimerization For review see [2]. This indicates that inhibitors may possibly interfere with FGF-2 or VEGF activity in two ways either directly by blocking the interaction of VEGF or FGF with their receptors or by disrupting the bystander effect of HSPGs for efficient growth factor binding.

Numerous angiogenesis endogenous inhibitor molecules such as thrombospondins, endostatin, angiostatin, or tumstatin have been identified [1–4]. In addition, a variety of inhibitor molecules obtained by chemical synthesis or by recombinant technology have been obtained some of which are in clinical development [4].

In this review, we aim to discuss some insights into angiogenesis inhibition obtained from our own studies. We will first discuss platelet factor-4 (PF-4) a member of the C–X–C chemokine family which is found in platelets and megacaryocytes [3]. We will then discuss another group of inhibitor molecules derived from studies of the VEGF/VEGFR system and give an example from our own investigations.

## 2. PF-4 and derived molecules

### 2.1. Anti-angiogenic activities

Platelet factor-4 (PF-4) is a tetrameric molecule made up of 4 subunits each of 70 amino acid length [5]. The four lysine

*Abbreviations:* BCE, bovine capillary endothelial cells; cyclo-VEGI, cyclic vascular endothelial growth inhibitor; CXCR3-B, CXC chemokine receptor 3 variant B; FGF, fibroblast growth factor; HSPGs, heparan sulfate proteoglycans; HUVEC, human umbilical vein endothelial cell; PF-4, platelet factor-4; VEGF, vascular endothelial growth factor

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residues required for heparin binding (lysines 61, 62, 65, and 66) are found on a single α-helix that spans from residue 61 to 70 found on each PF-4 monomer [6,7]. Together with the arginines, this forms, at PF-4's surface (with exception of lysines 14 and 50) a positively charged ring [7,8]. A stack of two β-sheets that are formed by the rest of the molecule constitutes the core of the PF-4 tetramer [8].

PF-4 inhibits potently the proliferation and migration of vascular endothelial cells in vitro [8–11]. In addition, PF-4 also blocks tubulogenesis in three-dimensional collagen type I cultures [11]. Furthermore, PF-4 also abrogates the formation of vascular channels from embryoid bodies [12]. Angiogenesis in vivo in matrigel or collagen plugs when implanted in mice is inhibited by PF-4. In addition, when recombinant PF-4 is injected in mice, it is targeted, in vivo, to endothelial cells that undergo active angiogenesis [13,14]. PF-4 has an antitumor effect in vivo through inhibition of angiogenesis [15,16]. This results in an decrease of vessels density in tumors is but not of tumor cell proliferation.

Furthermore, human glioma cells transduced with viral vectors containing PF-4 cDNA form only hypovascular tumors in vivo and only grow slowly [17]. These observations indicate that full length PF-4 exerts anti-angiogenic effects in vitro and in vivo.

A C-terminal PF-4 fragment (PF-4 CTF) of 24 amino acids from the C-terminus completely inhibits endothelial cell proliferation and angiogenesis in vitro induced by either FGF-2 or VEGF [18–20]. PF-4 also impairs vessel sprouting and tube formation in the ex vivo the aortic ring assay [20]. Angiogenesis in vivo in collagen sponges implanted subcutaneously in mice is also blocked by PF-4 CTF [20]. Finally, PF-4 CTF also inhibits tumor angiogenesis in intracranially implanted U87 glioma in immunodeficient mice.

We have also generated a derivative from PF-4 CTF named PF-4 DLR through domain-swapping of the DLQ motif which has been replaced by DLR. PF-4 DLR blocks more potently than PF-4 CTF, endothelial cell proliferation and migration FGF-2 or VEGF binding [21].

It is also a more potent inhibitor in various angiogenesis assays ex vivo and in vivo such as the CAM assay or tumor angiogenesis assays. For example, it inhibits tumor angiogenesis, tumor growth and tumor recurrence in intracranial glioma models in immunodeficient or syngeneic mice [21]. Furthermore, it is able to synergize in these models with other anti-invasive molecules such as the hemopexin fragment of MMP2 (PEX) and is active in inhibiting tumor recurrence [22].

Other PF-4-derived molecules such as PF-$4^{17–70}$ or the PF-4 heparin-binding-deficient mutant (rPF-4 241) will not be discussed here. The reader may consult the literature related to the subject [23].

### 2.2. Mechanisms of action of PF-4 molecules

PF-4 inhibits endothelial cell growth by slowing-down passage through the S phase [10], inhibits MAP kinases but not PKB/Akt and induces, in HUVECs, p21(Cip1/WAF1) [24,25]. Three potential mechanisms may be invoked to explain the effect of PF-4 on endothelial cells (Fig. 1):

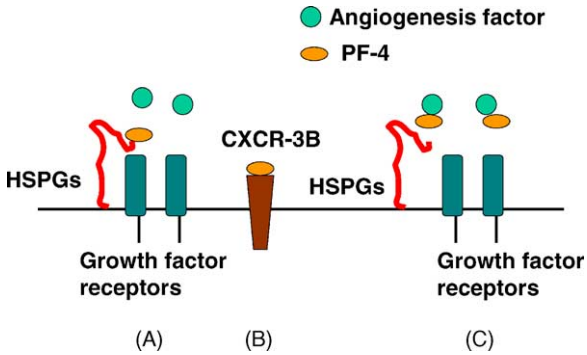


Fig. 1. Mechanisms of action of PF-4 and derived molecules. Three mechanisms are proposed: (A) direct binding of PF-4 to heparan sulfate proteoglycans (HSPGs) and inhibition of the cooperative effect; (B) activation of the chemokine receptor CXCR-3B; (C) direct binding of PF-4 to angiogenesis factors and inhibition of receptor activation.

(1) Competition of the cooperative effect of heparan sulfate proteoglycans on FGF or VEGF binding: PF4 binds to heparin and heparan sulfate and is able to displace growth factors from their proteoglycan binding sites.

    PF-4, PF-4 CTF or PF-4 DLR also block binding of FGF-2 to high affinity receptors and internalization. It has been shown that receptor binding of FGF-2 is facilitated by HSPGs [2]. Thus, the inhibitory effect of PF-4 molecules is partially due to blockade of the cooperative effect of HSPGs on the binding of FGFs to receptors. Nevertheless, it has also been shown that high affinity binding to growth factor receptors is, to a significant extent, independent of heparan sulfates [2]. Therefore, other mechanisms of inhibition also exist [20].

(2) Direct interaction with FGFs or VEGFs: we have demonstrated that PF-4 or PF-4 CTF and FGF family members physically associates and form a 1:1 complex. This changes the sphericity of the FGFs resulting in an inhibition of dimerization and receptor binding [11,19,20]. It seems that the minimal sequence for PF-4/FGF interactions is the 24 amino acid C-terminal sequence. Smaller fragment derived from this sequence are not active at concentrations at which PF-4 CTF shows activity [18–20]. It has been recently shown that the direct interaction between FGF-2 and PF-4 may be reinforced by heparin that additionally bridges between the two molecules [26].

    Interaction with PF-4 is not restricted to FGF family members. Indeed, full length PF-4 also binds surface immobilized $VEGF^{165}$ [27] (and our unpublished observations).

(3) Activation of chemokine receptors: recently, a cell surface receptor for PF-4 has been identified [28].

This receptor, CXCR3-B, is an alternative splice variant of CXCR3 which is expressed in human microvascular endothelial cells. PF-4 binds this receptor with high affinity ($IC_{50}$ value of 1.85 nM) which leads to an increase of cAMP and cell cycle inhibitor p21CIP1/WAF. CXCR3-B not only binds PF-4 but also other chemokines including Mig, IP10 or I-TAC. In addition, CXCR3-B is not specific for endothelial cells because other cell types such as the renal carcinoma ACHN cell line express CXCR3-B.

Thus, PF-4 is able to exert its activity by three independent mechanisms. Two are extracellular and involves binding to the ligands or co-receptors (HSPGs). One is cellular and involves the activation of specific cell surface receptors (CXCR-B). It is not clear which mechanism is predominant. The different mechanisms are possibly concurrent and are dependent on the environment of the vasculature. It is also not established, as yet, whether PF-4-derived fragment or molecules are also able to induce CXCR3-B receptor activation.

## 3. Inhibitors of VEGF receptor activity

An increasingly growing number of molecules are identified or developed presently that inhibit the activity of the VEGF receptors [29–31].

Besides Avastin, a humanized monoclonal antibody that has shown remarkable results in phase III clinical trials, promising candidates are receptor tyrosine kinase inhibitors for the VEGFR2 receptor tyrosine kinase, anti-VEGFR antibodies (imclone) or VEGF Trap, a decoy receptor that combines binding domains from VEGFR1 and R2 and possess very high affinity for VEGF-A [29,30]. Small chemical molecules that impair the interaction of VEGF with VEGF receptors haven also been described [31].

In our laboratory, we have devised a strategy to inhibit VEGF activity by designing a series of cyclic peptides that comprises the interacting loop of VEGF-A with VEGF-R2 (KDR) (residues 79–93 within β5–β6) [32].

The peptides were screened for inhibitory activity using the VEGFR binding assay on CHO cells transfected with

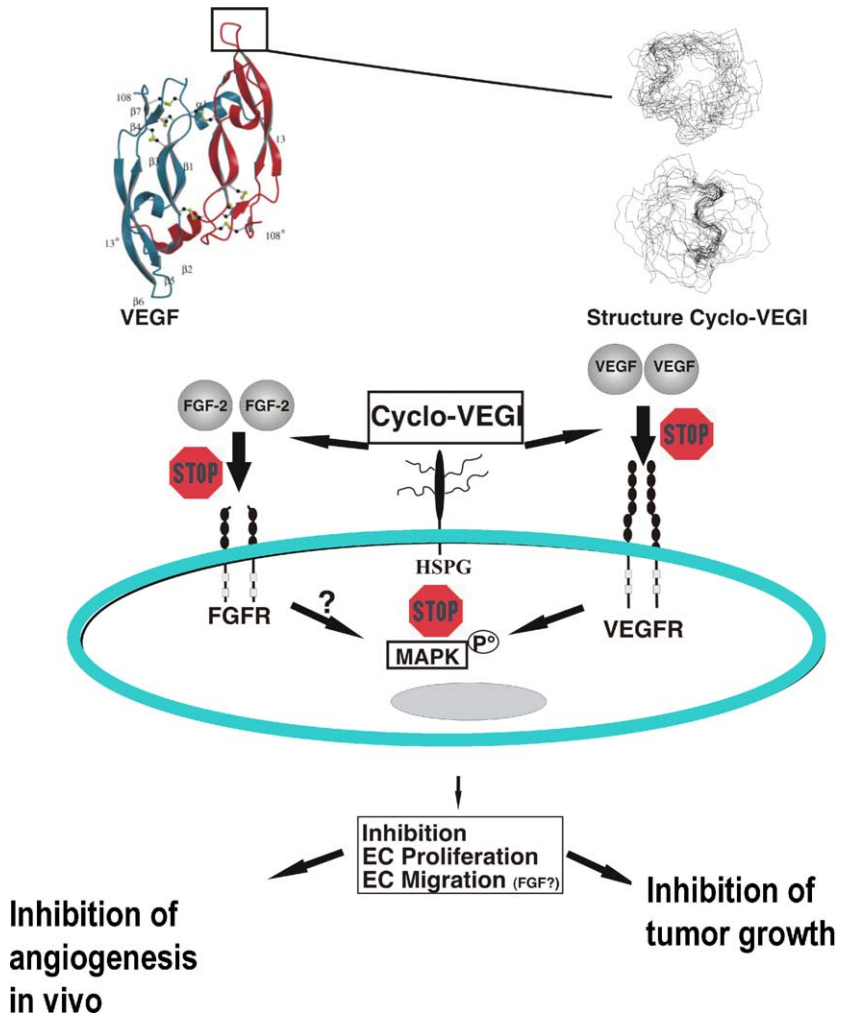


Fig. 2. Mechanisms of action of cyclo-peptidic angiogenesis inhibitors derived from the structure of VEGF.

VEGFR2. We found that only cyclic peptides but not linear peptides competed for VEGF binding in CHO VEGFR2 positive cells. One of these cyclic molecules of the size of 17 amino acids, we designated as cyclic vascular endothelial growth inhibitor (Cyclo-VEGI), exhibited the most potent inhibitory activity. Interestingly, cyclo VEGI also inhibited the binding of VEGF to VEGFR1 and also that of fibroblast growth factor-2 (FGF-2) to FGF receptor-1 to a lesser extent (Fig. 2).

Cyclo VEGI potently inhibited endothelial cell proliferation of bovine capillary endothelial cells (BCE) and human umbilical vein endothelial cells (HUVECs) and endothelial cell migration in monolayer migration assay (wounding assay). Furthermore, the molecule also inhibited in vivo angiogenesis in the differentiated chicken chorio-allantoid membrane assay (CAM assay).

To better understand the inhibitory effect of cyclo-VEGI, we resolved its three-dimensional structure by nuclear magnetic resonance (NMR) spectroscopy. NMR analysis revealed that cyclo VEGI possesses a unique structure. It is a cyclic peptide that contains an α-helicoidal domain within its structure. The three basic amino acids that are involved in the interaction of VEGF with KDR are located at the α helicoidal structure ($Arg^{82}$, $Lys^{84}$, $His^{86}$). This may create an amphipathic surface that is likely to be involved in the inhibitor effects of cyclo-VEGI. Studies are now underway to optimize the structure of cyclo VEGI to increase activity (increase of affinity, stability, etc.).

Cyclo-VEGI shows inhibitory activity in vivo. Cyclo VEGI inhibit growth of intracranial transplanted glioma U87 when given continuously via minipumps. Furthermore, growth of syngeneic intracranial GL 261 tumors in Balb/c mice are also inhibited by the molecule.

All in all, cyclo-VEGI seems to be a promising candidate for further development. Studies are now underway to optimize cyclo VEGI to increase activity (increase of affinity, stability, etc.).

## 4. Concluding remarks

In this article, we discussed results from our ongoing studies on the inhibition of angiogenesis that include platelet factor-4 and inhibitors derived from studies of the VEGF/VEGFR system.

The role and the mechanism of action of PF-4 is now better understood. Mechanisms involving binding to growth factors, co-receptors or chemokine receptors have been identified. PF-4 may play a role in tumor angiogenesis and other neoangiogenic processes. PF-4 may also be developed into a therapeutic agent by the optimization of C-terminal-derived fragments.

Blockade of VEGF is a major avenue for inhibition of angiogenesis. We have developed cyclopeptidic inhibitors of VEGF that are derived from the interacting loop of VEGF with KDR. One of these molecules, named cyclo-VEGI, has unique structural features and constitutes a promising candidate for further development.

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