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Mini review

# Molecular pathways of angiogenesis inhibition

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**Abstract**

A large body of evidence now demonstrates that angiostatic therapy represents a promising way to fight cancer. This research recently resulted in the approval of the first angiostatic agent for clinical treatment of cancer. Progress has been achieved in decrypting the cellular signaling in endothelial cells induced by angiostatic agents. These agents predominantly interfere with the molecular pathways involved in migration, proliferation and endothelial cell survival. In the current review, these pathways are discussed. A thorough understanding of the mechanism of action of angiostatic agents is required to develop efficient anti-tumor therapies.


*Keywords:* Angiogenesis; Angiostatic agent; Signaling; Endostatin; Angiostatin; Tumstatin; PF4; 16K hPRL; Apoptosis; Proliferation; Migration

## Angiogenesis as a therapeutic target

Angiogenesis is considered a promising target in the treatment of cancer [1,2]. Over the last 15 years, major progress has been made in the development of therapies based on targeting tumor angiogenesis. Currently, several angiogenesis inhibitors are approved for the treatment of cancer and many are in late-stage clinical testing. Most of these compounds act indirectly either by clearing angiogenic growth factors from the circulation or by blocking or preventing the receptors/signaling pathways of these growth factors. Another group of angiogenesis inhibitors are the direct angiostatic compounds with a direct effect on the endothelium, affecting cellular regulatory pathways, independently of the tumor cells. It is likely that a therapy which directly targets the genetically stable endothelial cells (EC) will reduce the risk of developing drug-induced resistance. Nevertheless, the development of this promising class of agents is lagging behind, maybe because of the lack of sufficient knowledge on the mechanism of action of these compounds. The main purpose of this review is to summarize the recent progress made in decrypting the cellular signaling pathways induced by this class of angiogenesis inhibitors. Global comprehension of the mechanisms of action of these direct inhibitors is required to develop efficient angiostatic therapies.

## Molecular pathways used by angiostatic agents

Over the last decade, the mechanisms of action of angiogenesis inhibitors have been subject of considerable attention and significant advances have been made. In this review, we focus on several angiogenesis inhibitors that can be categorized in the group of direct inhibitors of angiogenesis, and of which the signaling pathways are now partially identified (Table 1).

### *Endostatin*

Endostatin is an angiostatic 20 kDa internal fragment of type XVIII collagen [3]. The mechanism of action of endostatin has been extensively studied over the last years. Many cell surface proteins have been identified to interact with endostatin. These included integrins ($\alpha_5\beta_1$ and to an extent also to $\alpha_v\beta_3$ and $\alpha_v\beta_5$, [4]) and glyplican −1 and

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Table 1
Biological processes affected by angiostatic factors

| Biological processes | Angiostatic factors | References |
|---|---|---|
| *Migration* | | |
| MMPs | Endostatin, PF4 | [7,32] |
| Plasmin | 16K hPRL | [45] |
| Integrins | Endostatin, tumstatin, angiostatin, TSP-1 | [4,13,18,29] |
| *Proliferation/cell cycle arrest* | | |
| MAPK pathway | Endostatin, 16K hPRL | [10,47] |
| G1/S arrest | Tumstatin, angiostatin | [14,19] |
| Cip/Kip family | PF4, I6K hPRL | [34,46] |
| Cyclin D | Endostatin, 16K hPRL | [9,49,46] |
| G2/M arrest | 16K hPRL | [46] |
| *Survival/apoptosis* | | |
| Extrinsic pathway | Angiostatin, TSP-1, 16K hPRL | [20,31,49] |
| Intrinsic pathway | Endostatin, angiostatin, TSP-1, 16K hPRL | [12,20,30,49] |
| Miscellaneous | Turnstatin, angiostatin | [16,21] |

−4 [5] resulting in altered adhesion and migration. It has also been demonstrated that endostatin interferes with VEGF-R2 (KDR/FLK1) leading to reduced cell motility, proliferation and survival [6]. In addition, endostatin forms a stable complex with matrix metalloproteinase (MMP) −2 and inhibits its activity by masking MMP-2 catalytic domain [7]. To impair EC migration, endostatin is also capable of disturbing cell–matrix interaction by inducing tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin [8]. An other mechanism of endostatin mediated inhibition of angiogenesis is through arrest of EC in cell cycle. Endostatin blocks cell cycle progression at the G1/S transition by down-regulating the transcriptional activity of the cyclin-D1 promoter via the lymphoid enhancer-binding factor 1 site [9]. It has been demonstrated that endostatin interferes with proliferation by reducing the mRNA level of several proliferative genes including MAPK1, MAPK2 or c-myc [10]. In addition, endostatin disturbs the survival/death balance by inhibiting the anti-apoptotic signal induced by the phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase B (PKB) pathway, and inducing phosphorylation of PKB [11]. Alternatively, endostatin activates pro-apoptotic pathways through induction of caspase-9 activation by reducing the level of the anti-apoptotic proteins Bcl-2, Bcl-Xl, and Bad [12]. By gene array- and proteomic-analysis Abdollahi et al. have provided insight in the molecular signaling induced by endostatin in human dermal microvascular EC. This study has shown that a very large number of genes (12% of the 74,834 genes represented on the chip) has a significant alteration of expression in response to endostatin treatment. Among these genes, many known pro-angiogenesis genes (VEGF-A, MMP-9, HIF-1α) are up-regulated while anti-angiogenic genes, such as TSP-1, TSP-2, kininogen, and vasostatin, are down-regulated.

### *Tumstatin*

Collagen XVIII is not the only collagen of which degradation fragments were found to display angiostatic activity. Tumstatin is an angiogenesis inhibitor that is composed of the 28 kDa fragment of type IV collagen [13]. The binding to αVβ3 integrin is pivotal for the anti-angiogenic activity, leading to inhibition of proliferation of EC (G1 arrest) and induction of apoptosis by up-regulation of caspase-3 activity [14]. It is proposed that through the interaction with αVβ3 integrin, tumstatin inhibits activation of FAK, PI3K, PKB, and mammalian target of rapamycin (mTOR). This cascade of signalling leads to prevention of the dissociation of the eukaryotic initiation factor 4E protein (eIF4E) from the 4E-binding protein 1, resulting in inhibition of protein synthesis and finally to cell death [15]. Based on these results, tumstatin appears to acts as an EC specific protein synthesis inhibitor.

### *Angiostatin*

Angiostatin is a cleavage product of plasminogen and is composed of the first four-kringle domains [16]. Angiostatin has been shown to bind to several EC cell surface proteins. Moser et al. have described that the ATP synthesis by ATP synthase F1F0 is inhibited by angiostatin. This attack of the energy system ultimately leads to a caspase-mediated apoptosis [17]. In addition, angiostatin binds multiple cell surface targets including αVβ3 integrin and angiomotin [18]. Treatment with angiostatin inhibited migration in angiomotin-expressing cells but not in control cells, suggesting that angiostatin inhibits cell migration by interfering with angiomotin activity in endothelial cells. Angiostatin inhibits the proliferation of EC by down-regulating the protein level of cyclin-dependent kinase (cdk) 5, a cdk absent in quiescent endothelial cells but induced after treatment with bFGF [19]. Angiostatin also acts by up-regulating the mRNA level of FasL and reducing the level of c-Flip which activates the extrinsic apoptotic pathway (death receptor pathway). In addition, treatment of HUVEC with angiostatin also results in induction of the intrinsic pathway (mitochondrial pathway) by inducing p53, Bax, and tBid. The up-regulation of these molecules leads to the cytochrome-*c* release from the mitochondria and the activation of caspase-9 [20]. In addition to this well described mechanism, angiostatin also induces apoptosis by anoikis. Anoikis is the detachment of cells from the matrix which results in execution of apoptosis. Upon the binding of angiostatin to EC, RhoA is activated. This activation is followed by a transient increase in ceramide level which leads to an actin stress fiber reorganization and finally to the cell detachment [21]. To illustrate the close relationship between proliferation and apoptosis, it has been demonstrated that the angiostatin-induced cell death is strictly restricted to proliferating EC [22]. Finally, Chen et al. have compared the global action of angiostatin in EC. By microarray techniques, they found that the expression of

189 genes is altered by treatment with angiostatin (less than 1% of the genes represented on the chips). These genes are mainly involved in growth, apoptosis, and migration but also in inflammation [20]. Interestingly, angiostatin induces expression of adhesion molecules (ICAM-1, E-Selectin) while microarray analyses suggest that endostatin down-regulates this category of genes [23]. In addition, while the apoptotic pathways activated by angiostatin and endostatin are roughly similar, there is a high discrepancy between the identity and the percentages of genes regulated by these two factors.

*Thrombospondin-1*

Thrombospondin-1 (TSP-1), a multifunctional extracellular protein, was the first naturally occurring angiostatic agent to be discovered [24]. Unlike many angiostatic agents (endostatin, angiostatin, and the 16K fragment of prolactin or tumstatin), TSP-1 is not a cleavage product and is effective as native, full-length protein. It is stored in α-granules of platelets, where it is complexed with TGFβ1 [25]. The angiostatic activity of TSP1 has been localized to the procollagen domain and type-1 repeat (TSR) sequence of the molecule. This region has been found to interact with several candidate receptors. One of these receptors is CD36, which mediates TSP-1 inhibition of migration [26] and induction of apoptosis [27]. A recent report has shown that TSP-1 is also able to inhibit VEGF-induced migration in HUVEC lacking CD36. In this report, β1-integrins were identified as important receptors in TSP-1 mediated inhibition of EC migration. It was also found that PI3K was essential to mediate this inhibition [28]. In addition to the TSR region, TSP-1 also contains an RGD motif that allows the binding to αvβ3 integrin [29], as has also been described for endostatin, tumstatin, or angiostatin. Finally, the induction of apoptosis in EC is correlated with the up-regulation of Bax, the decrease of Bcl-2, and the activation of caspase-3 via the intrinsic pathway [30]. As well as described for angiostatin, Volpert et al. have demonstrated that TSP-1 activates an extrinsic apoptotic pathway by up-regulating expression of Fas-ligand [31].

*Platelet factor-4*

Platelet factor-4 (PF4) is a chemokine known to inhibit angiogenesis. Naturally secreted by platelets, it binds to and blocks heparin-like glycosaminoglycans on the EC surface. PF4 is also able to inhibit the EC migration by blocking the up-regulation of MMP-1 and MMP-3 induced by thrombin [32]. On the other hand, expression of tissue inhibitors of metalloproteinase (TIMP) TIMP-1 and TIMP-2 is not affected by PF4 [33]. The PF4 interference with the cell cycle has also been studied. Treatment with PF4 impairs pRB phosphorylation by reducing cyclin E-CDK2 activity. This reduced activity has been linked with the p21cip1-induced overexpression in PF4 treated EC [34]. In addition, PF4 promotes the expression of E-Selectin in HUVEC. Data provide direct evidence that the nuclear factor κB (NF-κB) activation is required for PF4 expression of E-Selectin [35].

Based on the β-sheet structure of PF4 and other angiostatic agents such as the bactericidal/permeability increasing protein-1 (B/PI), our laboratory has developed the *de novo* designed angiostatic peptide, called anginex [36]. This peptide is able to target tumor endothelial cells and inhibits tumor growth in animal models [37,38]. In addition, it has been demonstrated that anginex induces permeabilization of the endothelial cell membrane and its subsequent disruption [39]. In addition, we have demonstrated that anginex induces up-regulation of adhesion molecules (ICAM-1, VCAM-1, and E-Selectin). Furthermore, we have recently described that galectin-1 functions as the receptor for anginex, and that this protein is crucial for tumor angiogenesis [40]. It remains to be seen whether PF4 can signal through this receptor also.

*16-kDa N-terminal fragment of prolactin*

In 1991, Ferrara et al. showed that the 16-kDa N-terminal fragment of human prolactin (16K hPRL) can inhibit the growth of endothelial cells [41]. This property is not shared with the full-length prolactin and is not mediated by the PRL receptor. So far, it is unknown how 16K hPRL interacts with EC. Very recently, Nguyen et al. have demonstrated that 16K hPRL possesses a 14-amino-acid sequence having the characteristics of a tilted peptide [42]. Tilted peptides are short helical peptides characterized by a peculiar distribution of hydrophobic residues: they are amphipathic and their net hydrophobicity increases from one end of the helix to the other [43]. This sort of peptide is known to be able to destabilize the cellular membrane. One could speculate that this tilted peptide could interfere with the endothelial membrane and trigger an intracellular message. The mechanism of the anti-migratory properties of the 16K hPRL has been partially characterized. Plasmin is a broad-spectrum protease that also hydrolyzes many extracellular proteins, the most notable of which is fibrin. Plasmin is produced as an inactive precursor called plasminogen. uPa and tPA (tissue-type plasminogen activator) are two proteases with high affinity for plasminogen. The activation of plasminogen into plasmin could be negatively regulated by physiological inhibitors, namely plasminogen activator inhibitor (PAI)-1 and PAI-2 [44]. 16K hPRL inhibits uPa by increasing the expression of PAI-1 [45]. Like endostatin and angiostatin, 16K hPRL has also been shown to induce endothelial cell cycle arrest at the $G_0$–$G_1$ transition but also, interestingly, at the $G_2$–M checkpoint. This broad cell cycle effect results from combined effects on positive and negative regulators of cell cycle progression (down-regulation of cyclin-D1 and cyclin-B1 and up-regulation of p21cip1 and p27kip1) [46]. The 16K hPRL-induced cell-cycle arrest has also been correlated with the inhibition of Ras activation causing a subsequent MAPK pathway inhibition [47]. 16K hPRL specifically induces

endothelial cell apoptosis. Signaling events associated with 16K hPRL-induced apoptosis include increased DNA fragmentation and activation of caspase-3 [48]. As described for angiostatin and TMP-1, 16K hPRL activates both intrinsic and extrinsic apoptotic pathways. Furthermore, NF-κB activation is required for 16K hPRL-induced apoptosis: it is necessary for activation of caspase-8 and -9, which in turn trigger caspase-3 activation and DNA fragmentation [49]. It is interesting to note that the NF-κB activation appears to be a very proximal event and seems to play a central role in the angiostatic properties of 16K hPRL.

## Future directions

Inhibition of angiogenesis is a promising therapeutic approach to fight cancer. In recent years, many angiostatic compounds have been discovered and the angiostatic therapy has been shown to be effective as cancer treatment. In addition, important advances have been made towards the comprehension of the molecular mechanisms used by angiostatic agents to act on endothelial cells. In addition to block cell cycle progression, most of the angiostatic agents induces intrinsic and/or extrinsic apoptotic pathway. Nevertheless, up to now, a clear and precise view of how these proteins trigger angiostatic properties remains to be defined. For example, initial events, like transcription factor activation are poorly understood. Several recent findings from our and other groups suggest that activation of NF-κB may be a common mechanism of angiostatic agents to induce EC apoptosis and to improve immune response [35,49–51]. Further research should be required to confirm this hypothesis. In addition, the emergence of research based on the analysis of the global transcriptome and proteome will undoubtedly improve our comprehension of angiostatic mechanisms and will highlight common molecular angiostatic pathways. This information is of crucial importance for the rational design of clinical trials and therapies.

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