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REVIEW

Tumor Angiogenesis Inhibitors

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Abstract—Formation of the blood supply system is a critical step in malignant transformation of neoplasms which results in the penetration of tumor cells into neighboring tissues and metastatic growth. Significant progress in the elucidation of mechanisms underlying tumor angiogenesis and the discovery of a great diversity of biomolecules involved in its regulation have culminated in the development of a radically new approach to antitumor therapy based on the search for efficient inhibitors of tumor angiogenesis. This review is devoted to the analysis of action mechanisms and expression of the major endogenous inhibitors involved in regulation of tumor and physiological angiogenesis. The antiangiogenic effects of the majority of currently known synthetic inhibitors are considered in the context of their roles in the main steps of tumor angiogenesis. Possible applications of antiangiogenic therapy in the chemotherapy of cancer diseases are discussed.

Key words: tumor angiogenesis inhibitors, angiogenic growth factors, endothelial cell proliferation, endothelial cell migration, proteolytic enzymes, extracellular matrix, cell adhesion molecules

Recent studies have revealed that the antitumor effect of various drugs may be associated with their direct toxic action on tumor cells or it may also be realized via modulation of functioning of various systems involved in the tumor formation process. The putative biological target for chemotherapy by anticancer drugs should meet at least two criteria: 1) it should play a crucial role in the metabolism of a tumor cell (or the whole neoplasm); 2) the biochemical process of tumor cells which are intended to be influenced by chemotherapeutic agents should have some characteristic features which differ from normal counterparts of corresponding physiological processes occurring in the organism. Using these criteria, we consider angiogenesis as a target for antitumor chemotherapy.

Angiogenesis is a complex morphogenetic process that consists of the formation of blood capillaries from preexisting capillaceous processes and arrangement of capillaries into the vascular network [1]. Under normal conditions angiogenesis in most tissues and organs in characterized by low intensity (with exception of such physiological processes as tissue repair and the menstrual cycle). The intensity of angiogenesis is controlled by concerted expression of positive and negative modulators of angiogenesis. Imbalance in expression of these modulators is accompanied by changes in the intensity of tissue vascularization, which promotes the development of various pathological processes (e.g., retinopathy, psoriasis, rheumatoid arthritis, cardiovascular diseases, etc.).

The development and growth of malignant tumors are closely related to their intensive vascularization. Folkman and D'Amore demonstrated that subsequent growth of tumors reaching 2-3 mm^3 ultimately requires induction of angiogenesis and further enlargement of such tumors are accompanied by increase in their vascularization [2]. Subsequent studies revealed that induction of angiogenesis is a characteristic feature of malignant tumors [3, 4].

A chain of events leading to formation of blood capillaries in the tumor reflects the main steps of physiological angiogenesis. It consists of activation of endothelial cells of perineoplastic vessels, induction of proteolytic activity responsible for degradation of the basic membrane of the precursor vessel and adjacent extracellular matrix, and proliferation and migration of endothelium into the adjacent tissue followed by capillary structure formation resulting in the onset of blood circulation.

Abbreviations: Ang-1, -2) angiopoietins; AGF) angiogenic growth factors; bFGF) basic fibroblast growth factor; EGF) epidermal growth factor; ECM) extracellular matrix; HGF) hepatocyte growth factor; IGF) insulin-like growth factor; MMP) matrix metalloproteinase; PDGF) platelet derived growth factor; sFGFR) soluble receptor of bFGF; sVEGFR) soluble receptor of VEGF; TGF-β) transforming growth factor-β; Tie-1, -2) angiopoietin receptors; COX-2) cyclooxygenase-2; TIMP) tissue inhibitors of metalloproteinases; TNF-α) tumor necrosis factor-α; VEGF) vascular endothelial growth factor.

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Inhibition of angiogenesis at these stages causing inhibition of growth of blood capillaries is the basic principle of antiangiogenic therapy (Fig. 1). Popularization of Folkman's idea on the direct dependence of intensity of tumor growth on the tumor vascularization resulted in the development of a new trend in antitumor chemotherapy based on the search for effective inhibitors of tumor angiogenesis.

Endothelial cells, the main structural and functional component of blood vessels, are one of the main targets of antiangiogenic therapy. Endothelial cells lining blood vessels spanning a tumor are in an activated state; these cells are characterized by some morphological and biochemical features which differ them from normal endothelial cells [5]. For example, tumor endothelial cells express certain specific markers on the cell surface, e.g., flt-1, KDR (VEGF receptors), Tie-1, Tie-2 (angiopoietin receptors), $\alpha_v\beta_{3,5}$-integrins, etc. The presence of the tumor markers allows the use of selective chemotherapeutic treatments of endothelium of tumor

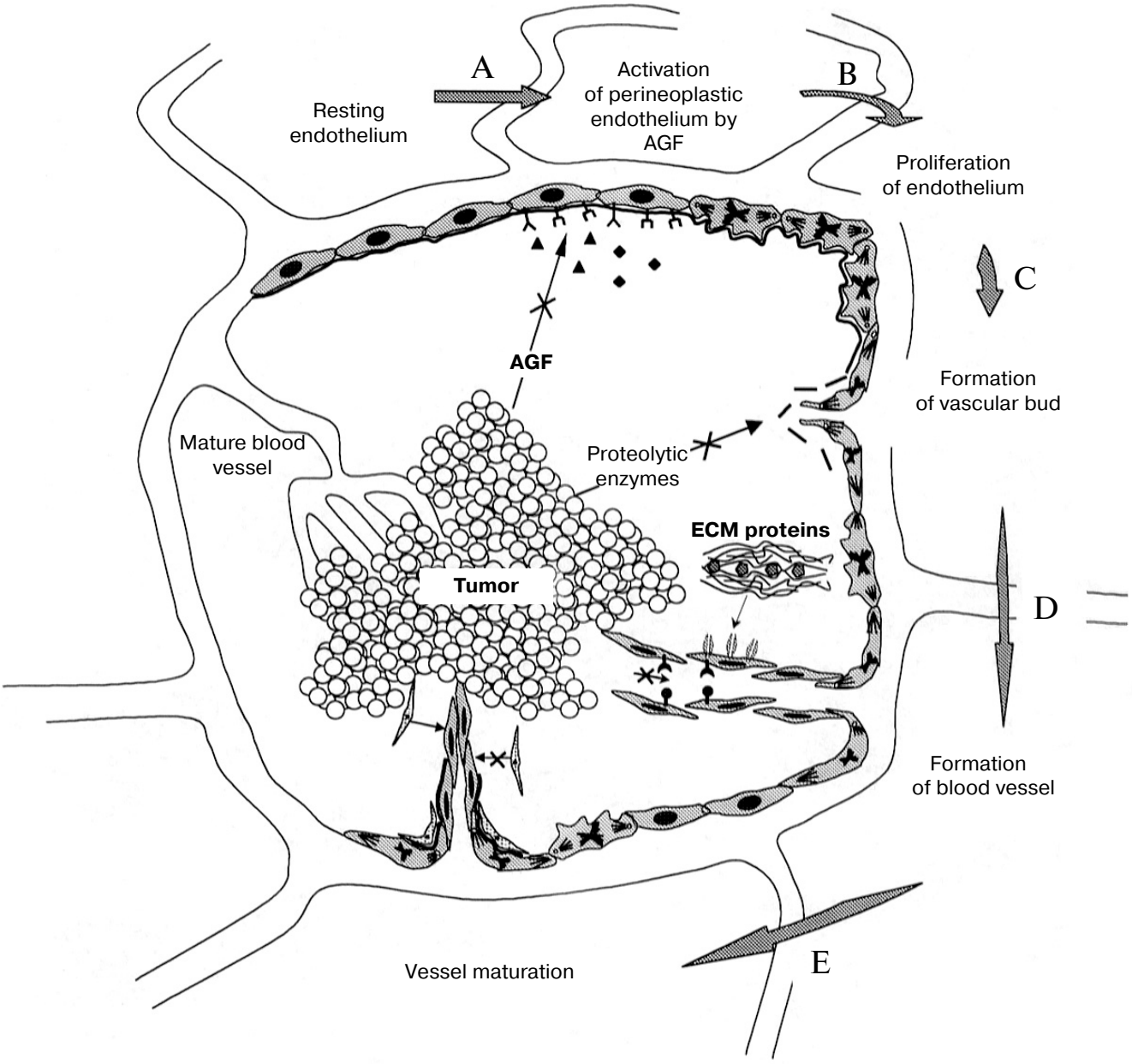


Fig. 1. The main strategies of antiangiogenic therapy based on inhibition of various stages of tumor angiogenesis. A) Inhibition of perineoplastic vascular endothelial cell activation induced by angiogenic growth factors (AGF) produced by tumor cells. B) Inhibition of proliferation of AGF-activated endothelial cells. C) Inhibition of proteolytic activity of tumor tissue promoting degradation of the basic membrane of the precursor vessel and endothelium migration into extracellular matrix (ECM). D) Inhibition of interaction between endothelial cells and components of ECM during formation of blood capillaries. E) Inhibition of the blood capillary maturation process.

vessels. In this connection it should also be mentioned that in contrast to tumor cells normal endothelial cells represent a homogenous cell population characterized by low frequency of mutations and stability of their genetic apparatus. This significantly reduces risk of possible development of drug resistance of normal endothelial cells to antiangiogenic drugs and significantly increases effectiveness of the therapeutic treatment.

The development of rapid methods for evaluation of the effects of putative inhibitors of angiogenesis on various stages of angiogenesis and overall growth of blood vessels was the key moment in the search for such inhibitors and study of mechanisms of their antiangiogenic effects. Experimental models developed for study of angiogenesis represent the basis of diagnostic systems widely used for testing of antiangiogenic activity. One such system employs a transparent chamber implanted into experimental animals for monitoring the development of blood vessels in various tissues [6]. The transparent chamber method for monitoring changes in vascularization of both normal and pathologically altered tissues implanted into the chamber allows qualitative determination of the effectiveness of tested compounds. Now antiangiogenic activity of drugs is often evaluated using a chorioallantois membrane [7]. This simple and highly reproducible method is based on the ability of tumor cells to induce vascular growth in the chorioallantois membrane. Addition of antiangiogenic preparation results in reduction in density of tumor-induced blood capillaries which can be qualitatively or semi-quantitatively evaluated.

The development of methods for endothelial cell cultivation opened new possibilities for expressing determination of antiangiogenic activity by evaluating inhibitory effect(s) of various compounds on proliferation and migration of the endothelial cells. Use of three dimensional substrates like collagen gel, gelatin, etc. allows evaluation of effects of angiogenesis inhibitors on processes of endothelial cell organization into capillary-like structures and blood vessel formation [8]. The diversity of endothelial cell cultures employed for evaluation of the effectiveness of angiogenesis inhibitors is related to morphological and biochemical features of endothelial cells lining blood vessels in various organs and tissues.

The intensive search for effective inhibitors of angiogenesis has resulted in the introduction of more than 300 compounds (of different structure and biological activity) into preclinical and clinical trials.

ENDOGENOUS INHIBITORS OF ANGIOGENESIS

Physiological inhibitors of angiogenesis. As mentioned above, angiogenesis in the adult organism is controlled by concerted expression of inducers and inhibitors of angiogenesis. The first evidence for the existence of physiological inhibitors of angiogenesis was obtained in 1990 when the antiangiogenic activity of thrombospondin (TSP-1) was recognized. This glycoprotein with molecular mass of 450 kD was found to be secreted by cultivated normal fibroblasts [9]. TSP-1 induces collagenase activity in cells; this leads to proteolysis of the basic membrane of precursor vessel and inhibition of proliferation and migration of endothelial cells. Subsequent strategy in searching for angiogenesis inhibitors employed screening of various tissues characterized by low vascularization for antiangiogenic activity determined by inhibition of proliferative and migration activity of vascular cells. Analysis of biological liquids isolated from the eye cornea and cartilaginous tissue resulted in elucidation of antiangiogenic activity of known physiological proteins troponin I and pigment epidermal derived factor (PEDF) [11, 12]. Substances exhibiting marked antiangiogenic activity have also been identified in eye lens and vitreous body [13]. Inhibitors of angiogenesis were among many biomolecules exhibiting a wide range of biological activities (Table 1). For example, steroids lacking glucocorticoid and mineralocorticoid activity (tetrahydrocortisol, 2-methoxyestradiol, progesterone derivatives) possess significant antiangiogenic activity, and the highest effect was observed under combined administration of these steroids with sulfated glucosaminoglycans [14]. Cortisone, dexamethasone, adrenocorticotropin, and estrogen were also active as inhibitors of vascular growth [15]. It has recently been found that biological molecules involved in regulation of immune response inhibit endothelial cell proliferation. These include interferons (α, γ), interleukin-12, members of CXC-cytokines (such as platelet factor 4 (PF4), protein inducing expression of interferon-γ (IP-10), and proteins produced by immune system cells (Gro-β, MIG)) [16-18]. Some authors believe that leukocytes and lymphocytes circulating in the vascular system and secreting these biomolecules play an important role in the maintenance of vascular endothelium in the resting state [19].

Besides the above mentioned biomolecules, the major components of extracellular matrix, proteoglycans, glucosaminoglycans, and heparin exhibit high antiangiogenic activity [20]. Hyaluronic acid (present in high concentrations in vitreous body) exhibits marked antiangiogenic properties; chondroitin sulfate localized in bovine aorta connective tissue membrane is an effective inhibitor of angiogenesis [21, 22]. The mechanisms underlying the manifestation of antiangiogenic properties of these compounds remain unclear; it is suggested that these compounds reversibly bind angiogenic growth factors and thus regulate the level of angiogenic stimulation of endothelial cells.

Most physiological inhibitors of angiogenesis are compounds playing important regulatory roles in various systems of the organism. Consequently, use of physiolog-

Table 1. Physiological inhibitors of angiogenesis

Inhibitor	Mechanism of antiangiogenic action	Reference
	Soluble receptors of growth factors	
sVEGFR sFGFR	competitive inhibition of AGF interaction with plasma receptors of endothelial cells	[66, 67]
	Steroid hormones	
Tetrahydrocortisol Methoxyestradiol Cortisone Dexamethasone Adrenocorticotropin Estrogen	inhibition of proliferation and migration of endothelial cells; activation of apoptosis of epithelial cells; inhibition of collagen deposition onto basic membrane of forming capillaries	[14, 15]
	Cytokines	
Interferon-α	inhibition of endothelial cell proliferation; activation of apoptosis of endothelial cells; inhibition of bFGF-mediated stimulation of angiogenesis	[121]
Interferon-γ	inhibition of endothelial cell proliferation; stimulation of IP-10 secretion	[16]
Interleukin-2	stimulation of secretion of IFN-γ and IP-10	[10]
	Chemokines	
GRO-β MIG IP-10 PF-4	inhibition of proliferation and bFGF and IL-8 mediated migration of endothelial cells; inhibition of blood capillary formation	[18]
	Components of extracellular matrix	
Heparin Hyaluronic acid Chondroitin sulfate	inhibition of activation of endothelial cells by angiogenic growth factors	[21, 22]
	Other inhibitors	
Thrombospondin	inhibition of collagenase activity, proliferation and migration of endothelial cells	[9]
Troponin I	specific inhibition of endothelial cell proliferation	[11]
PEDF	inhibition of migration and bFGF-mediated proliferation of endothelial cells	[12]
Ang-2	prevention of maturation of blood vessels, Ang-1 antagonist	[122]
TIMP	inhibition of matrix metalloproteinase activity	[90]
PAI-1	inhibition of urokinase activity	[96]

ical inhibitors as antitumor antiangiogenic preparations is complicated by evident (side) effects on various important physiological processes. Nevertheless, study of mechanisms and targets of their antiangiogenic action is an important precondition for the design of effective inhibitors of angiogenesis.

Tumor-induced inhibitors of angiogenesis. Until recently the phenomenon of restraining of growth and development of regional tumors (metastases) by the primary tumor remained unresolved. Much clinical experience confirms the fact of restraining of secondary tumor growth by the primary tumor. On the contrary, various therapeutic aggressions leading to regression or suppression of primary tumor growth cause sharp activation of metastasizing. Taking into consideration all these facts, Folkman suggested that, besides vascularization of the primary tumor tissue, primary tumors may induce secretion of angiogenesis inhibitors which inhibit vascularization of secondary tumors and therefore restrain their growth and development. The discovery of potent endogenous inhibitors of angiogenesis—angiostatin and endostatin—provides convincing evidence supporting this hypothesis.

Angiostatin. The first experimental evidence for the existence of tumor-induced inhibitors of angiogenesis was obtained during isolation of urinary protein from mice bearing Lewis solid carcinoma tumors [23]. This factor inhibited proliferation of endothelial cells.

In 1994 O'Reilly et al. isolated and characterized this factor. Analysis of the amino acid sequence of this protein revealed that this 38-kD protein shared 96% homology with the N-terminal fragment of plasminogen. It consists of four highly homologous kringle domains (K1-4). Later O'Reilly et al. obtained the same fragment after limited proteolysis of plasminogen with elastase; the authors named this fragment angiostatin [23] (Table 2). Subsequent studies revealed that suppression of growth of regional metastases in lung of Lewis carcinoma-bearing mice is associated with increased level of blood angiostatin. It was found that removal of the primary tumor caused a sharp decrease in angiostatin in blood circulation and the development of secondary tumors. On the basis of these results, it was suggested that the primary tumor induces a specific inhibitor exhibiting antiangiogenic activity and effectively suppressing vascularization of secondary tumors, preventing their rapid development and death of the tumor-bearing organisms.

Study of biological effects of angiostatin revealed that this polypeptide is a specific inhibitor of proliferation and migration of endothelial cells and blood capillary formation [24]. The discovery of the first specific inhibitor of angiogenesis attracted much attention of many scientists to the study of mechanisms of its formation and the realization of its biological effects.

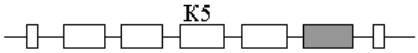
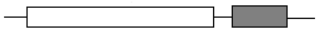
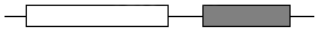
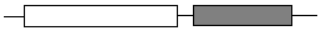
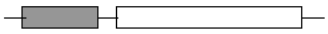
Attempts to obtain angiostatin by treatment of plasminogen with various proteases (e.g., plasmin, plasmin reductase, elastase, MMP-2, MMP-7, and MMP-9) resulted in isolation of several proteolytic fragments of plasminogen (K1-3, K1-4, K1-5, K5) exhibiting endothelium-specific antiangiogenic activity but differing in inhibitory potency [25-28] (Table 2). Study of conditioned media of various human tumor cell lines revealed the existence of a number of proteolytic fragments of plasminogen. They exhibited specific inhibitory activity with respect to proliferating endothelial cells.

Increased angiostatin content in the blood was observed at many tumors and the blood level of angiostatin varied depending on type and localization of the tumors. The highest level of angiostatin was observed in patients with various carcinomas (of urinary bladder, large intestine, prostate, etc.) and melanomas [29, 30]. This may be related to the nature of proteolytic enzymes (determined by tumor type) which form angiostatin during limited proteolysis of plasminogen. This hypothesis is supported by some experimental data. For example, Westphal et al. [28] found that angiostatin formation in urinary bladder and prostate carcinomas is closely correlated with the level of plasminogen activator, urokinase; melanomas are characterized by high level of tissue plasminogen activator (tPA) produced by the tumor cells. Macrophages infiltrating tumors also play an important role in angiostatin formation; they secrete MMP catalyzing plasminogen cleavage [31].

In spite of intensive studies on angiostatin our current knowledge is not sufficient for comprehensive characterization of the molecular mechanisms responsible for manifestation of inhibitory effect of angiostatin on endothelial cells. Specific binding of angiostatin with ATP-synthase located on the cell surface of endothelial cells of human fetus umbilical vein caused inhibition of ATP synthesis and impairments of endothelial energy supply under hypoxic conditions followed by apoptotic cell death [32]. Several authors have demonstrated that angiostatin blocked activation of endothelial cells by angiogenic growth factors (VEGF, bFGF) [33, 34]. This effect was mediated by inhibition of MAP-kinases (ERK-1 and ERK-2). Angiostatin is also involved in regulation of the cell cycle by inhibiting cyclin-dependent CDK-2 kinase [33, 34]. Angiostatin also inhibits plasminogen proteolysis by tPA; this prevents formation of plasmin, a potent inducer of angiogenesis [35]. It has recently been demonstrated that angiostatin selectively binds to $\alpha_v\beta_3$ integrins of endothelial cell surface, and this binding prevents interaction between endothelial cells and extracellular matrix playing an important role in angiogenesis [36].

Experiments on animals with various tumors revealed that daily angiostatin administration (in the range of therapeutic doses from 20 to 100 mg per kg) significantly suppressed growth of such tumors as melanoma B16, hemangioma, carcinomas of different localization, fibrosarcomas, etc. [37]. High effectiveness of angiostatin was also observed during administration of low doses

Table 2. Peptide inhibitors of angiogenesis that are proteolytic fragments of physiologically active proteins (dark rectangles designate fragments exhibiting antiangiogenic properties)

Precursor molecules	Proteolytic fragment	Molecular mass, kD
	Proteins involved in regulation of the blood coagulation system	
Plasminogen	NH_2- Angiostatin -COOH 	38
	K1-5 	55
	K5 	10
Antithrombin	Serpin 	55
	Extracellular matrix proteins	
Collagen XVIII	Endostatin 	20
Collagen XV	Restin 	22
Collagen IVα-1	Aresten 	26
Collagen IVα-2	Tumstatin 	28
Collagen IVα-3	Canstatin 	30
Fibronectin	Proteofib 	29
	Cytoplasmic protein of skeletal muscle cells	
Calreticulin	Vasostatin 	20
	Pituitary hormone	
Prolactin	Prolactin fragment 	16
	Proteolytic enzymes of extracellular matrix	
MMP-2	PEX 	25

(0.5-5 mg per kg daily) of this peptide as the main component of adjuvant therapy during postoperative course for prevention of growth and the development of regional metastases [24]. The main antitumor effect of angiostatin consists of suppression of tumor growth. Initially this results in reduction of tumor sizes, and later the tumors "fall into a sleeping state" characterized by the absence of growth and invasion into adjacent tissues. This angiostatin effect induced by suppression of tumor vascularization is related to increased apoptotic death of tumor cells without changes in their proliferation rate [38]. Taking into consideration mechanisms of angiostatin effects, this peptide might be used clinically in combined therapy together with traditionally employed chemotherapeutic agents directly acting on the tumor cells. Several authors have noted a synergic effect of angiostatin and radiotherapy on prostate carcinoma and Lewis lung carcinoma [39, 40]. Several schemes for combined chemotherapy including angiostatin have been developed [41]. We showed that angiostatin used in combination with directed antitumor preparations based on α-fetoprotein and doxorubicin significantly inhibited mouse melanoma B16 growth and increased the lifespan of the animals [42].

Endostatin. Studying cultured hemangioendothelioma tumor cells producing factors which influence proliferation of endothelial cells O'Reilly et al. isolated a new potent inhibitor of angiogenesis, endostatin [43]. Endostatin is a 20-kD polypeptide consisting of 184 residues. It represents the C-terminal globular domain (NCl) of collagen XVIII [43] (Table 2). The latter is a member of the family of extracellular matrix collagen proteins. Collagen XVIII is preferentially localized in the perivascular space of blood vessels. Endostatin shares certain similarity with the carbohydrate-binding domain of C-type lectin, but in contrast to lectin endostatin can bind zinc ions [44]. It seems unlikely that zinc ion is required for manifestation of antiangiogenic activity of endostatin; it is apparently involved in structural stabilization of the endostatin molecule and facilitation of collagen proteolysis [44].

The mechanism of endostatin formation in tumor tissue is similar to that of angiostatin formation; it consists of proteolysis of initial collagen by the proteolytic enzymes elastase and cathepsin L secreted by tumor cells [45, 46]. In contrast to angiostatin, endostatin has been found in tissues and body fluids of healthy subjects. It was shown that by analogy with the precursor protein endostatin is closely associated with elastic fibers lining walls of large blood vessels [47]. The highest endostatin content was found in walls of aorta and some large veins, the lowest content of this protein was detected in arterioles and blood capillaries. Apparently, particular endostatin localization reflects its important role in the regulation of physiological angiogenesis, which may consist of blockade of involvement of large blood vessels in vascular remodeling processes.

The biological effect of endostatin consists of specific inhibition of endothelial cell proliferation. The inhibitory effect is mediated by endostatin binding to heparin on the surface of endothelial cells. Some authors believe that heparin is a low affinity receptor of endostatin [48]. The mechanism of the inhibitory effect of endostatin consists of a significant increase in apoptosis of endothelial cells due to simultaneous repression of antiapoptotic bcl-2 and bcl-x_1 protein expression and activation of intracellular caspase-3 triggering a cascade of intracellular events leading to apoptotic cell death [49]. It was recently demonstrated that endostatin specifically inhibits intracellular signal transduction from angiogenic growth factors (VEGF and bFGF) at the stage of MAP-kinase (ERK-1, ERK-2) phosphorylation in endothelial cells [50]. Inhibition of catalytic activity of MMP catalyzing proteolysis of extracellular matrix and mediating tumor cell invasion into adjacent tissues and endothelial cell migration during formation of new capillaries is one of the mechanisms underlying antiangiogenic and antitumor effects of endostatin [37]. Angiostatin and endostatin share mechanisms of antiangiogenic activity which consist of inhibition of the key stages of angiogenesis: activation, proliferation, and migration of endothelial cells. Numerous experiments have revealed that endostatin is characterized by more pronounced and more stable suppressive effect on tumor growth than angiostatin. The mechanism of antitumor effect of endostatin is similar to that of angiostatin; it consists of mediated induction of apoptosis of tumor cells. Systemic administration of endostatin (in daily dose 0.3 mg per kg) prevented the development of local metastases in experimental animals subjected to resections of solid tumors [24]. Administration of higher doses (up to 20 mg per kg, daily) of endostatin caused potent inhibitory effect on Lewis and ovary carcinomas, hemangioma (EOMA), fibrosarcoma T241, melanoma B16F10, etc.; in some cases endostatin caused total regression of tumors [24].

High antitumor potential of endostatin and angiostatin together with lack of marked nonspecific toxicity and tumor resistance allows them to be considered as promising antitumor chemotherapeutic agents.

However, the development of a stable effect requires the use of high daily doses of angiostatin and endostatin (from 10 to 100 mg per kg) because of characteristic distribution and excretion of the administered compounds. This stimulated the use of gene engineering approaches for elaboration of recombinant forms of angiostatin [51] and endostatin [52]. Another approach involves gene therapeutic methods of administration of plasmids carrying genes encoding angiogenesis inhibitors into target tumor cells or administration of cells producing angiostatin and endostatin [53, 54].

Besides angiostatin and endostatin, other compounds exhibiting pronounced antiangiogenic activity

have recently been recognized. They represent proteolytic fragments of some known physiological proteins. For example serpin (specific inhibitor of endothelial cell proliferation), a proteolytic fragment of antithrombin III, was isolated from biopsy of small cell lung cancer. An inhibitor of angiogenesis vasostatin, 55-kD proteolytic fragment of calreticulin, was isolated from the supernatant of B-cells transformed with Epstein–Barr virus [55, 56] (Table 2).

The discovery of antiangiogenic activity of tumor-produced proteolytic fragments involved in regulation of physiological processes suggests the existence of similar mechanism of angiogenesis regulation in the healthy organism. This putative mechanism consists of controlled limited proteolysis of physiological proteins with formation of fragments exhibiting antiangiogenic properties. In fact a fragment of pituitary hormone, prolactin, and also proteolytic fragments of extracellular matrix proteins, fibronectin and various collagens [57-61], demonstrate some antiangiogenic properties [57-61] (Table 2).

SYNTHETIC INHIBITORS OF ANGIOGENESIS

Approaches used in the development of a new generation of synthetic antiangiogenic therapeutic preparations are based on the study of biochemical processes involved in angiogenesis in tumor tissue, identification of the main regulatory biomolecules, and design of specific inhibitors blocking their action. However, screening of various natural compounds and traditional drugs for possible antiangiogenic activity also represents an important direction in the search for effective inhibitors of angiogenesis.

Synthetic inhibitors of endothelial cell activation. A high level of proliferative activity maintained by constant stimulation by tumor-secreted angiogenic growth factors is a characteristic feature of blood vessel endothelial cells infiltrating the tumor. For example, proliferative activity of tumor endothelium is more than one order of magnitude higher than that of endothelium of "normal" vessels [5]. In this connection therapeutic strategies of inhibition of biological processes underlying mitogenic activation of endothelial cells are especially interesting because their realization would promote selective treatment of vascularization of tumor tissue and therefore tumor growth in general.

Proliferative activity of endothelial cells of tumor surrounding blood vessels is stimulated by various tumor-secreted growth factors: VEGF, bFGF, PDGF, EGF, TGF-β, IFG, etc. Among them VEGF and bFGF are the main angiogenic factors produced by most tumors. These factors are directly involved in induction and development of tumor angiogenesis: they activate endothelium of tumor surrounding blood vessels and stimulate proliferation, migration, and differentiation of endothelial cells [62, 63]. In progressive tumors high level of expression of VEGF and bFGF is an important criterion of intensity of tumor vascularization. The rate of VEGF and bFGF expression can also be used as a prognostic factor in clinical practice.

Elucidation of the regulatory role of VEGF and bFGF in tumor angiogenesis stimulated the search for therapeutic agents blocking expression of angiogenic growth factors and study of signaling pathways mediating their biological effects (Fig. 1A).

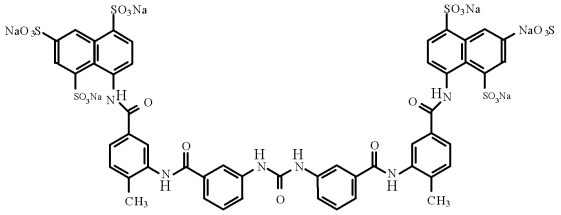
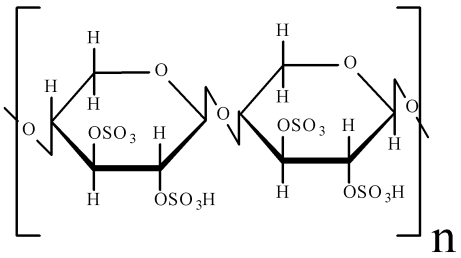
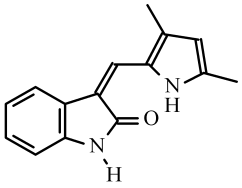
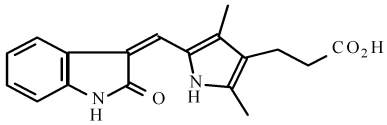
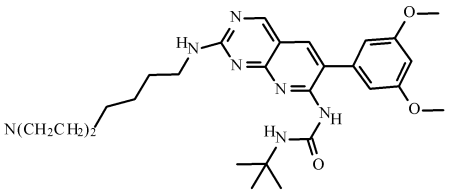
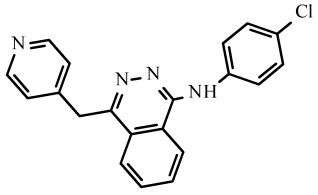
Results of clinical studies identified several effective inhibitors significantly reducing vascularization and suppressing tumor growth. These include monoclonal antibodies against VEGF [64] and its receptor [65] and soluble receptors of VEGF (sflt-1) blocking ligand–receptor interaction on the surface of endothelial cells [66, 67]. The regulatory effect of some angiogenic growth factors (VEGF, bFGF, PDGF, etc.) is known to be mediated via binding to heparin sulfate proteoglycans (HSPG) presented on the endothelial cell surface [68]. Blockade of interaction between growth factors and HSPG attenuates growth factor receptor binding and inhibits signal transduction. This represents the basic mechanism of angiogenesis inhibition by such polyanionic synthetic inhibitors of angiogenesis as pentosan and suramin, which are now under clinical trial [69, 70] (Table 3, Fig. 2).

The strategy of search for new antiangiogenic preparations based on inhibition of intracellular signal transduction after formation of a ligand–receptor complex resulted in the development of effective angiogenesis inhibitors blocking mitogenic effect of angiogenic growth factors (VEGF, PDGF, bFGF). The main action mechanism of these inhibitors consists of inhibition of tyrosine phosphorylation of the intracellular domain of the receptors; this results in blockade of growth factor intracellular signal transduction [71-74] (Fig. 2).

Recent trends in antiangiogenic therapy also involve the search for mechanisms of inhibition of secretion of angiogenic growth factors and their receptors produced by tumors and tumor vessel endothelial cells. At the present time angiozymes, catalytically active nucleic acids blocking secretion of VEGF receptor at the stage of its mRNA, are undergoing preclinical trials [75]. Other therapeutic possibilities which are intensively investigated now include: 1) transfection of endothelial cells with genes encoding inactive AGF receptors; 2) use of antisense oligonucleotide sequences complementary to mRNA and blocking expression of bFGF, VEGF, and other angiogenic growth and/or their receptors [76] (Fig. 2).

Synthetic inhibitors of endothelial cell proliferation. In spite of significant progress in the development of inhibitors blocking mitogenic stimulation of endothelial cells by AGF, other directions also attract much attention. These include the search for compounds exhibiting antiproliferative activity with respect to activated

Table 3. Synthetic inhibitors of endothelial cell activation by AGF

Inhibitor	Chemical structure	Mechanism of antiangiogenic effect	Phase of clinical trial
Suramin		AGF binding and prevention of interaction with growth factor receptor on the cell surface	II (prostate cancer)
Pentosan		AGF binding and prevention of interaction with growth factor receptor on the cell surface	I/II
SU5416		blockade of intracellular signal transduction from VEGF receptor	III (Kaposi sarcoma), I/II (large intestine cancer)
SU6668		blockade of intracellular signal transduction from receptors of VEGF, PDGF, and FGF	I
PD 173074		blockade of intracellular signal transduction from bFGF receptor	preclinical trials
PTK787		blockade of intracellular signal transduction from receptors of VEGF and PDGF	I/II

endothelial cells (Fig. 1B). Now several natural compounds and their synthetic low molecular weight analogs inhibiting proliferation of endothelial cells are recognized.

Fumagillin, an antibiotic secreted by *Aspergillus fumigatus fresenius* [77] is one such natural inhibitor. However, due to high toxicity its practical use is very limited. Nevertheless, on the basis of fumagillin several derivatives have been synthesized, and TNP-470 (Table 4) is one of the most promising derivatives for introduction to clinical practice. High antiproliferative effect of TNP-470 is associated with blockade of bFGF-induced

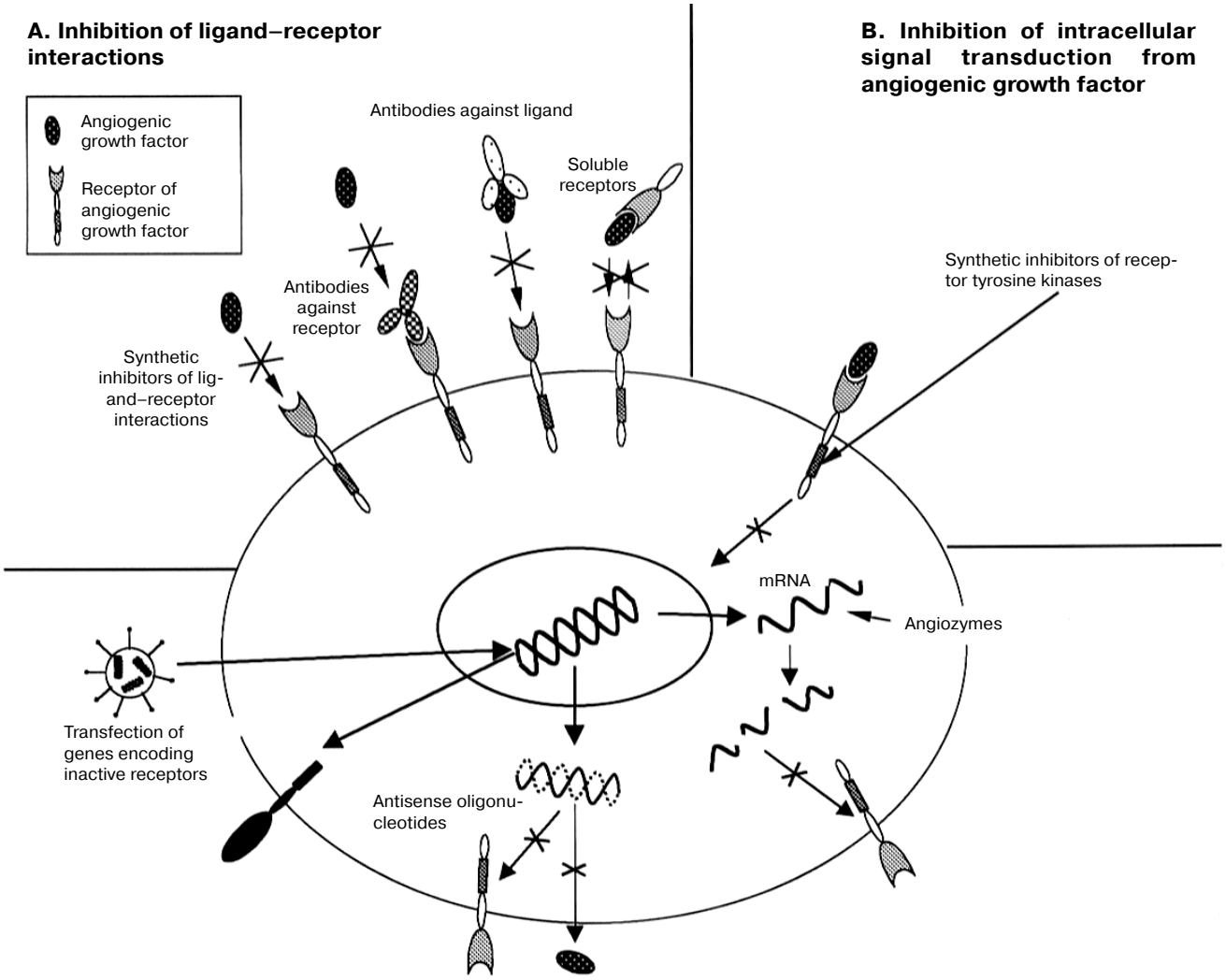


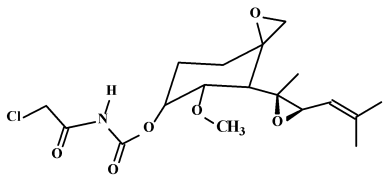
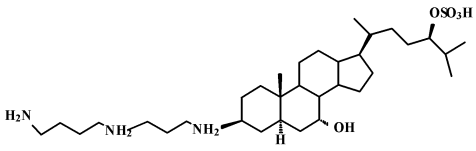
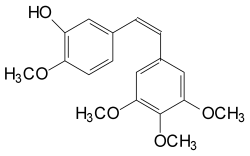
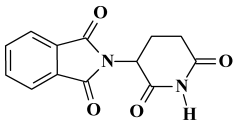
Fig. 2. Therapeutic strategies for inhibition of effects of angiogenic growth factors.

activation of endothelial cell proliferation [78]. Several studies have revealed various mechanisms responsible for inhibition of proliferation. It was shown that TNP-470 causes apoptosis of endothelial cells by inhibiting phosphorylation by cyclin-dependent kinase p21(CIP/WAF) [79]. Other authors found that TNP-470 can bind to methionine aminopeptidase (Met AP-2) and inhibit its activity [80]; this results in impairment of synthesis of some intracellular proteins required for endothelial cell proliferation. In spite of some reversible side effects, TNP-470 is highly effective in chemotherapy of uterine cervix carcinoma and is under clinical trial [81].

Squalamine (aminosterol) is the other low molecular weight inhibitor employed clinically as an antibacterial drug (Table 4); it was originally isolated from shark liver [82]. Study of its antiangiogenic activity revealed that squalamine inhibits proliferation of various endothelial cell lines induced by some angiogenic growth factors (VEGF, bFGF, PDGF, HGF). Clinical studies reveal that this preparation in combination with traditional chemotherapeutic agents cisplatin and carboplatin demonstrates effective antitumor activity.

Combretastatin A-4P (CA-4P) is a phosphorylated derivative of combretastatin (CA-4); the latter was isolated from tissue of the South African tree *Combretum caffrum* (Table 4) [83]. This preparation is under the second phase of clinical trial. The mechanism of action of CA-4P

Table 4. Synthetic inhibitors of endothelial cell proliferation

Preparation	Chemical structure	Mechanism of antiangiogenic effect	Phase of clinical trial
TNP-470 (AGM1470)		induction of apoptosis of endothelial cells via inhibition of cyclin-dependent kinases	I/II/III kidney carcinoma, uterine cervix carcinoma, Kaposi sarcoma
Squalamine		inhibition of proliferation and migration of endothelium	I/II non-small cell lung cancer, ovary carcinoma
Combretastatin A-4		inhibition of endothelial cell proliferation by impairment of actin–tubulin interactions	I/II
Thalidomide		suppression of expression of cell adhesion molecules (VCAM-1, E-selectins, $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins) by endothelial cells	II multiple myeloma, Kaposi sarcoma, glioblastoma, carcinomas of prostate, lung, breast, head, and neck

involves binding to tubulin accompanied by impairment of actin–tubulin interactions during endothelial cell division. CA-4P significantly impairs tumor blood supply due to disintegration of the vascular network and sharp reduction in blood circulation in the tumor tissue. Combined administration of CA-4P and inhibitors of NO synthase has synergic effect on the inhibition of tumor vascularization and growth [84].

The discovery of marked antiangiogenic activity of thalidomide (Table 4) initially employed as a sedative antiinflammatory drug was quite unexpected [85]. Thalidomide inhibits proliferation of endothelial cells induced by bFGF and VEGF. The mechanism of this effect remains unknown. Some authors suggest [86] that inside cells thalidomide undergoes nonenzymatic hydrolytic cleavage resulting in formation of an epoxy derivative that is responsible for the antiangiogenic effect of this drug. At the present time thalidomide is under the second phase of clinical trial as a potential antitumor preparation for chemotherapy of myeloma, prostate cancer, and also as an effective drug for treatment of various diseases characterized by imbalance of vascularization [87].

Inhibitors of proteolytic activity of extracellular matrix components. Secretion of proteolytic enzymes (serine and cysteine proteinases, matrix metalloproteinases, etc.) by tumor cells is an important step providing tumor invasion into surrounding tissues and its metastasizing potential. This process also required for tumor vascularization also plays a key role in angiogenesis. For example, local increase of proteinase activity leads to partial degradation of a connective tissue base of tumor-infiltrating blood vessels. This results in initiation of migration and proliferation of endothelial cells and finally in formation of new capillaries. Inhibition of proteolytic activity causes inhibition of tumor vascularization and suppression of tumor growth and metastasizing [88]. So inhibition of proteolytic enzymes is a rather promising strategy for antitumor therapy (Fig. 1C).

Matrix metalloproteinases (MMP) play a central role in the regulation of remodeling of tumor tissue. These enzymes contain zinc ions in the active site. High level of MMP expression is typical for many tumors [89]. In the human body MMP activity is regulated by four specific tissue metalloproteinase inhibitors (TIMP 1-TIMP 4) [90]. The development of a neoplasm is accom-

panied by inhibition of TIMP secretion and stimulation of MMP secretion by tumor cells. This causes evident imbalance between the proteolytic enzymes and their inhibitors and results in sharp increase in proteolytic activity followed by local proteolysis of extracellular matrix.

Increase in level of physiological inhibitors of proteolytic enzymes (TIMP) was initially considered as one of the possible variants of suppression of tumor MMP activity. In spite of certain effectiveness of exogenous TIMP in the inhibition of vascularization of primary tumors and inhibition of metastasizing in some experimental models, clinical trials were interrupted because of short half-life underlying low effectiveness of these inhibitors.

Several synthetic low molecular weight metalloproteinase inhibitors are known to date. They inactivate MMP by blocking zinc ions in the active site. On the basis of hydroxamic acid, effective MMP inhibitors (prinomastat, marimastat, metamastat, etc.) have been synthesized. Some of these inhibitors are now under clinical trial as antitumor preparations [88] (Fig. 3). Retinoic acid and its analogs were also used for synthesis of angiogenesis inhibitors. They specifically inhibit enzymatic activity of MMP-2, which is characterized by high level of expression in tumors [91]. Use of MMP inhibitors for chemotherapy of various cancers (prostate, ovary, stomach, pancreas, lung, and breast) has given promising results; these inhibitors stabilized tumor process by suppression of vascularization and growth of primary tumors and regional metastases [88]. In some cases combination of MMP inhibitors and traditional chemotherapeutic agents (e.g., carboplatin and doxorubicin) produced more effective antitumor action causing partial tumor regression [92].

Plasminogen activators, urokinase (uPA) and tissue plasminogen activator (tPA), are other proteolytic enzymes playing an important role in the induction of remodeling of tumor tissues [93]. Both enzymes are serine proteinases catalyzing plasminogen cleavage, which results in formation of active serine proteinase, plasmin. The latter exhibits a wide range of proteolytic activity: it can catalyze degradation of fibrin and also connective tissues components of extracellular matrix, laminin and fibronectin. Plasmin can also activate MMP and elastases [94].

Vascular remodeling of tumor tissue is accompanied by high expression of urokinase and its receptor (uPAR) on the surface of endothelial cells of tumor vessels [95]. Urokinase is secreted by endothelial cells as an inactive form (pro-uPA), which binds to urokinase receptor; urokinase activation also requires a catalytic amount of plasmin. The interaction of urokinase with its receptor localized on certain sites of endothelial surface leads to sharp increase in local proteolytic activity of urokinase. Subsequent proteolytic degradation of plasminogen by urokinase results in formation of plasmin, which in turn catalyzes directed proteolysis of extracellular matrix. One of the physiological pathways of regulation of endogenous plasmin level involves changes in expression of urokinase and its cell membrane receptor. Inhibitors of plasminogen activators (PAI-1, PAI-2) play an important role in this regulation. These inhibitors interact with uPA/uPAR followed by formation of a ternary complex, its internalization, and subsequent hydrolytic degradation of urokinase in lysosomes [96]. Several effective angiogenesis inhibitors acting at the level of urokinase interaction with its receptor have been developed. These include soluble urokinase receptor suPaR [97], monoclonal antibodies against urokinase and its receptor [98, 99], *p*-aminobenzamidine, a synthetic competitive inhibitor of urokinase binding with its receptor [100], and also amiloride derivatives (B428 and B623), which are promising antitumor chemotherapeutic agents [101].

Inhibitors blocking interaction of endothelial cells with extracellular matrix. The interaction of endothelial cells activated by AGF with extracellular matrix components is required for migration of endothelial cells, adhesion, and formation of the architecture of blood vessels (Fig. 1E). The interaction of endothelial cells with matrix is mediated by integrins localized on the outer surface of endothelial cells. Integrins represent a family of heterodimeric transmembrane proteins that consist of various combinations of 15 α and 8 β subunits [105]. Being membrane proteins integrins are involved in migration and adhesion of cells via interaction with many proteins of the extracellular matrix such as vitronectin, fibronectin, laminin, collagen, fibrinogen, etc. [105]. Specific expression of some integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the surface of endothelial cells of tumor capillaries allows using these integrins as a convenient target for selective treatment of tumor angiogenesis [106].

Vitaxin, a preparation of humanized antibodies against $\alpha_v\beta_3$-integrins, is now under phase II of clinical trial for treatment of Kaposi sarcoma, melanoma, and lung carcinoma [107]. A low molecular weight antagonist (3-oxo-1,4-benzodiazepine) selectively blocking $\alpha_v\beta_3$ integrins on the endothelial cell surface is at the initial stage of clinical study [108]. It is known that integrins recognize the Arg-Gly-Asp (RGD) sequence, which is widely present in various proteins of extracellular matrix [109]. Based on this fact new inhibitors, cyclic peptides containing RGD-sequence, have been synthesized. The mechanism of their action includes blockade of integrin binding with extracellular matrix components, and this results in apoptosis of endothelial cells [106]. Arap et al. demonstrated [110] that use of RGD-peptides as vector molecules for delivery of cytotoxic agents to the surface of tumor endothelium results in effective suppression of angiogenesis and growth of some tumors. Recently, it was found that one of the mechanisms of antitumor effect of cytokines TNF-α and interferon-γ employed for chemotherapy of various tumors consists of impairment

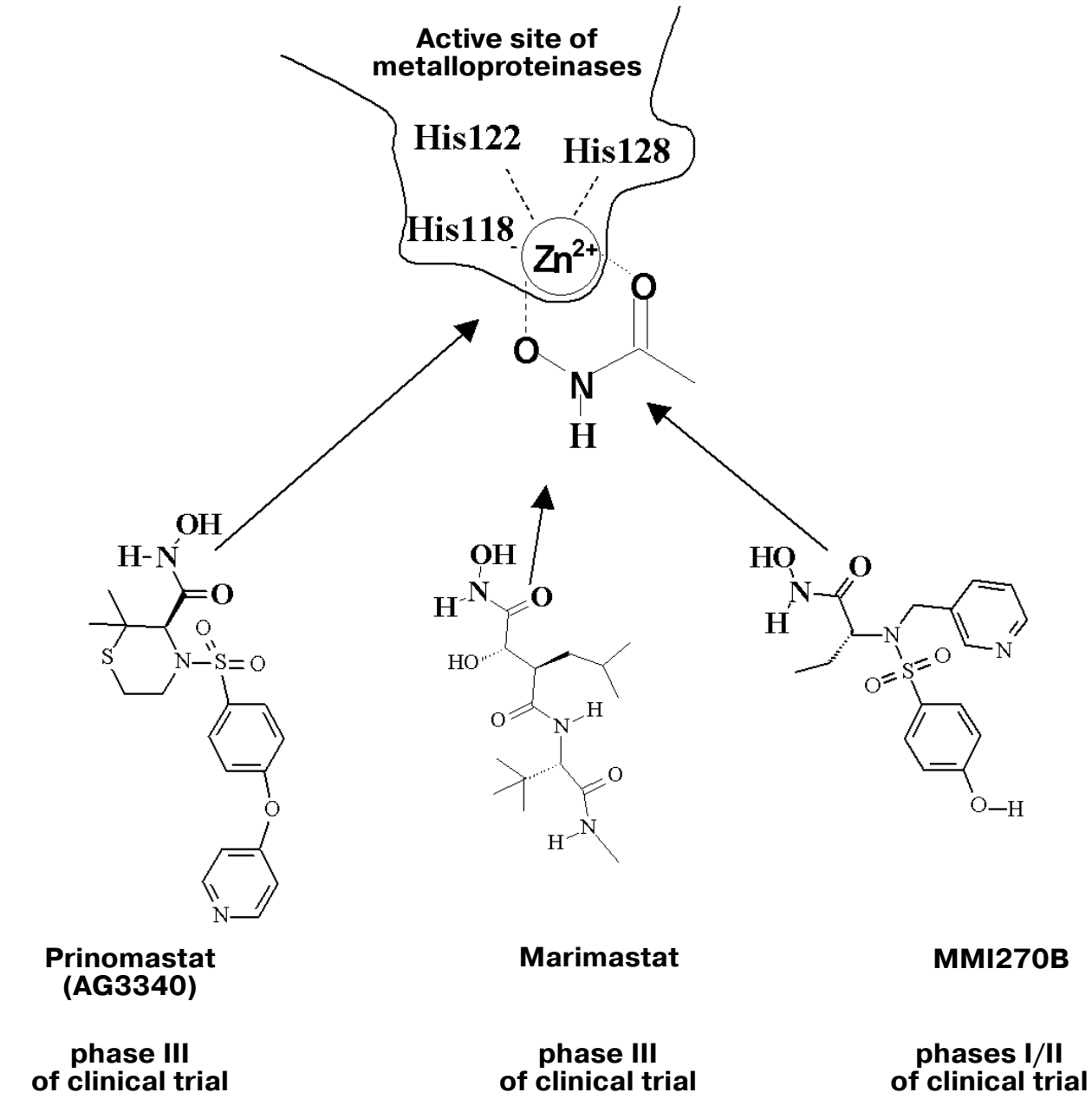


Fig. 3. The main synthetic MMP inhibitors and mechanism of their action. Inhibition of MMP activity by the synthetic inhibitors is based on the blockade of the active site of the enzyme due to binding of active site zinc ion into a tight complex with hydroxamic acid derivatives.

of tumor blood supply due to inactivation of $\alpha_v\beta_3$-integrins on the surface of endothelial cells accompanied by their apoptosis [111].

OTHER INHIBITORS OF ANGIOGENESIS

The list of compounds involved in angiogenesis inhibition is constantly increasing. It includes compounds of various chemical structure and biological activity.

Several synthetic low molecular weight compounds blocking intracellular messengers necessary for the metabolic activity of proliferating endothelial cells are now studied clinically. These include inhibitors of MAP-kinase (PD98059), protein kinase C (PKC412, SPC100097), and also inhibitors of calcium metabolism (CAI) [112-114].

Interleukins regulating various steps of tumor angiogenesis play an important role in the vascularization of tumor tissue. Therapeutic strategies of regulation of secretion of interleukin inducing and inhibiting angiogenesis in the tumor have been realized in two antitumor preparations. The first is a dipeptide (L-glutamyl-L-tryptophan). Its antiangiogenic effect is associated with stimulation of interleukin-12 expression (an endogenous angiogenesis inhibitor). Clinical studies revealed that this preparation reduces Kaposi sarcoma and metastasizing melanoma [115]. The second preparation, monoclonal antibodies against interleukin-8 (which is a potent angiogenesis stimulator overexpressed in certain tumors [116]), is under the first stage of clinical trial.

Since cyclooxygenase-2 (COX-2) and thymidine phosphorylase are highly expressed in certain tumors and there is a correlation between the expression of these enzymes and intensity of tumor vascularization, these enzymes represent attractive targets for the development of chemotherapeutic agents. COX-2 produced by inflammatory response cells infiltrating a tumor is involved in formation of prostaglandin PGE_2 (potent vasodilatator) and thromboxane A_2, responsible for COX-2-dependent stimulation of endothelial cell migration. A specific inhibitor of COX-2—1,5-diarylpyrazole (Celecoxib)—exhibits potent antiangiogenic action and suppresses vascularization of certain tumors [103]. Indomethacin and aspirin, nonsteroid antiinflammatory drugs widely used in clinical practice, also inhibit COX-2 [104]. So they might be used in complex chemotherapy of tumors as potential antiangiogenic drugs.

Thymidine phosphorylase (TP) catalyzing reversible hydrolysis of pyrimidine deoxynucleotides is a potent stimulator of angiogenesis [102]. High level of TP expression was noted in the zone of active vascularization under physiological (menstrual cycle) and pathological conditions including various cancers (breast, ovary, pancreas, etc.) [125, 126]. Inhibition of this enzyme by synthetic inhibitors (6-aminothymine, 6-amino-5-bromouracil) is accompanied by inhibition of tumor vascularization [127]. The therapeutic potential of these compounds is now being investigated.

Good evidence exists that the antitumor effect of many chemotherapeutic agents employed clinically includes not only direct cytotoxic effect on tumor cells but also suppression of angiogenesis. For example, doxorubicin (an antitumor anthracyclin antibiotic) exhibits not only cytotoxic effect; it also demonstrates antimitotic action on endothelial cells [117]. Taxol, vinblastin, and dolastatin are tubulin-binding preparations which effectively suppress endothelial cell proliferation [118-120]. Interferon-α, a preparation of wide spectrum of biological activity, is employed for the treatment of some tumors; it also inhibits angiogenesis due to blockade of bFGF mediated migration and proliferation of endothelial cells [121].

PROSPECTS FOR USE OF ANTIANGIOGENIC AGENTS FOR TREATMENT OF ONCOLOGICAL DISEASES

Vascularization is a critical step in tumor malignization, which is accompanied by tumor metastasis into surrounding tissues and distant organs. At this stage traditional antitumor therapy may be ineffective and in some cases it even deteriorates the course of the disease. For example, radiotherapy initiates tumor vascularization in response to damage of peritumor vascular network and peritumor stroma cells. Surgical operations often activate regional metastases and lead to formation of multiple metastasizing foci. The effectiveness of a complete course of chemotherapy is often limited by the development of drug resistance of tumor cells and appearance of numerous toxic side effects. New strategies for chemotherapy based on blockade or suppression of tumor vascularization are very promising in terms of prevention of tumor growth and reduction in risk of possible activation of metastasizing foci.

In contrast to traditional chemopreparations widely used in practical oncology, angiogenesis inhibitors have several important advantages in therapeutic use. First of all, in contrast to cytotoxic chemopreparations which act on various (including tumor) cells characterized by high proliferative activity, most of the antiangiogenic preparations selectively inhibit proliferation of endothelial cells of tumor vessels. Particular localization of the treating target (tumor blood vessels) during administration of antiangiogenic preparations reduces possible risk for the development of side effects compared with traditional chemopreparations characterized by rather low selectivity. As mentioned above, use of endothelial cells of tumor vessels as the main object for chemotherapeutic treatment also avoids rapid development of drug resistance of tumor cells to the chemotherapeutic preparations. This significantly extends the therapeutic range of antiangiogenic preparations because it allows doses and time-course of chemotherapy to be varied without significant loss of effectiveness of their antitumor activity.

Pilot results obtained during clinical studies of antiangiogenic compounds for chemotherapy of various malignant tumors in man revealed that manifestation of antitumor effects is often expressed as stabilization of the neoplastic process, which is characterized by termination or attenuation of growth of primary tumors and suppression of their metastasizing activity [123]. This is due to selective cytotoxic effect of antiangiogenic preparations on the tumor, which is caused by blockade of tumor supply with nutrients and oxygen.

Since manifestation of antitumor activity of a new class of chemotherapeutic agents is rather specific, there is active discussion on the need for possible changes in criteria traditionally employed for evaluation of effectiveness of new chemotherapeutic preparations during clini-

cal trials. For example, clinical observations on the time-course of the tumor process during various modes of therapeutic treatments demonstrate that in contrast to rapidly induced cytotoxic effects of chemopreparations, which are usually detected as size reduction of tumors, appearance of antitumor effect of antiangiogenic preparation requires longer therapeutic treatment (from several months to several years) [123]. The main parameter of effectiveness of antiangiogenic preparation is the time required for attenuation or arrest of tumor growth suggesting stabilization of the tumor process in the body.

Differences in the targets of antitumor effect of cytotoxic preparations (which directly inhibit growth of tumor cells) and antiangiogenic preparations (which inhibit processes mediating tumor vascularization) imply significant differences in therapeutic schemes of their employment. In contrast to the traditional strategy of chemotherapy, which consists of short repeated courses with high doses of chemotherapy followed by short periods of rehabilitation, the highest effectiveness of antiangiogenic treatment is usually observed during long-term courses of therapy with relatively low doses of antiangiogenic preparation [124].

Now there is no doubt that combination of these two different approaches used in the therapy of malignant tumors (direct cytotoxic effect on tumor cells and indirect cytotoxic effect realized via inhibition of blood supply to tumor tissue) will significantly increase effectiveness of antitumor therapy and consequently improve the results of chemotherapy of oncological patients.

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