

# Natural Products as Angiogenesis Inhibitors

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**Abstract:** Angiogenesis is a strictly controlled process in the healthy, adult human body. It is regulated by a variety of endogenous angiogenic and angiostatic factors. It is only switched on, e.g., during wound healing. Pathological angiogenesis occurs, for example, in cancer, chronic inflammation, or atherosclerosis. Angiogenesis inhibitors are able to interfere with various steps of angiogenesis, like basement destruction of blood vessels, proliferation and migration of endothelial cells, or the lumen formation. Among the known angiogenesis inhibitors compounds derived from natural sources, like flavonoids, sulphated carbohydrates, or triterpenoids are playing a prominent role.

**Key words:** Angiogenesis inhibitor, tumour, chronic inflammation, atherosclerosis, plants, microorganisms.

## Abbreviations:

Ara:	arabinose
CAM:	chorioallantoic membrane
DS:	degree of sulphation
FBHE:	foetal bovine heart endothelial cell
FGF-1:	acidic fibroblast growth factor
FGF-2:	basic fibroblast growth factor
Flk-1:	VEGF-receptor
G-CSF:	granulocyte colony-stimulating factor
Glc:	glucose
GM-CSF:	granulocyte-macrophage colony-stimulating factor
IGF-1:	insulin-like growth factor 1
IL-1:	interleukin 1
IL-2:	interleukin 2
IL-8:	interleukin 8
IL-12:	interleukin 12
IP-10:	inducible protein 10
MW:	molecular weight
PAF:	platelet activating factor
PDGF:	platelet-derived growth factor
PF4:	platelet factor 4
PGE <sub>1</sub> :	prostaglandin E <sub>1</sub>
PGE <sub>2</sub> :	prostaglandin E <sub>2</sub>
PKC:	protein kinase C
PMA:	phorbol 12-myristate 13-acetate
RIF:	radiation-induced fibrosarcoma
SPARC:	secreted protein acidic and rich in cysteine
TGF- $\alpha$ :	transforming growth factor $\alpha$

TGF- $\beta$ :	transforming growth factor $\beta$
TNF- $\alpha$ :	tumour necrosis factor $\alpha$
tPA:	tissue plasminogen activator
TSP-1:	thrombospondin
UFH:	unfractionated heparin
uPA:	urokinase
VEGF:	vascular endothelial growth factor

## Introduction

### Definition

The term "angiogenesis" was introduced in 1935 by Hertig to describe the formation of new blood vessels (1). The process of neovascularisation (angiogenesis) is a complex process, which comprises the activation, adhesion, proliferation and transmigration of endothelial cells from preexisting blood vessels (1). In this process the endothelial cells are playing the leading part. In the healthy, adult organism, the turnover of endothelial cells and angiogenesis is rather slow (3 months to one year). Only in certain conditions, e.g., wound healing, embryogenesis or in the female reproductive system (e.g., formation of the corpus luteum) is angiogenesis switched on for a short, defined time period (2). The regulation of the formation of new capillaries is regulated by a complex interaction of growth factors, cell adhesion molecules and specific signal transduction pathways. This generates very efficient control mechanisms, which keep the endothelial cells quiescent or activate them (3).

### Process of angiogenesis

According to Folkman and Brem the formation of new capillaries takes place in a series of sequential steps (Fig. 1) (4, 5). Under certain conditions cells or tissues secrete angiogenic stimuli like, e.g., FGF-2 (Table 1) (6). The stimulated endothelial cells secrete proteases – especially plasminogen activators and collagenases – which lead to the local degradation of the basement membrane of the parental blood vessel and of the extracellular matrix (7, 8). Then endothelial cells migrate through the formed gaps of the basal membrane towards an angiogenic stimulus, which is a chemotactic signal as well (9, 10). Furthermore angiogenic stimuli induce the proliferation of endothelial cells. By endothelial cells aligning in a bipolar configuration capillary sprouts are formed (11, 12). These

layers of endothelial cells form a lumen and finally the tips of two neighbouring capillary sprouts connect and form a new capillary loop, through which blood begins to flow (4, 13). The formation of a new blood capillary is finished by the formation of a new basal membrane and the accumulation of pericytes and other perivascular cells near to the newly formed capillaries. These pericytes inhibit the proliferation of endothelial cells and maintain them dormant under normal, physiological conditions (14, 15).

**Mechanisms and mediators of angiogenesis**

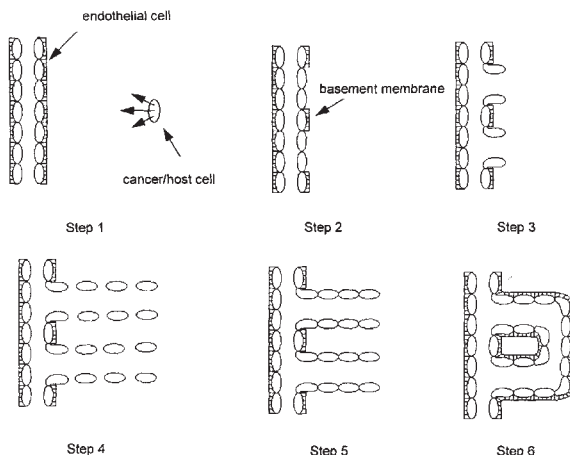
The process of angiogenesis is not only controlled by the quantity and quality of angiogenic stimuli, but it depends on the coordinated production of endogenous angiogenic and angiostatic factors (Table 1). Therefore angiogenesis can be induced by either the increased production or, respectively, activation of angiogenic factors or by reduction of the production or, respectively, inactivation of angiostatic factors. The physiological mechanisms and mediators of angiogenesis are manifold and reviewed in more detail elsewhere (16, 17). In Table 1 some of these angiogenic and angiostatic factors are listed.

**Angiogenesis-dependent diseases**

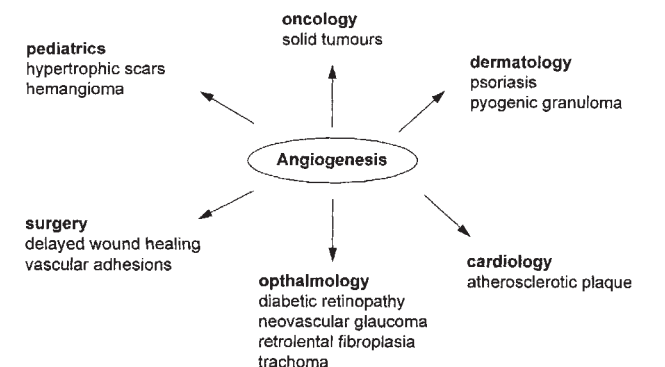
If the control mechanisms fail, angiogenic activity stays permanent or angiogenesis is activated in the wrong tissue or at the wrong time. The resulting pathological growth of new blood capillaries is a characteristic of the so called angiogenesis-dependent diseases (3, 18). Practically every branch of medicine is concerned by such diseases (Fig. 2). The outcome of these illnesses can be rather severe and lead, e.g., to blindness or death (19). In many diseases like, e.g., psoriasis, rheumatoid arthritis and cancer there is an overproduction of angiogenic factors in combination with a lack of angiostatic factors. Pathological angiogenesis can be the basic disease, like in the case of the more than 20 diseases of the eyes, where neo-vascularisation of the cornea leads to blindness (20) or it is the prerequisite for the maintenance of the basic disease, like in the case of the development of solid tumours. The importance of angiogenesis for tumour development is described briefly.

**Table 1** Some angiogenic and angiostatic factors.

Angiogenic factors	Ref.	angiostatic factors	Ref.
<i>growth factors</i>		<i>cytokines</i>	
FGF-1	(102)	interferon $\alpha$	(103)
FGF-2	(104, 105)	platelet factor 4	(106)
G-CSF	(107)	IL-12	(106)
GM-CSF	(107)	inducible protein 10	(106)
IGF-1	(102)	TGF- $\beta$ (at high doses)	(24)
hepatocyte growth factor	(102)	TNF- $\alpha$ (at high doses)	(108)
PDGF	(24)	<i>TIMPs (tissue inhibitors of metalloproteinases)</i>	(109)
TGF- $\alpha$	(24)		
TGF- $\beta$ (at low doses)	(24)	<i>serine proteases</i>	(109)
TNF- $\alpha$ (at low doses)	(108)		
VEGF	(110, 111)	PAI	(109)
<i>cytokines</i>		NO	(112)
IL-1	(113)	<i>cleavage product of plasminogen</i>	(102)
IL-6	(114)		
IL-8	(103, 115)	angiostatin	(102)
<i>prostaglandins</i>		<i>breakdown products of collagen</i>	
PGE <sub>1</sub>	(116)	endostatin	(117, 118)
PGE <sub>2</sub>	(119)	SPARC peptides 2.1 and 4.2	(102)
<i>matrix metalloproteases</i>		<i>thrombospondin-1</i>	
elastase	(109)	(102)	
gelatinase	(109)	<i>16 kD prolactin fragment</i>	
collagenase	(120)		
stromelysin	(121)		
<i>serine proteases</i>			
plasmin	(109)		
urokinase	(121)		
t-PA	(121)		
<i>SPARC and bioactive SPARC peptides</i>		(102)	
PAF	(122)		



**Fig. 1** Sequence of events in angiogenesis. Step 1: release of angiogenic stimuli, step 2: dissolution of the basement membrane of a vessel and the interstitial matrix, step 3: migration of endothelial cells, step 4: proliferation of endothelial cells, step 5: lumen formation, step 6: sprouts form loops [adapted from (123)].



**Fig. 2** Selection of angiogenesis-dependent diseases [modified according to (124)].

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## Tumour and angiogenesis

Without angiogenesis there is no progressive growth of solid tumours and no shedding of metastatic tumours from the primary tumour (21, 22). Angiogenesis is an absolute requirement for tumours to grow beyond the maximal size (1–2 mm) which can be reached when the tumour solely depends on diffusion for an adequate supply of oxygen and nutrients. For the further development to a solid tumour it has to be connected to the blood stream. The process of tumour angiogenesis follows the already described steps. Neovascularisation of a tumour is induced by a shift of the equilibrium between the above mentioned regulators of angiogenesis. Growth of capillaries in tumours cells can be promoted, for example, via overexpression of one or several stimulators of angiogenesis (16, 17), recruitment of host cells, like macrophages, which produce their own angiogenic proteins (23, 24), or upregulation of cell adhesion molecules (25).

Based on this knowledge Folkman postulated in 1972 that the development of angiogenesis inhibitors might be a therapeutic approach for the treatment of tumours (26, 27).

In oncology the primary goal of angiosuppression is not the cure, but the delay of tumour growth. The stop of tumour growth reduces the tumour burden of the body, which might increase the efficacy of another, simultaneously applied, adjuvant therapy (5). Chemotherapy, hyperthermia, radiation, and immune therapy are known to be much more effective, if the number of tumour cells is low. A combination of angiosuppression with such therapies might therefore increase the survival time and increase the chance of cure.

## Assays

In screening for new angiogenesis inhibitors different strategies can be used (28). As shown above angiogenesis provides a number of pathogenic steps that can be blocked or modified in an effort to inhibit tumour-associated angiogenesis (Fig. 1 and Table 1). *In vitro* the downregulation of angiogenic pathways: e.g., growth factor antagonism (FGF-2, VEGF), interference with cytokines, e.g., IL-8, interference with receptors, e.g., Flk-1 or inhibition of collagen I production, the upregulation of angiostatic pathways: e.g., increase of TSP-1, angiostatic C-x-C chemokines, angiostatin (29, 30), the increase of collagens IV and V, the inhibition of heparinases, the direct or indirect inhibition of proteases like, e.g., matrix metalloproteases, the inhibition of integrin expression ( $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ), inhibition of chemotaxis or interference with macrophages can be tested (16, 19).

As *in vivo* assays, e.g. the test on the chorioallantoic membrane of the fertilized hen egg (CAM-assay), the corneal micropocket assay in rabbits, rats or mice (31) can be used.

However, caution must be exercised in the extrapolation of *in vitro* observations to the *in vivo* situation. Therefore in this review results obtained in only *in vitro* screening systems are not discussed in further detail (31).

In this review natural products are listed which have proven their anti-angiogenic effect in at least one *in vivo* assay. Also many inhibitors of angiogenesis – at the moment about 200 – are not covered here. These inhibitors include synthetic as

well as endogenous compounds, such as angiostatin. This review puts emphasis on natural products and some derivatives mainly derived from plants and microorganisms with anti-angiogenic activity.

## Angiogenesis Inhibitors

### Angiostatic steroids

Angiostatic steroids are a class of steroids, lacking glucocorticoid and mineralcorticoid activity, with anti-angiogenic activity when administered in the presence of heparin, heparin fragments or heparin analogues (32, 33).

Tetrahydrocortisol (1) and tetrahydrocortisol-3 $\alpha$ -glucuronide (2), both metabolites of glucocorticoids in humans, inhibit in combination with heparin angiogenesis in the CAM-assay (34).

Heparin can be substituted with heparin derivatives or defined heparin fragments, lacking anticoagulant activity (35, 36) and also with suramin (37) or inhibitors of arylsulfatase (38). The mechanism of action is not known exactly, but the angiostatic steroids seem to influence the turnover of collagen in the basal membrane of the blood vessels (39).

### Calphostin C

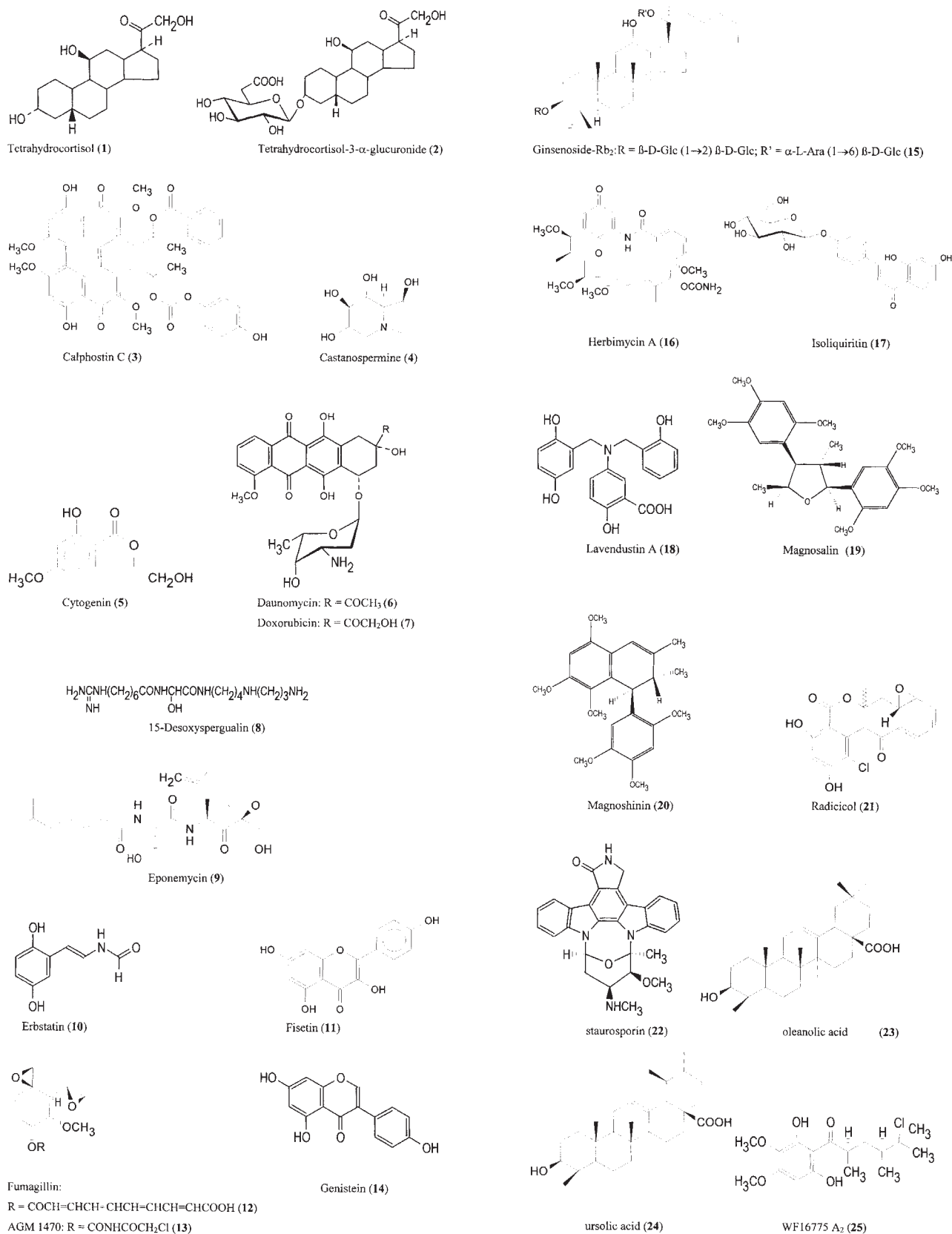
The PKC inhibitor calphostin C (3), isolated from *Cladosporium cladosporioides*, blocks angiogenesis potentiated by integrin  $\alpha_v\beta_5$ , but not by integrin  $\alpha_v\beta_3$  (40). 4  $\mu$ g calphostin C/sponge/day also inhibit neovascularisation induced by FGF-2 (100 ng/sponge/day), IL-1 $\alpha$  (50 ng/sponge/day), TNF- $\alpha$  (50 ng/sponge/day) or the PMA (30  $\mu$ g/sponge/day) in the rat sponge model (41, 42).

### Castanospermine

The  $\alpha$ -glucosidase I inhibitor castanospermine (4), an alkaloid isolated from seeds of *Castanospermum australe* and pods of *Alexa leipetala*, inhibits angiogenesis and tumour growth in C57/Bl mice after systemic application of 2.5 and 5.0 mg/mouse/day (43). Castanospermine (4) does not inhibit the proliferation of endothelial cells or their ability to attach to various extracellular matrix molecules, but it reduces their ability to migrate and invade basement membrane gels *in vitro*. It also prevents morphological differentiation of endothelial cells *in vitro*, probably by shifting the extracellular surface oligosaccharides towards high mannose chains, which prevents lumen formation (43).

### CM 101

A group B streptococcus polysaccharide toxin CM 101 that is responsible for pulmonary disease in infected human neonates binds preferentially to the capillary endothelium of a variety of carcinomas but not to normal, mature endothelium. CM 101 treatment of mice implanted with human or murine tumours results in an intense intratumoural inflammatory reaction associated with necrosis, hemorrhage, thrombosis and the release of large amounts of cytokines including TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and MIP-1. CM 101 reduces tumour volume and prolongs survival of the treated mice. Preliminary results of a phase I trial suggest the drug is well tolerated. Objective



**Fig. 3** Structure of angiogenesis inhibitors (1 – 25).



responses are noted in three of 15 patients with classical Kaposi's sarcoma, hepatocellular carcinoma, and metastatic small bowel adenocarcinoma (44).

### Cytogenin

Cytogenin (**5**) is a fungal metabolite isolated from the broth of *Streptomyces eurocidicum*. Cytogenin (**5**) suppresses the angiogenic response induced by malignant tumour cells when administered orally, but does not inhibit embryonic angiogenesis. That means cytotenin (**5**) is inactive in the CAM-assay but active *in vivo* models for tumour associated angiogenesis and rheumatoid arthritis (45).

### Cytotoxic agents

Some compounds, known as cytotoxic agents, have a substantial effect both on endothelial and tumour cells (46, 47). In contrast to the following listed cytotoxic agents with anti-angiogenic activity cyclophosphamide, melpham, 5-fluorouracil, methotrexate and *cis*-platin are inactive in this respect (48).

The anthracycline antibiotics daunomycin (**6**) and doxorubicin (**7**), derived from *Streptomyces caeruleorubidus* or *peuceticus* var. *caesius*, inhibit angiogenesis in the CAM-assay at doses of 5–20 µg/pellet (48, 49). At non-cytotoxic concentrations (IC<sub>50</sub>-values 2.5–15 µg/ml) they inhibit the lumen formation of endothelial cells plated on matrigel in a dose-dependent manner (49).

Bleomycin, a complex of glycopeptide antibiotics, isolated from the broth of *Streptomyces verticillus*, inhibits angiogenesis in the CAM-assay in a dose-dependent manner with and without copper [IC<sub>50</sub> (without copper) = 75 ng/pellet and IC<sub>50</sub> (with copper) = 1.5 ng/pellet] (50).

The alkaloid colchicine, derived from *Colchicum autumnale*, inhibits angiogenesis in the CAM-assay (51). However, it does not inhibit the FGF-2 or VEGF induced corneal neovascularisation in C57BL/6 mice at the dose of 0.25 mg/kg, *i.p.* (48).

The corneal neovascularisation in C57BL/6 mice is also reduced by taxol, derived from, e.g., *Taxus brevifolia*. Taxol (6 mg/kg, *i.p.*) inhibits the FGF-2 and VEGF-induced neovascularisation by 45% and 37%, respectively (48).

Vinblastine and vincristine, derived from *Catharanthus roseus*, inhibit angiogenesis in the CAM-assay (51). But vincristine does not inhibit the FGF-2 or VEGF induced corneal neovascularisation in C57BL/6 at the dose of 0.2 mg/kg, *i.p.* (48).

### 15-Deoxyspergualin

15-Deoxyspergualin (**8**), a synthetic analogue of spergualin isolated from the broth of *Bacillus laterosporus*, inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The IC<sub>50</sub>-value was 480 ng (960 pmol) per egg (52). It is an inhibitor of tyrosine kinase and it inhibits the proliferation of vascular endothelial cells by 40% at a concentration of 10<sup>-5</sup> M in a three-dimensional culture system involving collagen gel, but not in a two-dimensional culture system (28, 53).

### Eponemycin

Eponemycin (**9**), isolated from the fermentation broth of *Streptomyces hydroscopicus*, is one of the most potent angiogenesis inhibitors in the CAM-assay. The IC<sub>50</sub>-value is 0.1 ng (250 fmol) per egg. It inhibits migration and proliferation of endothelial cells. It also shows antitumour activity against various solid tumours, e.g., B16 melanomas (28, 54).

### Erbstatin

Erbstatin (**10**), a specific tyrosine kinase inhibitor, isolated from *Streptomyces* sp., inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The IC<sub>50</sub> value is 80 ng (450 pmol) per egg. It inhibits proliferation of endothelial cells (IC<sub>50</sub> = 3.6 µM) (55).

### Fisetin

Fisetin (**11**), a plant flavonoid, inhibits angiogenesis in the CAM-assay. At the concentration of 100 ng per egg it shows an anti-angiogenic effect in 43% of the eggs (56).

### Fumagillin and derivatives

The microbial antibiotic fumagillin (**12**), isolated from *Aspergillus fumigatus*, inhibits at the concentration above 2 µg per pellet angiogenesis in the CAM-assay (5, 28). It also suppresses tumour-induced neovascularisation in the mouse dorsal air sac model. However, fumagillin produces excessive toxicity, like severe weight loss. Therefore fumagillin derivatives were developed that retain anti-angiogenic activity with reduced side-effects.

TNP-470 (formerly called AGM-1470) (**13**) is a more potent, less toxic fumagillin analogue, which after oral administration inhibits growth of solid tumours, e.g., Lewis lung carcinoma in mice (5, 57). In the mouse sponge implantation assay TNP-470 (**13**) inhibits neovascularisation induced by FGF-2 (5). *In vitro* TNP-470 (**13**) selectively inhibits proliferation (IC<sub>50</sub> about 10 pg/ml), migration of endothelial cells (IC<sub>50</sub> about 100 pg/ml) and also inhibits *in vitro* capillary tube formation at concentrations that were cytostatic (57, 58). Anti-angiogenic activity was shown *in vivo* using a variety of assays (58). In the rat TNP-470 (**13**) inhibits capillary-like tube formation of endothelial cells with a minimal effect on non-endothelial cell growth (5). TNP-470 (**13**) inhibits *in vivo* growth of a variety of human xenografts and murine tumours in the absence of direct *in vitro* growth inhibition of the same tumour cell lines (57, 59). It also suppresses metastasis in both human xenograft and murine tumour models (59). TNP-470 (**13**) is currently undergoing phase I and early phase II clinical trial testing, e.g., in Kaposi's sarcoma and other tumours. Preliminary results indicate that the agent is well tolerated, but its CNS-toxicity is dose-limiting (44).

### Genistein

The isoflavonoid genistein (**14**) inhibits the FGF-2-induced corneal neovascularisation in NZW rabbits (0.04 mg/day, subconjunctival). In this experiment the number of vessels is reduced from 63 to 36 and the vascularised area is reduced from 21.4 mm<sup>3</sup> to 10.4 mm<sup>3</sup> (60). The genistein (**14**) inhibits tyrosine kinase, uPA and PAI-1 (61, 62). It also inhibits

migration and proliferation of endothelial cells (61, 62). Clinical cancer trials are ongoing (61).

#### Ginsenosides

20(R)-, 20(S)-Ginsenoside-Rg<sub>3</sub> and ginsenoside-Rb<sub>2</sub> (**15**), isolated from the roots of red ginseng (*Panax ginseng*), significantly decrease the number of blood vessels oriented toward the tumour mass in a dose-dependent manner in the highly metastatic tumour cell B16-BL6 melanoma in syngenic mice after oral administration of 10–1000 µg saponin/mouse. This anti-angiogenic effect of these saponins may partly contribute to the inhibition of metastases in the forementioned tumour model (63, 64).

In contrast a mixture of saponins derived from ginseng enhances angiogenesis in *in vivo* wound healing models. Furthermore this mixture of saponins significantly stimulates tube formation by endothelial cells in a dose-dependent manner (10–100 µg/ml), decreases the activity of PAI-1 (50–100 µg/ml), slightly enhances endothelial cell proliferation (1–100 µg/ml) and significantly enhances migration of endothelial cells (10–100 µg/ml) (65).

#### Herbimycin A

Herbimycin A (**16**), an ansamycin antibiotic isolated from *Streptomyces hygroscopicus*, inhibits angiogenesis in the CAM-assay and in the corneal rabbit assay in a dose-dependent manner (66, 67). The ID<sub>50</sub> value is 150 ng (260 pmol) per egg (67). It might selectively reduce the activity of certain oncogene products related to tyrosine kinase (5). It is an inhibitor of tyrosine kinase, which is part of a domain in receptors of, e.g., FGF-1 or FGF-2 (28).

#### Isoliquiritin

Isoliquiritin (**17**), isolated from a water extract of licorice root, inhibits granuloma angiogenesis in adjuvant-induced chronic inflammation at the dose of 0.31–3.1 mg/kg. It has a potency for anti-tube formation at the concentration between 0.1–100 µM (68).

#### Krestin

Krestin is a protein-bound polysaccharide isolated from *Coriolus versicolor* strains (5). In the mouse dorsal air sac assay it inhibits neovascularisation of tumours (69). Krestin has no direct effect on the proliferation or migration of endothelial cells (69), but it reduces the synthesis of collagen by endothelial cells (70).

#### Lavendustin A

Lavendustin A (**18**), isolated from *Streptomyces griseolavendus*, is a potent and selective inhibitor of protein tyrosine kinase (61). It inhibits VEGF- and FGF-2-induced angiogenesis in the sponge implants in rats at the concentration of 10 µg/sponge/day (61, 71).

#### Magnosalin and magnoshinin

The neolignans magnosalin (**19**) and magnoshinin (**20**), derived from *Magnolia salicifolia*, inhibit angiogenesis and

granuloma formation in the adjuvant-induced mouse pouch granuloma after oral and intra-pouch administration (72). The anti-angiogenic effect of magnosalin (**19**) is greater than that of magnoshinin (**20**) (72). They inhibit fetal bovine serum-stimulated [IC<sub>30</sub> (magnosalin) = 0.51 µM and IC<sub>30</sub> (magnoshinin) = 8.14 µM] and IL-1α-stimulated [IC<sub>50</sub> (magnosalin) = 1.22 µM and IC<sub>50</sub> (magnoshinin) = 0.74 µM] tube formation of endothelial cells in a dose-dependent manner (73).

#### Radicalol

The macrolide antibiotic radicalol (**21**), derived from *Nectaria radicola*, has a differentiation modulating effect and in addition inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The IC<sub>50</sub> value is 200 ng (540 pmol) per egg. It has an inhibitory effect on uPA production and proliferation of endothelial cells. It exhibits antitumour effect in several tumour models (28).

#### Staurosporin

Staurosporin (**22**), isolated from a *Streptomyces* strain, inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The IC<sub>50</sub> value is 33 ng (71 pmol) per egg (56). It is an inhibitor of tyrosine kinase. Vascular endothelial cells are inhibited with an IC<sub>50</sub> = 0.88 nM (28).

#### Sulphated carbohydrates

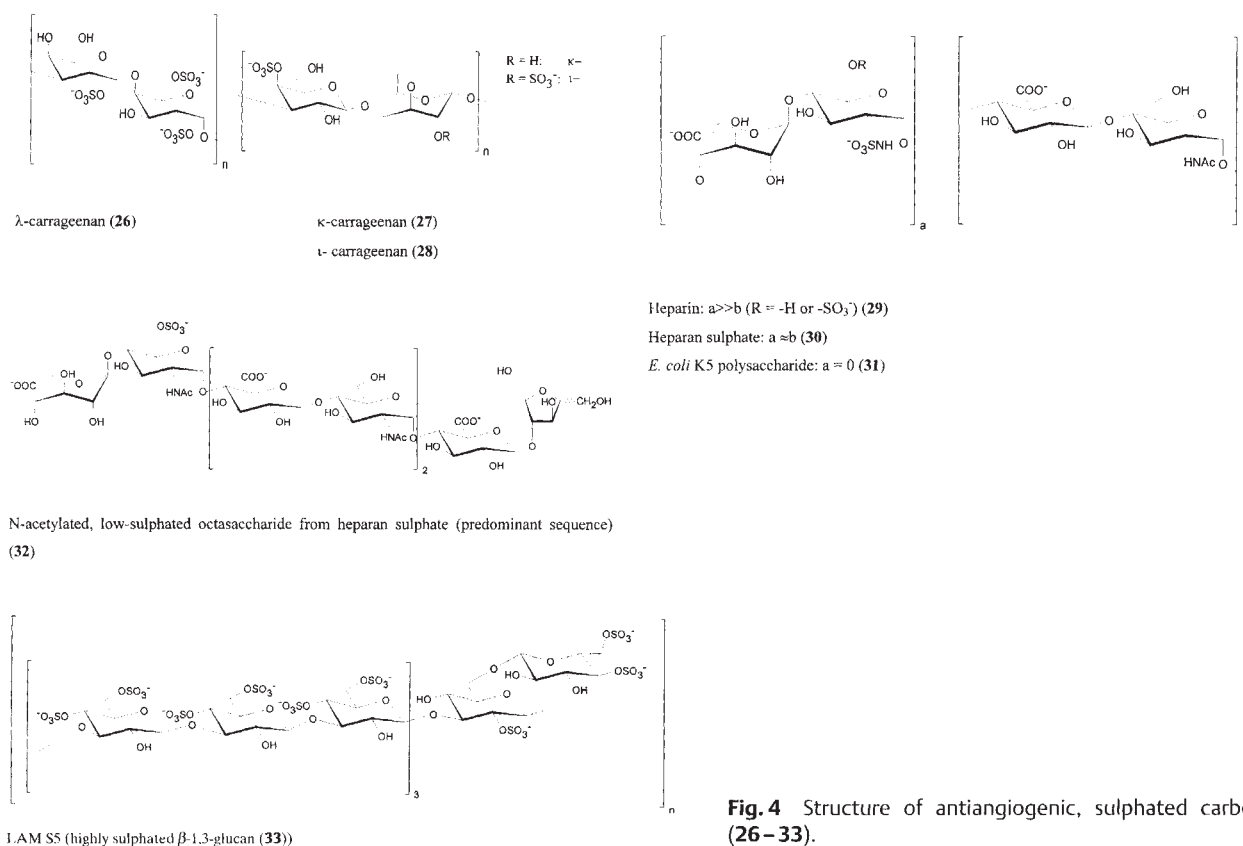
Sulphated carbohydrates like heparin (**29**) and heparan sulphate (**30**) can modulate a multitude of cellular functions (e.g., growth, morphology, migration, etc.) (74), which are related to angiogenesis, by interacting with extracellular matrix proteins (17), growth factors (75, 76), growth factor receptors (77), enzymes (78, 79), and proinflammatory mediators (80).

Despite the tremendous amount of data – obtained *in vitro* – on the structural requirements for heparin and heparan derivatives and fragments for binding to growth factors and activating endothelial cells (75, 81), less is known about the *in vivo* activity of these compounds (82).

In the following is given a description of the few sulphated carbohydrates which have been evaluated *in vivo* for their anti-angiogenic activity:

SCM-chitin III is a group of chemically related chitin derivatives containing 6-O-sulphate and 6-O-carboxymethyl groups. It inhibits tumour-associated angiogenesis in mice and significantly inhibits lung tumour colonisation of B16-BL6 melanoma in experimental and spontaneous lung metastasis in mice (5, 83). SCM-chitin is an inhibitor of collagenase type IV and it prevents the invasion of endothelial cells through matrigel and migration of endothelial cells (5, 83).

λ- (**26**), ι- (**28**), and κ-carrageenan (**27**), sulphated polysaccharides derived from various red algae, are better angiogenesis inhibitors than suramin in the CAM-assay. At the concentration of 50 µg/pellet the anti-angiogenic activity of λ-carrageenan (**26**) was best, followed by ι- (**28**) and at last κ-carrageenan (**27**) (84). The carrageenans are selective growth factor antagonists for, e.g., FGF-2 (85).



**Fig. 4** Structure of antiangiogenic, sulphated carbohydrates (26–33).

Methylated or acetylated  $\iota$ -carrageenan are anti-angiogenic in the CAM-assay at the concentration of 50  $\mu$ g/pellet (84). In contrast to  $\iota$ -carrageenan (28) they do not act as growth factor antagonist for FGF-2 (86).

The highly sulphated  $\beta$ -1,3-glucan LAM S5 (DS = 2.3, MW = 12500 D) (33), obtained by sulphation of the  $\beta$ -1,3-glucan laminarin, derived from *Laminaria digitata*, inhibits angiogenesis in the CAM-assay in a dose-dependent manner (80% inhibition at the dosage of 10  $\mu$ g/pellet and 100% inhibition at 50  $\mu$ g/pellet) and has antitumour activity against RIF-1 tumour (87). Combining LAM S5 (33) with tetrahydrocortisol (1) or the cytotoxic agent mephalan resulted in a slight increase in anti-tumour activity. *In vitro* endothelial cells are inhibited at a lower concentration of LAM S5 (33) than RIF-1 cells since the  $IC_{50}$  value for inhibition of the proliferation of the endothelial line FBHE is 1  $\mu$ /ml (88) and 3  $\mu$ g/ml LAM S5 starts to inhibit tubule formation of endothelial cells, whereas the proliferation of RIF-1 cells is inhibited with an  $IC_{50}$  of 30  $\mu$ g/ml (87). Therefore the antitumour activity of LAM S5 (33) will be due to inhibition of tumour neovascularisation instead of a direct effect on RIF-1 cells (87).

In contrast to these findings, a laminarin sulphate of unknown structure mimics the action of FGF-2 and is postulated to be used as wound healing reagent (89). This discrepancy again stresses the need to reveal at least basic structural information on the tested sulphated carbohydrate.

Certain batches of heparin (29), in dependence on their source, are able to inhibit angiogenesis in the CAM-assay (74, 90). UFH can obtain fractions, which inhibit or stimulate

angiogenesis. So the angiogenic response of porcine heparin, examined with the mesenteric-window assay in adult male rats, can depend on the mean-molecular weight of heparin. The 2.4-kDa fraction suppresses angiogenesis by 46%, whereas the 22-kDa fraction stimulates angiogenesis by 123% as compared with UFH (91).

In an attempt to define the fine structure of heparin/heparan sulphate, experiments with the *Escheria coli* K5 capsular polysaccharide (31) and fragments thereof down to octasaccharide size revealed that their possible anti-angiogenic effect is not strictly related to sulphate groups, because low- or non-sulphated heparin/heparan fragments with a  $[-Glc\ \beta 1,4-GlcNAc\ \alpha 1,4]_n$ -sequence have an anti-angiogenic effect in the CAM-assay. The anti-angiogenic activity of  $[-Glc\ \beta 1,4-GlcNAc\ \alpha 1,4]_n$ -containing saccharides is potentiated by the presence of  $\iota$ -uronic acid and one or two *O*-sulphate groups in the non-reducing-terminal disaccharide unit, as demonstrated by comparison of the anti-angiogenic activity of the low sulphated octasaccharide from heparan sulphate (32), derived from pig mucosa with the non-sulphated octasaccharide (75). Low molecular weight heparan sulphate also inhibits neovascularisation in the chemical cauterized rat cornea (92).

Desulphated heparin derivatives, like the most effective heparanase inhibitors 2,3-*O*-desulphated heparin and periodate-oxidized, borohydride-reduced heparin inhibit angiogenesis in the CAM-assay at least as efficaciously as heparin in combination with cortisone. 2,3-*O*-Desulphated heparin significantly decreases tumour growth of certain tumours and prolongs survival times of C57BL/6N mice in a B16-F10 melanoma experimental lung metastasis assay (93).



Pentosan polysulphate (xylan polysulphate) was the first anti-angiogenic agent to enter clinical trials. *In vitro* it inhibits FGF-2-induced endothelial cell migration and proliferation, growth of an adrenal cancer cell line transfected with a FGF gene inducing constitutive FGF-2 production; and it inhibits the paracrine effects of heparin-binding growth factors secreted by a variety of malignant tumour cell lines. In an initial phase I clinical trial the administration of pentosan polysulphate via continuous intravenous infusion and subcutaneous injection to patients with acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma, the agent proved toxic producing anticoagulation, thrombocytopenia, and increased transaminase levels without evident clinical activity. Similar results were obtained when pentosan polysulphate was administered subcutaneously to patients with solid tumours (44, 94).

#### Protamine

Protamine is a 43 kD arginine-rich cationic protein, which binds heparin and inhibits migration and proliferation of endothelial cells (61). *In vitro* it interferes with growth factors (growth factor antagonist), it decreases the production of angiogenic factors and it inhibits the migration of endothelial cells (95). *In vivo* it inhibits tumour angiogenesis and growth in a number of animal models after systemic administration (61, 73). Subcutaneous administration of protamine inhibits embryonic vascularisation in a rat model, which results in significantly lower capillary densities and a larger variability in capillary spacing than in untreated controls (73). However, protamine is toxic for reasons other than its biological effect on endothelial cells (73).

#### Tecogalan (DS-4152, SP-PG)

Tecogalan is a sulphated polysaccharide-peptidoglycan complex, isolated from the cell wall of an *Arthobacter* species (96). In the CAM-assay the anti-angiogenic activity of tecogalan is enhanced by cortisone acetate or tetrahydro S (97). It inhibits FGF-2-induced neovascularisation in the corneal pockets of rabbits in a dose dependent manner at the concentration of 100 µg and 200 µg per pellet (96). In addition, tecogalan, combined with tetrahydro S, inhibits angiogenesis induced by the ovarian ascites tumour M5076 in the murine dorsal airsac assay (98). Tecogalan also possesses *in vivo* antitumour activity against human xenografts and murine tumours when co-administered with either cortisone acetate or tetrahydro S (97, 98). It inhibits *in vitro* endothelial cell growth at 250 µg/ml and the FGF-2-induced migration is inhibited at 64 µg/ml (95, 99). This inhibition is increased when combined with tamoxifen (44, 98). Furthermore tumour angiogenesis in Kaposi's sarcoma is markedly inhibited at the concentration of 2.5 mg/kg in nude mice.

In combination with cortisone acetate tecogalan inhibits angiogenesis in the CAM-assay and growth of solid Sarcoma 180 tumour in mice (97, 98).

This agent has recently undergone phase I clinical testing in patients with solid tumours, including AIDS-associated Kaposi's sarcoma at doses between 125 mg/m<sup>2</sup> in a single *i.v.* infusion every three weeks. The dose-limiting toxicity was anticoagulation. Other toxicities included fever and rigors. The serum half-life of tecogalan sodium was between 1–1.5

hours. The recommended phase II dose is 390 mg/m<sup>2</sup> over 24 hours (44, 100).

#### Oleanolic acid

The triterpene aglycone oleanolic acid (**23**) inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The minimum dose required for producing an avascular zone in the CAM is 5 µg (10 nmol). The ID<sub>50</sub> value is 40 µg (80 nmol) per egg. It inhibits the proliferation of endothelial cells in a concentration-dependent manner (IC<sub>50</sub> = 20 µM) (101).

#### Ursolic acid

The triterpene aglycone ursolic acid (**24**) inhibits angiogenesis in the CAM-assay in a dose-dependent manner. The minimum dose required for producing an avascular zone on the CAM is 2 µg (4 nmol). The ID<sub>50</sub> value is 5 µg (10 nmol) per egg. It inhibits the proliferation of endothelial cells in a concentration-dependent manner (IC<sub>50</sub> = 5 µM) (101).

#### WF16775 A<sub>2</sub>

WF16775 A<sub>2</sub> (**25**) is an antibiotic which is isolated from the fungus *C. erysiphoides* (61). It is preferentially cytostatic to endothelial cells (61). *In vivo* it inhibits tumour angiogenesis and growth (61).

#### Conclusions

After about 25 years of research in angiogenesis inhibitors the data on positive results obtained in various animal models are accumulating and the first agents have entered clinical trials phase I–III. The approach of angiosuppression for the treatment or even prevention of certain diseases like cancer, chronic inflammation or atherosclerosis looks very promising. In most cases a long-term treatment will be necessary. Despite the large number of existing angiogenesis inhibitors there is still a great demand to look for new anti-angiogenic compounds, because the known ones are not perfect. For long-term chronic uses, these agents should possess lower side effects, especially little acute or chronic toxicity. They should be administered orally. Less frequent administration requires agents with long half-lives (44). Also angiogenesis inhibitors with different modes of action will be necessary in the clinic, because the combination of various angiogenesis offers a better chance to interfere with the very complex and redundant process of angiogenesis.

As shown in this review many of the known angiogenesis inhibitors are derived from natural products. Plants and microorganisms are and will be a good source to provide new leads for the development of new or improved angiogenesis inhibitors.

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